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Review

Zebrafish disease models in hematology: Highlights on biological and translational impact

Daniela Zizioli[a,*], Marina Mione[b], Marco Varinelli[a], Michele Malagola[c], Simona Bernardi[c], Elisa Alghisi[c], Giuseppe Borsani[d], Dario Finazzi[a,e], Eugenio Monti[a], Marco Presta[f], Domenico Russo[c,**]

[a] Unit of Biotechnology, Department of Molecular and Translational Medicine, University of Brescia, Italy
[b] CIBIO, Center for Integrative Biology, University of Trento, Italy
[c] Unit of Blood Disease and Stem Cell Transplantation, Department of Clinical and Experimental Sciences, University of Brescia, ASST-Spedali Civili di Brescia, Italy
[d] Unit of Biology and Genetic, Department of Molecular and Translational Medicine, University of Brescia, Italy
[e] Clinical Chemistry Laboratory, ASST, Spedali Civili di Brescia, Italy
[f] Unit of Oncology and Immunology, Department of Molecular and Translational Medicine, University of Brescia, Italy

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ABSTRACT

Zebrafish (*Danio rerio*) has proven to be a versatile and reliable *in vivo* experimental model to study human hematopoiesis and hematological malignancies. As vertebrates, zebrafish has significant anatomical and biological similarities to humans, including the hematopoietic system. The powerful genome editing and genome-wide forward genetic screening tools have generated models that recapitulate human malignant hematopoietic pathologies in zebrafish and unravel cellular mechanisms involved in these diseases. Moreover, the use of zebrafish models in large-scale chemical screens has allowed the identification of new molecular targets and the design of alternative therapies. In this review we summarize the recent achievements in hematological research that highlight the power of the zebrafish model for discovery of new therapeutic molecules. We believe that the model is ready to give an immediate translational impact into the clinic.

1. Introduction

Zebrafish (*Danio rerio*) is a freshwater teleost fish that has emerged as a model organism in many research fields. In the early 1980s, the zebrafish was used as a classical developmental and embryological model thanks to the combination of external fertilization and optical transparency of embryos and larvae [1]. In the 1990s thousands of developmental zebrafish mutants were identified through forward genetic screens showing a high conservation of disease genes among species [2]. The strong conservation of key developmental processes of the blood system allowed the identification of several genes that are involved in human hematopoietic disorders [3]. In the last years, the development of genetic techniques, such as transgenesis, highlighted the power of zebrafish to model human acquired disease and different transgenic strategies have been developed with the aim to induce gene and oncogene expression in specific tissues and organs [4]. Additionally, forward genetic approaches (ENU-mutagenesis) have identified zebrafish mutants phenocopying human disorders; these approaches have given a great contribution to the discovery of several key genes involved in a biological pathways and molecular mechanisms of different malignant hematopoietic disorders [5]. Recently, novel genome editing technologies have been applied to establish animal models with increasing rapidity. Compared with methods using Zinc Finger Nucleases (ZFN) or Transcription activator-like Effector Nucleases (TALEN), a more recent technology named Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) has played an important and appreciating role in specific gene targeting to generate mutant lines and animal models. The CRISPR/Cas9 system is highly efficient and specific in generating heritable gene-inactivating mutations and knock-in (KI) alleles in zebrafish with negligible off-target effects [6]. Moreover, due to the optical transparency and the ease of manipulation, zebrafish provides a unique opportunity for studying human cancer cell behavior in xenotransplantation assay [7]. Finally, given the size and the permeability to small molecules, zebrafish embryos are suitable to test a variety of different compounds in high-throughput, large-scale chemical screens [8]. Although murine models

* Corresponding author.
** Correspondence to: D. Russo, Chair of Hematology, Unit of Blood Disease and Cell Therapies, University of Brescia, ASST-Spedali Civili di Brescia, Italy.
E-mail addresses: daniela.zizioli@unibs.it (D. Zizioli), domenico.russo@unibs.it (D. Russo).

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represent the hallmark of hematological research, zebrafish, with its considerable advantages, is becoming a valuable tool for understanding physiological and pathological mechanisms involved in human hematopoiesis. This review summarizes the important insights obtained from studying hematopoiesis in zebrafish, its contribution in understanding the molecular mechanisms underlying malignant hematopoiesis and discovering new potential therapeutic compounds.

2. Studying the hematopoietic niche from zebrafish to mammals

2.1. Hemangioblast theory and HSC emergence

During the past years several animal models have been used to study the process by which blood cells are formed, namely hematopoiesis. Indeed, different authors have demonstrated that there is a certain degree of conservation of the overall hematopoietic process among vertebrates, despite few differences existing. Zebrafish shares, with other vertebrates, all major blood cell types which are generated by similar developmental pathways. Additionally, many of the genes known to regulate erythropoiesis in mammals have been identified in zebrafish. There is also a high conservation of transcription factors and major signaling pathways involved in hematopoietic stem cell (HSC) differentiation and maturation [9,10]. Notably, zebrafish possesses blood-forming marrow, spleen and thymus which exist only in jawed vertebrates and it lacks lymph nodes. In mammals, primitive hematopoiesis is largely erythropoietic and occurs outside the embryo in the blood islands of the yolk sac. In the later stages of development, hematopoiesis moves to the aorta-gonad-mesonephros region and the fetal liver, whereas in adult definitive hematopoiesis occurs in the bone marrow where all blood cell lineages are produced [11]. By contrast, primitive hematopoiesis in the zebrafish occurs in the intermediate cell mass (ICM, a tissue derived from ventral mesoderm), anteriorly in the paraxial mesoderm over the yolk, and posteriorly in a small ventral cluster of cells located in the developing tail referred to as the posterior blood island (Fig. 1a). The ICM is a region analogous to the blood islands of the yolk sac in mammals. Hematopoietic progenitor cells from the posterior blood island enter the circulation slightly later than those in the ICM and may represent the end stage of primitive hematopoiesis. As in mammals, zebrafish blood development involves three sequential waves: the primitive one, the erythromyeloid progenitor (EPM-derived) wave and the definitive wave that occurs in different embryonic locations (Fig. 1b) [12]. The primitive wave starts at 11 h post-fertilization (hpf) and it is characterized by the presence of “primitive” macrophages and the first circulating erythrocytes. Studies of gene expression and fate mapping showed that the myeloid and erythroid progenitors are anatomically separate. The myeloid progenitors were found in the anterior lateral-plate mesoderm (ALM), whereas erythrocytes precursors were located in the posterior lateral-plate mesoderm/intermediate cell mass (PLM/ICM) [13]. The formation of the PLM/ICM has been studied in a number of different fish species [14] and it is characterized by the migration of bilateral stripes of cells that differentiate into “primitive” erythroblasts and endothelial cells, indicating the presence of a bi-potential precursor for blood and vasculature, named *hemangioblast* [15]. The bi-potential precursor theory has been confirmed by experiments *in vitro* and *in vivo* showing that *hemangioblasts* differentiated from human embryonic stem cells (hESCs) were able to generate blast colonies with both hematopoietic and endothelial-vascular potential [16]. In this frame, zebrafish represented a powerful tool to investigate the complex cellular mechanisms of the stem cell niches that generate the *hemangioblasts* during development. Through the use of caged dextran in single cell-resolution-fate maps experiments, Vogeli et al. [17] demonstrated *in vivo* the presence of bipotential progenitors in the zebrafish blastula. These cells showed both endothelial and hematopoietic potential and clustered specifically in dorsal area of the yolk extension that represents the site of definitive hematopoiesis in the developing embryo. After almost a century from the first observation of the *hemangioblast* [18], the zebrafish had finally provided the live imaging evidence of this highly conserved process. Most of the core regulators of hematopoiesis are evolutionary conserved between teleosts, such as zebrafish and mammals; different authors having defined genes and transcription factors involved in primitive and definitive hematopoiesis. The earliest molecular marker of primitive hematopoiesis is the bHLH transcription *factor stem cell leukemia* (*scl/tal1*) that is expressed at early somite stages (10.5–11 hpf) in the PLM [11,19]. When the mesoderm cell fate become more defined to endothelial and hematopoietic lineages, the hematopoietic transcription factor *LIM domain only 2* (*lmo*), the vasculogenesis genes GATA *binding protein 2a* (*gata2*) [20], the ETS family members *Friend Leukemia virus Integration 1a* and *1b* (*fli1a* and *fli1b*) [21] and the *ets-related protein* (*etsrp/etv2*) are all expressed. Recently, Reischauer et al. [22] identified the basic helix-loop-helix-Per-Arnt-Sim (bHILH-PAS) domain transcription factor *cloche* as a master regulator of endothelial and hematopoietic fate being expressed in a highly specific spatio-temporal pattern during late gastrulation. Epistasis experiments revealed that *cloche* functions upstream of *etsrp/etv2* and *tal1/scl*, the earliest expressed endothelial and hematopoietic transcription factor genes identified to date. At 12 hpf (5 somites) when ICM precursors adopt a cell fate they express three different transcription factors: *gata1*, considered a key regulator of erythroid cell fate [22,23]; *pu.1* (*spi1b*), a master regulator of myeloid lineage [23]; *kdr*, expressed in angioblasts [24]. Few identified factors have been shown to act upstream of *gata1* or *pu.1* to regulate the balance between erythroid and myeloid cell fate [25]. Definitive hematopoiesis in zebrafish has been shown to initiate around 28–30 hpf and gives rise to definitive adult-like hematopoietic stem cells (HSPc), which have both self-renewal capacity and erythroid, myeloid and lymphoid potential. HSPc emerge from *kinase insert domain receptor-like* (*kdrl*)-positive endothelium lining the ventral wall of the dorsal aorta, equivalent to the mammalian Aorta-Gonad Mesonephros region (AGM) [26,27]. The newly emergent HPSc co-express endothelial markers such as *kdrl*, *c-myb* or run-related transcription factor 1 (*runx1*) [19,28–30]. Burns et al. [31] highlighted the hypothesis that *runx1* is necessary in primitive hematopoiesis but it is also required for definitive hematopoiesis. In zebrafish, *runx1* expression begins at 5-somite stage in PLM, being expressed in dorsal aorta at 30 hpf. *runx1* is considered one of the earliest markers of HPSc, with *c-myb* acting downstream; analysis of zebrafish mutants has demonstrated that *c-myb* expression is *runx1*-dependent [32]. Although the mouse model had provided functional assays to study HSC identity and function [27,33], the development *in utero* of embryos hampered live imaging studies about HSC origin. Few years ago, the zebrafish allowed for the first time the visualization in a living organism, of the endothelial-to-hematopoietic transition (EHT) mechanism, an evolutionarily conserved process of HSC generation through the ventral wall of the dorsal aorta (DA) [26,34,35]. In the AGM region, endothelial cells were occasionally observed to transform into spherical shape cells and to bud into the lumen of the DA. In zebrafish these newly formed HSCs rapidly enter the circulation through the lumen of the caudal vein whereas in mammals and chick embryos they proliferate and differentiate locally [36]. In mammals, these HSCs created during embryogenesis sustain blood production throughout life and represent a landmark in regenerative medicine. In fact, deciphering the mechanisms of such signaling pathways, involved in the induction and migration of stem and progenitor cells, could bring us closer to understand the mechanism of HSC homeostasis and response to injury. Beside the known role of Notch signaling in the generation of hematopoietic stem cells during vertebrate development [28,37,38], the extrinsic signals that instruct cells to become blood-cell precursors and the interactions between HSCs and surrounding microenvironment are mostly unknown. Because of ease of manipulation and constant innovation in technologies, the use of the zebrafish in this field has facilitated the understanding of the early developmental processes leading to the programming of HSCs [39]. For instance, it has been reported that, upon *Notch* activation in endothelial progenitors that

a

Site in Zebrafish (Timepoint)	Lateral mesoderm/ICM (15 hpf/18 hpf)	Dorsal aorta/AGM (36 hpf)	CHT (3 dpf)	Kidney Marrow (4 dpf)
Site in Humans (Timepoint)	Yolk sac mesodermal cells (19 dpf)	Dorsal aorta/AGM (27 dpf)	Fetal Liver/ Placenta (35 dpf)	Spleen/Thymus/Bone Marrow

b

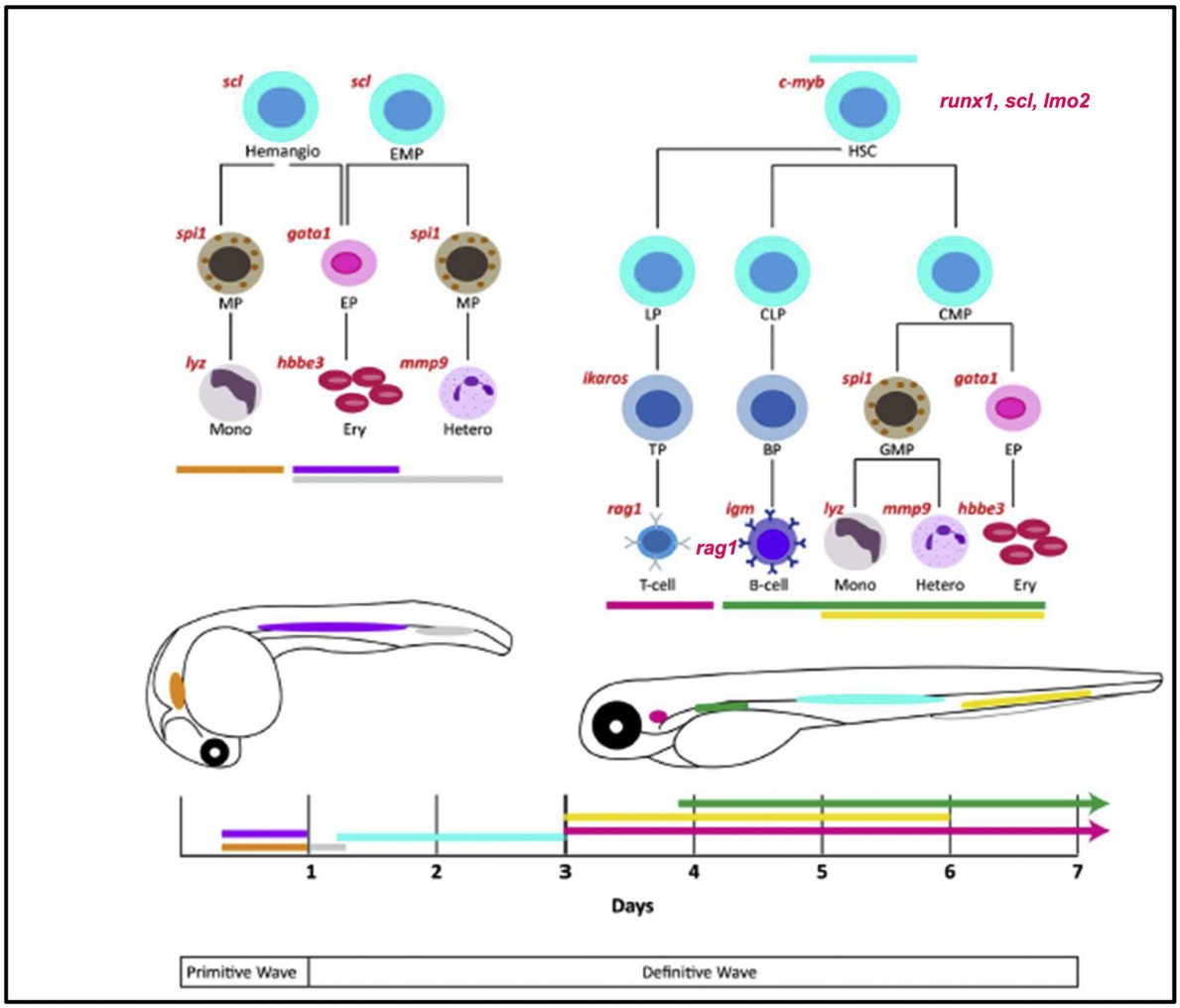

Fig. 1. a. Hematopoiesis in humans and zebrafish (reproduced from Martin et al. [122]). The stage of hematopoiesis is illustrated with mayor key regulators of the process. At the bottom: the sites and times of occurring events shown in human and zebrafish. Abbreviations: AGM, aorta gonad mesonephros; CHT, caudal hematopoietic tissue.

b. Zebrafish hematopoiesis and its key regulators (reproduced from reference: Rashigaemi P. et al. [12]). Schematic representation of hematopoiesis in zebrafish. The primitive wave commences in two locations, the anterior lateral mesoderm (ALM) (orange), which gives rise to primitive monocytes, and the intermediate cellular mass (ICM) (violet), which generates mostly primitive erythrocytes before 24 hpf. A transient 'intermediate' wave occurs in the posterior blood island (PBI) where both erythrocytes and heterophils are formed (grey). Definitive hematopoietic stem cells (HSCs) are initially formed by budding from the hemogenic endothelium on the ventral wall of dorsal aorta (blue). A subset of these HSCs migrate to the caudal hematopoietic tissue (CHT) (yellow) to produce several cell lineages, and also the thymus (purple), where T lymphocyte production occurs. Finally, HSCs seed the developing kidney (green), the final site of definitive hematopoiesis where erythroid, myeloid, and B lymphocyte production occurs. The lineage-specific transcription factors that serve to regulate this process are in red. Abbreviations: **BP**: B cell progenitor, **CLP**: common lymphoid progenitor, **CMP**: common myeloid progenitor, **EP**: erythroid progenitor, **Ery**: erythrocyte, **GMP**: granulocyte-monocyte progenitor, **Hemangio**: hemangioblast, **Hetero**: heterophil, **HSC**: hematopoietic stem cell, **Mono**: monocyte, **TP**: T cell progeny. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

generate HSCs, there is a direct interaction with somatic tissues [40]. The authors showed that migrating endothelial precursors that give rise to hematopoietic cells and muscle cells physically interacted through specific junction adhesion molecules (Jams). This indicated that the fate of HSCs precursor in zebrafish may be controlled even earlier than previously appreciated and that the involvement of these earlier factors may be essential in HSC expansion *in vitro*. In another report, Sawamiphak et al. [41] revised the role of pro-inflammatory *cytokine interferon-γ* (IFN-γ) as a positively modulator of the endothelial-to-HSC transition in zebrafish. The authors demonstrated that Notch signaling

and blood flow positively regulate the expression of IFN-γ and its receptor in the AGM, leading to consequent activation of *Stat3* which is required for HSC formation. The relevance of sterile inflammation in hematopoiesis was also confirmed by Li et al. who showed how the knockdown of the negative regulator of the interferon signaling, IFN regulatory factor 2 (IRF2), increased of HSCs in zebrafish, whereas mouse embryos lacking IFN-γ and IFN-α had significantly fewer HSCs in the AGM [42]. The modulation of inflammatory signaling and the close interaction with myeloid cells seem to play a key role in HSCs emergence in both mammals and zebrafish. CD68+ macrophages accumulate in the mesenchyme beneath the ventral part of the aortic endothelium in human embryos at 34 days of development and the number of myeloid cells increases concomitantly with HSPCs emergence [43]. Studies in zebrafish demonstrated that macrophages play an important role in definitive hematopoiesis by inducing metalloprotease-degradation of the extra cellular matrix (ECM) and by facilitating the migration of the emerging HSCs toward the other hematopoietic organs. Beside the inflammatory pathway, Carroll et al. [44] discovered that estrogens and estrogen-related compounds were capable of influencing the production of *Runx1*-positive HSPCs in the AGM by antagonizing VEGF signaling and prompted hemogenic endothelium identity. The authors hypothesized that a gradient of estrogens during vascular patterning enables the specification of hemogenic endothelium cells at the correct location and arterial/venous differentiation. On a more practical note, all these findings suggest the need to consider different signaling pathways and molecules to define new alternative strategies that could improve the current protocols used to generate *in vitro* HSCs from mouse or human embryonic stem cells.

2.2. Colonization of the hematopoietic organs

From 48 to 96 hpf, in zebrafish the newly emerged HSCs enter the circulation and migrate to colonize the Caudal Hematopoietic Tissue (CHT) in the posterior tail region during a transient wave of definitive hematopoiesis [45,46]. The zebrafish CHT is comparable to the mammalian placenta and fetal liver being the tissue where the nascent HSCs undergo proliferation and maturation before colonizing the kidney marrow (KM) which function as the adult hematopoietic niche similar to the mammalian bone marrow. In both fish and mammals, the adult niche is characterized by a complex system of vessels that interacts with HSCs and acts as interface between blood circulation and stromal cells. Despite the technical advances in imaging and the availability of several murine transgenic lines (reviewed in [47]) a dynamic live view of blood progenitors in the mammalian niche has not been achieved yet. In this context, the zebrafish has provided again an alternative tool to analyze endogenous blood progenitor cells and the interactions with the perivascular niche in a live embryo. In a recent publication Tamplin et al. [48] showed through live-imaging microscopy that newly emerged HSCs migrated to the CHT *via* circulation, extravasated and lodged at the external wall of the endothelium. Here the stem cells prompted a peculiar endothelial remodelling event that the authors refer to as “endothelial cuddling”: endothelial cells surround a single blood stem cell and form a pocket in association with the closer mesenchymal stromal cells. Most importantly, the authors also showed that this behavior is conserved throughout evolution, as live imaging of mouse fetal liver explants [49] revealed comparable interactions between hematopoietic cells and sinusoidal endothelial cells. After the intimate contact with the endothelial cells, the blood progenitors were seen to divide *via* asymmetric division, a process that seems dependent upon the mesenchymal stromal cells as observed in other stem niche system (reviewed in [50]). Within the zebrafish CHT, as in the mammalian fetal liver and spleen, the hematopoietic progenitors divide, proliferate and differentiate to sustain the developing embryos before the onset of the definitive hematopoiesis in the KM [13,45]. Around 5 dpf the HSPCs migrate directly from the AGM region to seed the thymus and the kidney marrow *via* a circulation-independent route along the pronephric tubules [19]. As in mammals, the SDF1-CXCR4 axis is responsible for HSPC homing to the kidney and high expression of zebrafish homologous *sdf-1* is reported in the renal tubule cells [51]. The KM represents the ultimate site of definitive hematopoiesis and produces mature myeloid and lymphoid cells by two weeks of development. In conclusion, the use of zebrafish in developmental hematopoiesis sheds light on HSC ontogeny: the conservation of genes and pathways regulating hematopoiesis between human and zebrafish makes this animal model an ideal system to investigate hematological malignancies to define critical regulators and underlying their genetic causes.

3. Modelling human malignant hematopoiesis

A primary objective in the oncology research is to identify which genetic lesions cause neoplasia and how mechanistically those mutations activate leukemogenesis. As described above, hematopoiesis in zebrafish is very similar to that in higher vertebrates and many mutated zebrafish orthologs of human disease genes have successfully phenocopied the human disease phenotypes. Abnormal differentiation and uncontrolled growth of blood cells lead to blood disorders or neoplasia and they are classified based on cell type involved in the disease. The majority of hematopoietic malignancies arise through chromosomal translocations and insertions that lead to abnormal activation of oncogenes in HSPCs [52]. Zebrafish is well suited for the generation of transgenic lines expressing human mutant genes, or carrying genomic alterations involved in leukemogenesis, and the easy way to generate transgenic animals contributed significantly to develop different malignant hematopoiesis models. Hundreds to thousands of embryos per day can be genetically manipulated to test the function of mutant genes that have been discovered in human leukemia studies. The conservation through evolution of the hematopoietic regulatory genes has led to the development of blood reporter lines that are often shared in the zebrafish research community. The combination of the emergence of new technological tools and the ease of zebrafish biology provide a unique opportunity to study *in vivo* human genes related to leukemogenesis. In the last decade, a significant number of human hematopoietic malignancies have been modelled in zebrafish (Table 1 lists a compilation of current zebrafish models of hematopoietic disorders). The models were developed by creating stable transgenic lines or through transient overexpression of human or zebrafish orthologs of oncogenes associated with hematopoietic malignancies. In addition, several research groups have used cell-type specific promoters to drive the expression of human oncogenes and fusion gene cDNAs in zebrafish. There are many types of malignancies, but in most cases they can be classified based on the rate of progression: acute or chronic conditions and the original cell type that is transformed: myeloid or lymphoid. Several categories of leukemias have been defined: acute myeloid leukemias (AML), chronic disorders such as myelodysplastic syndrome (MDS) and myeloproliferative neoplasm (MPN); chronic myeloid leukemia (CML); acute lymphoblastic leukemia (ALL); chronic lymphocytic leukemia (CLL).

We will highlight the recent advances in the understanding of blood diseases, such as T-cell Acute Lymphoblastic Leukemia and myeloproliferative disorders.

3.1. Insights into the T-cell acute lymphoblastic leukemia

The first zebrafish model of hematological malignancies was obtained by inducing the expression of the murine *c-Myc* oncogene, fused with Green Fluorescent Protein, and placed under the control of the zebrafish lymphoid tissue-specific promoter *recombination-activating gene-2 (rag2)* [53]. This model reproduced a T-cell Acute Lymphoblastic Leukemia (T-ALL) characterized by the proliferation of lymphoid compartment and blasts (immature T-cells) infiltration in different organs and tissues, suggesting complete penetrance of tumor induction upon successful integration of the *c-Myc* transgene. Moreover, zebrafish

Table 1
Most representative zebrafish models of human hematological disorders.

Disease	Genes	Promoter	Transgenic line	Pathological phenotype	Shared features with human pathology	References
Lymphoid neoplasm models						
T-ALL	c-Myc	*rag2*	*Tg(rag2:EGFP-Myc)* *Tg(rag2:LOXP-DsRed2-LOXP-EGFP-mMyc)*	Clonal expansion of T lymphocytes precursors	High number of circulating lymphoblasts	[53–55,63] [57,123]
			Tg(rag2:MYC-ERT2)	Extensive subcutaneous infiltration Transplantability of leukemic cells	*tal1/scl* and *lmo2* overexpression *Pten* inhibition Shared Copy Number Alterations	[58,60]
T-ALL	NOTCH1	*rag2*	*Tg(rag2:Hsa.NOTCH1-EGFP)*	Lymphoblasts infiltration in tissues and organs Transplantability of leukemic cells	Common gene signature (*hes1, il7r*)	[124,125]
pre B-ALL	TEL-AML1 (hs)	β-Actin	*Tg(actinβ:EGFP-Hsa.ETV6-RUNX1)*	Oligoclonal B-lineage ALL increased immature "blast like" lymphoid cells	Common gene signature (*rag2, ikaros, scl) deregulated expression of proapoptotic genes*	[126]
pre B-ALL		*rag2; lck*	Tg (*hMYC;GFP*)	Pre-B- ALL phenotype: histology and organ involvement		
Myeloid neoplasm models						
AML	AML-ETO (RUNX1-CBF2T1) (hs)	Cmv	*Tg(hsp70:AML1-ETO)*	Embryonic loss of circulating blood cells, disrupted definitive hematopoiesis	Impaired differentiation Comparable transcriptional changes	[30,77]
MPD	KRASG12D (hs)	β-Actin	*Tg(-actin-LoxP-EGFP-LoxP-kRASG12D;hsp70-Cre)*	Expansion of myeloid cells in the kidney	Compromised erythropoiesis	[127]
MPN	NUP98-HOXA9 (hs)	*spi1* (PU.1)	*Tg(spi1:loxP-EGFP-loxP:NUP98-HOXA9)*	Malignant myeloid infiltrates Kidney hypertrophy	Impaired differentiation of myeloid precursors bcl2 overexpression	[87]
AML	MYCN (mm)	HSE	*Tg(MYCN:HSE:EGFP)*	Increased of peripheral myeloid cells Myeloid blasts infiltrates Decreased TGFb signaling	Downregulated p27 and p21 in hematopoietic cells up-regulation of *scl*, *lmo2*, and *runx1*	[128]
MPD	HRASV12G (hs)	*fli1*	*Tg(fli1:Gal4FF;UAS:egfp-HRASV12)*	Arrest of differentiation Hyper proliferation of hematopoietic cells	Accumulation of blood cell in hematopoietic tissue	[80]
MS	KIT D816V (hs)	*Ubi*	*Tg(actb2:KIT-D816V:2AeGFP)*	Increase of PAS positive cells Kidney marrow expansion	Accumulation of mast cells in hematopoietic tissue	
Transient assays						
AML	MYST3/NCOA2 (hs)	*spi 1* (PU.1)	DNA injection	Myeloid blasts in the kidney	Leukemic infiltration of the bone marrow	[129]
AML/CML	*stat5.1 H298R/N714F*	Cmv	mRNA injection	Hyper proliferation of myeloid and erythroid cells	Constitutively active *Stat5*	[130]
PV	*jak2a V581F*		mRNA injection	increase in erythropoiesis	downregulation of *epo*	[131]
aCML	*tel-jak2a* (dr)	*spi1* (PU.1)	DNA injection	Accumulation of large white blood cells Anemia	Arrest of differentiation increase of blast cells	[132,133]
AML	NPMc(hs)	Cmv	mRNA injection in p53m/m line	Increased number of myeloid cells and progenitors	Abnormal cellular localization of the mutated protein expression of NPMc in multipotent or progenitor cell	[134]
AML	FLT3-ITD/FLT3-TKD (hs)	Cmv	mRNA and DNA injection	Expansion of myeloid populations	Resistance to AC220 treatment	[135]

Abbreviations: (mm) *Mus musculus*; (hs) *Homo sapiens*; (dr) *Danio rerio*; cmv = CitoMegaloVirus promoter; HSE = Heat Shock Element.

Myc-induced leukemia was characterized by the biallelic co-expression of the transcription factor *tal1/scl* and *lmo2* as in leukemic lymphoblasts of a subset of patients [54]. Unfortunately, the expression of murine *Myc* under the *rag-2* promoter was so disruptive that the majority of fishes died before reaching sexual maturity, thus requiring *in vitro* fertilization (IVF) to maintain the transgenic line. To avoid early lethality, the researchers switched to a *Cre-Lox* conditional system and a heat-shock promoter (*hsp70*) to temporally control *Cre* expression [55]. This improved model allowed the researchers to explore the molecular events governing the progression of disease from the localized T-lymphoblastic lymphoma (T-LBL) to disseminated T-ALL. All of the *Myc*-induced models of T-ALL in zebrafish begins as T-LBL with thymic hyperplasia and localized outgrowth before advancing to T-ALL and expanding into the circulation and other tissue. Feng et al. bred double transgenic (*rag2-LDL-mMyc*;*rag2-EGFP-bcl2*) heterozygotes with the *hsp70-Cre* inducible model and then monitored disease onset after inducing *Cre* expression in progeny. The triple transgenic fish (*Myc*:*Cre*:*bcl2*) accelerated T-LBL induction by suppressing *Myc*-induced apoptosis. When premalignant GFP-positive T cells were assayed by Annexin V staining, they found that *bcl-2* expression did indeed inhibit apoptosis in these T cells, providing a mechanism through which *bcl-2* collaborates with *Myc* in lymphomagenesis. The authors demonstrated that LBL cells with increased *bcl2* levels possess a distinct cellular phenotype, including impaired vascular invasion, metabolic stress and autophagy. It also promoted homotypic cell adhesion through *spi1* and *icam* that prevented intravasation into the vascular space and restricted the tumor to the thymus [56]. In order to modulate *c-Myc* expression the same research group generated a stable transgenic line in which the zebrafish *rag2* promoter drives the expression of human *c-MYC* gene fused to the ligand-binding domain of a modified estrogen receptor. The transgene was post-translationally induced by treatments with 4-hydroxytamoxifen (4-OHT) and developed T-ALL; upon cessation of treatment and loss of *c-Myc* expression, the tumor cells undergo apoptosis and the tumor rapidly regresses. These findings allowed the discovery of *pten* haplo-insufficiency and *akt* activation in zebrafish as co-factors in *Myc*-mediated oncogenesis by promotion of T-cell migration, suppression of autophagy and inhibition of apoptosis [57]. The importance of *Akt* signaling in zebrafish T-ALL progression is not surprising given the frequent disruption of the PTEN-PI3K-AKT pathway in human T-ALL.

The *Myc*-induced T-ALL model has given also a great contribution in understanding the molecular mechanisms involved in tumor initiation, leukemic dissemination and radiation sensitivity. For instance, the analysis of acquired copy number aberrations (CNA) through comparative genomic hybridization arrays of different zebrafish leukemic samples showed a remarkable overlap between T-ALL genes across species. Rudner et al. identified a group of recurring CNA [58] and a short list of genes shared by zebrafish and human T-ALLs, suggesting that the mechanisms governing oncogenesis and disease progression have persisted over evolution [59]. Recently, different zebrafish Myc-induced leukemia models have been created that express a variety of fluorescent proteins including AmCyan, GFP, zsYellow, dsREDexpress, and mCherry [60]. A combination of different transgenic lines in large-scale cell transplantation experiments allowed isolating single Leukemia Propagating Cells (LPCs) from heterogeneous primary T-ALL samples. Besides the identification of mutational events responsible for a specific cellular phenotype, transplantation experiments could answer fundamental questions regarding tumor cell heterogeneity and cancer progression. First, disease progression and relapse are strictly correlated to clonal evolution, which leads to reduced latency, increased frequency of LPCs, and chemotherapy resistance. Secondly, clonal evolution is related to the activation of the *AKT/mTORC1* pathway, and a subsequent involvement of *RAS* and *PI3K/AKT* pathways. Indeed, the activation of *AKT* signaling driven by *PTEN* inactivation occurs in > 18% of human T-ALL whereas the activation of *RAS* pathway promotes T-ALL in mouse models [61]. The zebrafish T-ALL model showed the importance of *RAS* and *PI3K/AKT* pathways in disease progression and chemotherapy resistance and provided a reason for utilizing *PI3K/AKT* inhibitors in preclinical testing. Finally, regarding resistance to therapy, Langenau et al. showed that the *bcl-2* zebrafish homologue was able to block irradiation- and dexamethasone-induced apoptosis in *c-Myc* T-ALL lymphocytes, thus indicating that the anti-apoptotic role of *bcl-2* in thymocytes is remarkably conserved between species [62,63]. A powerful complementary approach to the discovery of novel oncogenes and tumor suppressors has been the use of retroviral and transposase-based insertional mutagenesis screens [64]. This technique has been particularly effective in identifying novel oncogenes in T cell acute lymphoblastic leukemia (T-ALL). The first studies implicating Notch-1 as a major driver of T-ALL came from insertional mutagenesis screens using Moloney murine leukemia virus injected into neonatal mice. JDP2 is a transcription factor whose expression is recurrently up-regulated because of a common integration site in murine insertional mutagenesis models of T-ALL. This small bZIP protein contains an N-terminal domain that recruits cofactors, a basic domain that binds DNA, and a leucine zipper domain capable of heterodimerization with other bZIP proteins, such as c-JUN and DDIT3 [65]. The role of JDP2 in cancer is controversial because it can partially transform chicken embryonic fibroblasts and accelerate hepatocellular carcinoma in mice, yet it has a tumor-suppressor role in human prostate cancer. These features that may relate to its ability to both activate and repress AP-1 target sites, depending on the cellular context and bZIP binding partner [66]. Several authors showed that JDP2 is frequently aberrantly expressed in human T-ALL and established its oncogenic role by demonstrating that it can initiate T-ALL in transgenic zebrafish. JDP2 overexpression is associated with a poor outcome in patients and is required for survival of human T-ALL cells *in vitro*. Mechanistically, JDP2 transcriptional activity promotes cell survival through direct activation of the anti-apoptotic MCL1 protein. It was shown that *jdp2* overexpression leads to *mcl1* up-regulation and steroid resistance *in vivo*, providing a potential explanation for the poor survival of T-ALL patients whose leukemic blasts overexpress JDP2. Mansour et al. [67] support both viral and transposase-based insertional mutagenesis models that implicate JDP2 as a T-ALL oncogene. They proposed that the mechanism by which the JDP2 protein exerts its oncogenicity in this setting is through suppression of TP53, given that T-ALLs arising on a TP53 heterozygous background have a particularly high frequency of insertions at the Jdp2 promoter. Although this mechanism is intriguing, it is unlikely to be responsible for the ability of *jdp2* to transform thymocytes in zebrafish model, where loss of tp53 neither induces T-ALL nor collaborates with Myc in tumorigenesis [68]. Thus, *jdp2* represents one among few selected oncogenes (including *Myc*, *Notch*, and Myr-AKT) capable of initiating T-ALL in the zebrafish. The long disease latency and incomplete penetrance in the model suggests that as yet undiscovered secondary mutations are likely to be involved in transformation [69]. Although most studies support a repressive role for JDP2 through its ability to recruit histone deacetylases and its interaction with histones and the PRC2 complex, ChIP-seq data suggest that JDP2 can also have a role as a transcription activator in T-ALL cells. Given that bZIP proteins such as JDP2 are extremely challenging to target directly, our data suggest that inhibiting its downstream effector MCL1 would be a rationale alternative approach in patients with JDP2 overexpression, once specific and potent MCL1 inhibitors become available for clinical use.

Recently, Leong and collaborators identified *ARID5B* as a critical target directly regulated by TAL1 complex in T cells and assumed that *ARID5B* is a factor that stabilizes the gene expression program in malignant T cells [70]. They established a double transgenic lines (*rag2 ARID5Brag2*:*mCherry*) and analyzed the tumor cells that showed reduced expression of *lkc*, *tcra*, *cd4 or cd8*. The hypothesis is that ARID5B overexpression induces differentiation of thymocytes at an immature stage and promotes cell survival, which may predispose the cell to acquire additional genetic abnormalities that can induce leukemia. The

data suggest that ARID5B reinforces the oncogenic transcription program induced by oncogene *TAL1* and *MYC*.

In the past years several authors tried to understand the role of Hedgehog signaling in normal and malignant T-cell. It is known that Hedgehog signal transduction stimulates growth and proliferation in multiple cell types during embryonic development. Loss of-function of PTCH1, a negative regulator of Hedgehog signaling, results in aberrant Hedgehog pathway activation and drives oncogenic mutations in some tumor types. Burns et al. reported the higher frequency of Hedgehog pathway mutations in T-ALL cases that are resistant to induction chemotherapy and they showed that Hedgehog signaling represses apoptosis in *PTCH1* mutant T-ALL cells. Using the *ptch1* mutants the authors showed that *ptch1* mutations accelerate *notch1*-induced T-ALL, thus demonstrating that mutational activation of the Hedgehog pathway is a driver oncogenic lesion in the molecular pathogenesis of T-ALL [71]. Novel findings by Huiting et al. have highlighted the role of *udf1* in zebrafish. UFD1 is a critical regulator of the ER-stress response and a novel contributor to *MYC*-mediated leukemia aggressiveness, with implications for target therapy in T-ALL [72]. In zebrafish, *udf1* heterozygosity induced tumor cell-apoptosis and significantly impaired disease progression in the fish model of *Myc*-induced T-ALL with implication for target therapy in T-ALL and likely other *MYC*-driven cancer.

Finally, TOX has been identified as a collaborating oncogenic driver that synergizes with MYC and intracellular NOTCH1 to initiate early onset of T-ALL by expanding the number of transformed clones and increasing genomic instability [73].

In conclusion, past and recent discoveries in zebrafish improved our understanding of the molecular mechanisms that modulate leukemia aggressiveness and may provide new strategies for the development of target treatments.

3.2. Studying myeloid malignancies

Myeloproliferative disorders have been modelled in zebrafish through the expression of human oncogenes associated with myeloid disorders by transient over-expression assays or by the generation of stable transgenic lines (see Table 1). AML is the most common acute leukemia in adults, its incidence increasing with age [74]. AML results from the clonal expansion of undifferentiated myeloid precursors or blast cells, resulting in replacement of normal bone marrow cells and pancytopenia [75]. Transient over-expression assays represent one of the best-established systems to study gene function in zebrafish and in other animal models. Through this approach a variety of gene and fusion oncogenes associated with hematopoietic malignancies have been expressed in zebrafish embryos with the aim to perturb the blood compartment. Human genes and fusion oncogenes associated with myeloid disorder are often embryonic lethal and require tissue specific or inducible promoters to drive gene expression in the right compartment. The RUNX1 gene is commonly disrupted by chromosomal translocations in human myeloid malignancies. In a subset of AMLs, RUNX1 is often fused to the eight twenty-one gene (RUNX1-ETO) [76]. This is exemplified by the fusion oncogene *AML1-ETO* (*RUNX1-CBF2T1*) that causes disruption of normal hematopoiesis, aberrant circulation, internal hemorrhages and cellular dysplasia when expressed ubiquitously in zebrafish embryos. In 2008, Yeh et al. developed a stable zebrafish transgenic line characterized by the expression of the *AML1 (RUNX-1)-ETO* fusion oncogene under the control of the heat-shock responsive *hsp-70* promoter [77]. Heat treatment at 38 °C for 1 h between 14 and 19.5 hpf was sufficient to induce the *AML1-ETO* phenotypes and to avoid vascular defects. As in AML patients, the expression of *AML1-ETO* inhibited the normal erythroid development, promoting the expansion of myeloid progenitors. Tg(*hsp*:*AML1-ETO*) embryos exhibited an accumulation of immature non-circulating blast cells, downregulation of *tal1/scl* expression and a shift in myeloerythroid progenitor cell fate, suggesting that *tal1/scl* may contribute to *AML1(RUNX1)*-ETO-associated leukemia. Microarray analysis using blood cells from wild-type and *AML1-ETO* embryos showed down regulation of *scl*, *cmyb*, *runx1*, *nfe2* and *znfn1a1* genes and overexpression of *lmo1*, *hoxa9*, *hoxa10*, as observed in different human AML samples [77,78]. The common cytological and transcriptional features between humans and zebrafish *AML1(RUNX1)-ETO* models provided the opportunity to perform whole-organism chemical suppressor screens to identify compounds able to revert the phenotype that revealed surprising roles of different cellular pathways that will be discussed in the next paragraph.

Although the heat-shock inducible strategy has proven to be reliable to control gene expression at a chosen time, the *hsp70* promoter can be leaky and specific promoters are needed to activate transgenes in certain tissues or cellular subtypes. An alternative elegant method to spatially and temporally control transgene expression is represented by the GAL4-UAS binary system. Thanks to the development of highly efficient transgenic methods, a variety of Gal4 lines are now available. This system has proven to be highly versatile and successful in establishing disease models such as melanoma or pancreatic cancer [79]. We developed a zebrafish model of myeloproliferative disorder by inducing human oncogenic *HRAS* expression under control of the *fli1* promoter. In myeloid malignancies, *HRAS* mutations are more frequent than *KRAS* mutations, whereas *NRAS* mutations are rare [80,81]. However, even though *NRAS*, *KRAS*, and *HRAS* mutations have the potential to induce myeloid leukemia in mice, they differ in terms of potency and disease phenotype. Interestingly, *HRASV12* mutation induces an AML-like phenotype in a bone marrow transduction/transplantation murine model. The pathological phenotype is characterized by massive infiltration of leukemic cells, widespread pulmonary hemorrhages, anemia and short disease latency [82]. The *RAS*-expressing larvae showed a pathological phenotype characterized by the expansion of caudal hematopoietic tissue and severe vascular defects. The expansion of the caudal tissue is closely linked to the hyper-proliferation of the first circulating hematopoietic cells. The transgenic larvae also showed an increased expression of myeloid-erythroid genes (*gata1*, *c-mpl*, *pu.1*, *mpx*) and the arrest of erythroid and myeloid differentiation. The transient assay through the injection of mutant RAS showed the hyperproliferation of myeloid cells and arrest of differentiation in kidney marrow. Further studies demonstrated the down-regulation of the *Notch* pathway as a key mechanism involved in leukemogenesis and allowed the identification of new candidate genes associated with neoplastic transformation [80,81].

3.3. Forward genetic approaches: zebrafish mutants with high susceptibility to T-lymphoblastic lymphoma and T-cell acute lymphoblastic leukemia

Forward genetic screens in zebrafish are a powerful approach to the discovery of cancer-related genes that may prove relevant in human diseases [83]. Such screens rely upon randomly modifying the *Danio rerio* genome, which can be accomplished using ultra-violet light, chemical mutagenesis and insertional mutagenesis using transposons or retroviral vectors. Using the alkylating mutagen N-ethyl-N-nitrosourea (ENU)-mediated mutagenesis, a forward genetic screen was performed in which the native *p56 lck* promoter directs T-cell-specific expression of enhanced green fluorescent protein tg(*lck*:EGFP). Atypical GFP patterns could represent T-cell malignancies, benign lymphoproliferation, autoimmune T-cell infiltrations or non-T-cell GFP expression [84]. Three mutant lines, identified from this screen, hulk (*hlk*), *sherk* (*srk*) and *oscar the grouch* (*otg*) were able to develop transplantable T-ALL malignancies that phenotypically and histologically resemble oncogene-induced leukemia. Affected fishes of each mutant line showed lesions that were strikingly similar to T-ALL induced by oncogene overexpression; GFP + areas were solid, intensely bright masses resembling tumors, frequently arising from the thymic region. Neoplasms typically spread locally to gills and adjacent structures, generally in a cephalocaudal pattern through the entire fish over weeks-to months, until

generalized edema and circulatory collapse caused the death of the animal. In all three mutants, tumors firstly developed coincident with sexual maturity (3–4 months) and peaked at 5–8 months (young adult). These studies emphasize the feasibility of forward genetic screens with adult vertebrates to discover non-embryonic phenotypes relevant to human health, such as cancer predisposition.

To detect and monitor ALL-progression, Borga et al. [85] established a double transgenic fish by crossing tg(*rag2*:hMYC) with tg(*lkc*:*EGFP*) animals. They referred this double transgenic line as tg(*hMYC*;*GFP*). This zebrafish model of pre-B ALL, driven by human MYC, resembles the precursor-B cell acute lymphoblastic leukemia (pre-B ALL) that is the most common pediatric cancer. Different studies likewise demonstrate key roles for MYC in the molecular pathogenesis of B-lineage ALL. In terms of detecting pre-B ALL, dual-transgenic hMYC;GFP fish proved a powerful model because the *lck*:EGFP expression not only allowed the detection of pre-B ALL but pre-B and T-ALL could be distinguished on the basis of the differing GFP levels *in vivo*. As the only robust zebrafish pre-B ALL model in which T-ALL also develops, this model may reveal differences between *MYC*-driven pre-B *versus* T-ALL and be exploited to discover novel pre-B ALL therapies.

3.4. The gene editing technique for the creation of zebrafish models of hematopoietic disorders

Besides the traditional transgenic approaches, the novel gene editing tools have been used for the creation of animal models of hematopoietic disorders. The induction of targeted mutations in zebrafish has only become possible through the use of genome editing tools, which induce mutations through DNA double-strand breaks and error-prone repair by non-homologous end joining. A variety of alterations, including deletions and missense, nonsense, and frameshift mutations, inactivate the TET2 enzyme in different types of human myeloid malignancies, such as myelodysplastic syndromes (MDS) or myeloproliferative neoplasms (MPN), *de novo* acute myeloid leukemia (AML) and chronic myelomonocytic leukemia (CMML). *TET2* belongs to the *TET* (ten-eleven translocation) family of methylcytosine oxidases. *TET2* (like *TET1* and *TET3*) is a tumor suppressor gene and encodes a DNA methylcytosine oxidase that convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) to initiate the demethylation of DNA. In HPSCs, mutations in TET2 cause a premalignant state of clonal dominance that drives acquisition of additional mutations that culminate in MDS. MDS are characterized by an aberrant hematopoietic differentiation, leading to cytopenia, increased blasts, and often splenomegaly. In 2015, Gjini et al. [86] used the zinc finger nuclease technology to generate stable zebrafish lines with loss-of-function mutations in the *tet2* gene that truncate the encoded protein and disrupt the catalytic activity of the hydroxylase. Homozygous *tet2* mutant zebrafish were viable and fertile and had undetectable 5hmC content in blood cells of the KM but not in other tissues. This suggests that Tet2 is required for methylcytosine dioxygenation in hematopoietic cells, but Tet1 or Tet3 can supplant this function in other tissues, providing an explanation for the observation that *TET2* mutations are found only in hematologic malignancies. Moreover, the *tet2* mutant zebrafish developed myelodysplasia of the KM at 11 months of age, with a clonal marrow cell population harbouring decreased numbers of erythrocytes and increased numbers of myelomonocyte and progenitor cell populations. By 24 months of age, the mutant zebrafish had progressed to full-blown MDS, with a decrease in erythrocytes in the peripheral blood. Thus, zebrafish homozygous for the mutation provide a reliable model for studying myeloid malignancies with TET2 loss, with a view to identify specific small-molecule inhibitors and potential synthetic-lethal genetic interactions.

The *NUP98-HOXA9* oncogene was observed in MDS, CML and AML. Conditional expression of the human *NUP98-HOXA9* oncogene under the control of the zebrafish spi1 promoter was achieved by using a Cre/lox technique [87]. The zebrafish model resembles the human disease with perturbed hematopoiesis promoting myeloid fates with enhanced *spi1*+ precursor production at the expense of *gata1*+ erythroid precursors. A percentage (23%) of mutated fishes developed myeloproliferative neoplasms before 24 months of age. All the models presented here, provide solid collective evidence that zebrafish can serve as a model organism for the study of hematological malignancy and hematopoietic oncogenes. Although relevance to human disease phenotypes remains often undefined, the hematopoietic perturbations in these models indicate a conserved activity of the cellular pathways between zebrafish and humans. For these reasons, the zebrafish is a good model system to test specific interactions between collaborating oncogenes and to identify cellular pathways that may provide new therapeutic targets through large-scale chemical screenings.

4. The use of zebrafish in drug discovery

The final goal of building and studying zebrafish leukemia models is to develop effective and less toxic therapies. Because of the small size at embryonic and larval stages, zebrafish represents a powerful tool for identifying novel biologically active compounds and testing the effects of chemicals *in vivo* through large-scale chemical screens. The use of zebrafish allows carrying out high throughput screen (HTS) using 96-well plates and thousands of compounds can be tested in a single experiment. Due to the optical transparency, it is possible to analyze functional and morphological changes in the internal organs of living individuals. Given the similarities in function among vertebrate organs and tissues, the zebrafish provides a unique opportunity to identify molecules and pathways that are relevant to human disease or malignancies. In some instance, hits derived from these screens became drugs that have entered in clinical trials [88].

4.1. Identification of new therapeutic targets

Originally, chemical screens were designed using zebrafish mutants with the aim to identify pathways and potential therapies able to reverse a disease phenotype [89,90]. Functional changes can also be quantified using specific fluorescent transgenic lines and whole-mount RNA *in situ* hybridization may also be used to detect expression of cell differentiation markers. This was the case of the first “hematopoietic” screen in which 3 different libraries of 2357 FDA-approved compounds were tested to identify small molecules regulating HSCs formation in zebrafish embryos. By examining *cmyb* and *runx1* expression using RNA *in situ* hybridization, North et al. found 35 molecules able to increase the number of HSCs in the AGM region [91]. Among these substances, 10 of them affected the prostaglandin pathway whereas cyclooxygenase inhibitors decreased HSC numbers [92]. Since prostaglandin E2 (PGE2) is the main “promoter” of hematopoietic stem cell (HSC) growth, the authors treated zebrafish embryos with the PGE2 derivative dmPGE2 (16,16-dimethyl-PGE2) and observed increased of *runx1/cmyb* positive cells in the AGM regions. They also found that indomethacin inhibited HSC gene expression in 90% of embryos, pointing to a specific role of PGE2 in the formation of AGM HSCs. Using different zebrafish reporter and inducible lines, Goessling et al. [93] showed that PGE2 interacts with the *wnt* pathway at the level of *β*-catenin degradation to control HSC number and viability in the hematopoietic niche. Moreover, studies in zebrafish embryos identified the *cAMP/PKA* pathway as a PGE2 target responsible for *β*-catenin protein stability *in vivo*. This interaction occurs in the hematopoietic niche during embryogenesis and is conserved also in murine hematopoietic stem and progenitor cells. Treatments with dmPGE2 elevated *cAMP* activity in human Cord Blood cells *in vitro* and could enhance multi-lineage hematopoietic recovery in non-human primate (*rhesus macaque*) autologous transplantation experiments [94]. Based on the numerous data collected from different *in vitro* and *in vivo* assay, the FDA approved an Investigational New Drug application [95] for dmPGE2 in April 2009. An independent phase 1 clinical trial evaluating dmPGE2 in human Cord blood cells

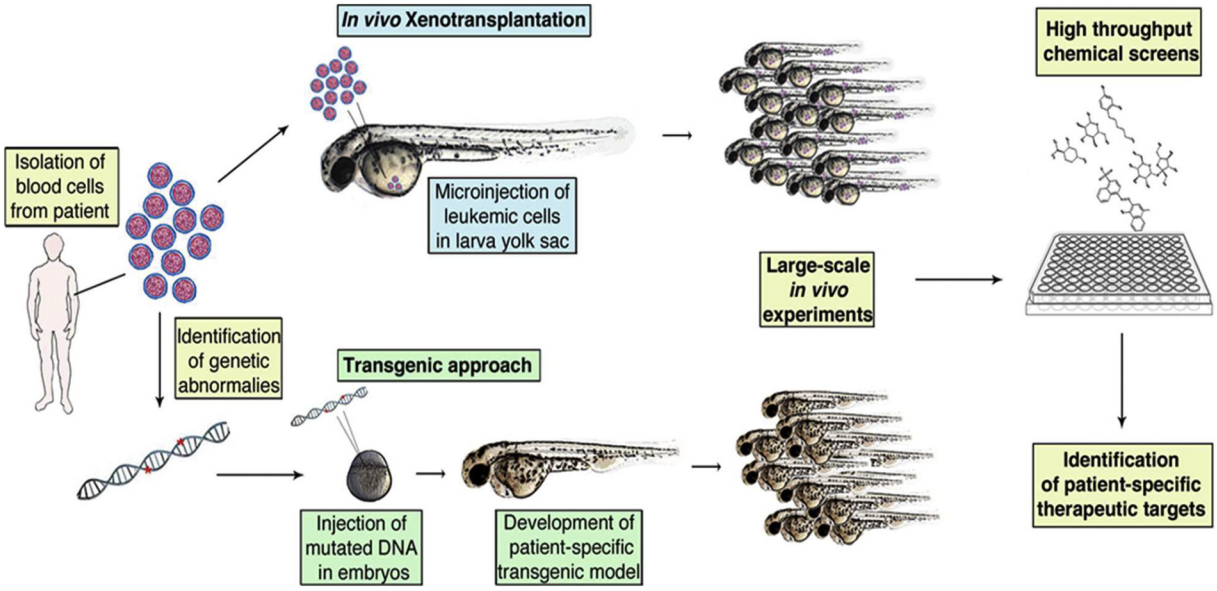

Fig. 2. Representation of different strategies to develop patient-specific therapies using the zebrafish. Leukemic blood cells can be isolated from patient and directly injected into the yolk sac of 2 dpf larvae (*In vivo* Xenotransplantation). On the other side, genetic abnormalities that drive hematopoietic malignancies (red dots) can be identified and used to develop zebrafish transgenic models of human disease (transgenic approach). Given the size and the ease of manipulation, both strategies are well suited for large-scale *in vivo* experiments. Finally, both xenotransplanted and transgenic larva can be used to test thousands of different chemical molecules with the aim to identify new therapeutic compounds.

transplantation has been completed, demonstrating clear safety with durable, multi-lineage engraftment of treated Umbilical CB units [96]. dmPGE2 represented the first compound discovered in zebrafish to be used in patients, thus confirming that zebrafish-based chemical screens can lead to therapeutic strategies in myeloid malignancies.

As described above, several studies have demonstrated that zebrafish leukemia/T-ALL models are highly translational with respect to human disease as they are strikingly similar to human leukemia at the genetic, molecular, and cellular level [12]. Gutierrez et al. developed a fluorescence-based screen using the Tg(*rag2*:MYC-ER) zebrafish line to identify small molecules with selective activity against MYC-overexpressing preleukemic thymocytes [68]. Three-dpf Tg(*rag2*:MYC-ER;-*rag2*:dsRed) embryos were treated with different drugs and analyzed for thymic fluorescence after four days of treatment at 7 dpf. Perphenazine (PPZ), an FDA-approved phenothiazine antipsychotic, was identified from this screen, and its selective toxicity against MYC-overexpressing thymocytes was confirmed in a complementary screen using human T-ALL cell lines (Fig. 2).

4.2. Large scale chemical screening to test therapeutic compounds

In the last years large scale chemicals screening were also performed using libraries of well-characterized compounds with the final aim to test therapeutic molecules in zebrafish models of hematopoietic malignancies. For instance, Yeh et al. [97] have conducted an *in vivo* chemical screen to identify compounds that could reverse the hematopoietic phenotypes of the human AML1-ETO fusion oncogene. As mentioned above, the expression of the human oncogene after heat-shock treatment induced the expansion of myeloid progenitors, inhibiting the normal erythropoietic development. The authors tested 2000 bioactive compounds for their capacity to restore the expression of the erythrocyte marker gene *gata1* in heat-treated *Tg(hsp:AML1-ETO)* embryos. They identified nimesulide, a selective cyclooxygenase-2 inhibitor, as a main compound that rescued *gata1* expression. Accordingly, *AML1-ETO* expression induced a β-catenin-dependent COX-2 up-regulation, leading to the observed hematopoietic differentiation defects. Studies on murine bone marrow cells showed that inhibition of COX-2 repressed β-catenin activation and also reduced serial replating capacity of AE positive cells. Nimesulide treatment suppressed the initiation and progression of tumor xenografts in mouse models, indicating a critical role for COX-2 activity in the tumorigenicity of human AML cell lines. Thus, the use of the zebrafish AML1-ETO model has allowed the identification of COX inhibitors readily available in clinic - as new therapeutic molecules that could be used as a part of AML treatment regimen to improve clinical outcomes in patients [98]. In conclusion, different screens in zebrafish identified cyclooxygenase-2 and β-catenin as key players involved in blood cell development in the hematopoietic niche.

Beside the discovery of new therapeutic compounds, the zebrafish proven to be a powerful tool also in finding new uses and different protocols for drugs that have already received FDA approval. Recently, Gutierrez et al. [99] screened libraries containing almost 5000 FDA-approved compounds and natural products using a zebrafish model of T-ALL. The authors combined the zebrafish screen with a complementary screen in human cell lines, and identified Perphenazine, a phenothiazine with a NOTCH-independent anti–T-ALL activity. Although phenothiazines have been used for over 50 years as antipsychotic drugs, they also showed an antiproliferative activity that had been attributed to a variety of cellular mechanisms [100]. Using different approaches the authors discovered the tumor suppressive serine/threonine phosphatase protein phosphatase 2A (PP2A) as a new target of phenothiazines. Further investigation clarified that the antipsychotic activity of phenothiazines was due to increased de-phosphorylation of different PP2A targets. The authors observed that phenothiazines were able to suppress lymphocytes proliferation in different zebrafish models of T-ALL. The data were then confirmed in human and murine cell lines showing an increased de-phosphorylation of PP2A targets, such as AKT, ERK, BAD and p70S6K. Perphenazine treatment induced also the inhibition of leukemic cell growth and decreased spleen weight in humanized mice transplanted with human primary T-ALL cells. Although phenothiazines have marked extra-pyramidal side effects limiting their use as chemotherapeutic drugs, these studies allowed the identification of PP2A as a new molecular target involved in T-ALL progression. In conclusion, drug discovery in zebrafish is characterized by a unique combination between the potential of high-throughput chemical screening, the possibility to develop complex disease models and the

Table 2
Main compounds identified by zebrafish chemical screens as possible drug candidates in hematology.

Library	Screen design	Purpose	Hit compound	Mechanism of action	Relevance to clinic	References
5000 synthetic compounds	GATA1-GFP embryos Analysis of fluorescence erythrocytes	Identify defects in primitive erythropoiesis	Phenylhydrazine	Directly denaturation of hemoglobin	Study erythroid maturation and morphogenesis in vertebrates	[136]
2357 biologically active compounds	Wild type embryos *In situ* hybridization for *runx.1/cmyb*	Identification of new pathways modulating HSCs formation	PGE2	Activation of cAMP/PKA pathway and induction of β-catenin protein stability	Enhancing multilineage hematopoietic recovery	[91,93,94,137]
2000 biologically active compounds	*Tg(hsp:AML1-ETO)* embryos *In situ* hybridization for *gata1*	Rescue of differentiation defects induced by AML1-ETO	COX-2 inhibitors	Inhibition of COX and repression of β-catenin pathway	Inhibiting AML cells tumorigenic potential	[77,97] [112]
26,400 pharmacophore compounds	*Tg(lck:EGFP)* embryos Analysis of thymic fluorescence	Elimination of developmentally arrested, immature T-ALL blasts	Lenaldekar	Inhibition of the PI3K/AKT/mTOR pathway and cell-cycle delay in G2/M	Highly specificity for lymphoblasts Low side effects	[92]
4880 FDA-approved drugs	*Tg(rag2:MYC-ER)* *(rag2:dsRed)* Analysis of thymus fluorescence	Identification of small molecules against MYC-overexpressing thymocytes	Phenothiazines	Stimulation of tumor suppressor PP2A activity in leukemia cells	Identification of new therapeutic target in T-ALL	[99]

ability to test side effects and toxicity in a whole organism [8]. Table 2 shows a list of the main compounds identified by the use of zebrafish models as possible drug candidates in hematologic malignancies.

5. Zebrafish xenotransplantation as a resource for drug optimization in leukemia and multiple myeloma

Xenotransplantation in immunocompromised mice of patient-derived tumor cells or tumor cell lines has represented a widely used *in vivo* approach to study the biology of malignant hematopoiesis and for the development of novel therapies against hematologic diseases [101,102]. However, several limitations, including timing of the experimental procedures and animal house facility costs, do not allow the use of these models for a rapid, cost-effective screening of potential drug candidates. Moreover, immunosuppression can modify the hematopoietic microenvironment, thus affecting tumor cell behavior and response to therapy [105]. When compared to other vertebrate model systems, tumor cell engrafting in zebrafish embryos may offer many advantages, including ease of experimentation, drug administration due to their permeability to small molecules, and amenability to *in vivo* manipulation. In addition, the optical transparency of the zebrafish embryo provides an *in vivo* system suitable for the analysis of the engrafted tumor at the single cell-level. Usually, human tumor cells are transplanted in the yolk sac of 2-day old embryos, a nutrient-rich environment for injected cells, and the maintenance of tumor-bearing embryos at 35 °C enables the growth of grafted cells without compromising zebrafish development [103]. In addition, the possibility exists to transplant tumor cells into different anatomical sites, including blastodisk, hindbrain ventricle, and bloodstream [104]. The combination between the limited number of cells required for the transplant (25–200 cells/embyo) and the possibility to perform high-throughput screening in 96-well plate enables the analysis of a high number of tumor grafts and compounds in a single experiment [105]. This strategy is now becoming popular also in hematology, due to the ease of collecting blood tumor cells from patients and the increasing number of molecules targeting specific pathways to be tested for personalized medicine [106–109].

Pruvot et al. [110] were the first authors to a establish zebrafish xenograft model to explore the efficacy and toxicity of different anti-leukemic drugs. To this aim, they performed a series of experiments by injecting different human leukemic cell lines (including K562, Jurkat and NB4 cells) and blast cells sorted from AML patients in the yolk sac of zebrafish embryos at 48 hpf. Notably, labeled fluorescent tumor cells remained in the circulation of zebrafish embryos for several days without affecting their development. Addition of imatinib mesylate to the fish water induced a decrease in the number of the target K562 cells whereas cyclophosphamide affected Jurkat cell burden, as confirmed by a decrease of expression of the human L32 and HPRT housekeeping genes in grafted embryos. To better quantify the reduction of the leukemic burden after drug treatment, Corkery et al. [111] developed an efficient procedure to measure the number of labeled tumor cells in treated embryos. Briefly, embryos were enzymatically dissociated to a single cell suspension after xenotransplantation and drug treatment. Then, the number of fluorescent cells in the suspension was counted at the microscope using a semi-automated macro after nuclear co-staining to confirm the enumeration of intact leukemic cells. Quantification of cancer cell proliferation and cell migration was further improved by Zhang et al. [112] using a phenotype-based chemical screening based on a high-content automated imaging system. The efficacy of selective clinical therapeutics (imatinib, dasatinib, parthenolide, TDZD-8, arsenic trioxide, niclosamide, salinomycin, and thioridazine) was assessed in xenotransplanted larvae by analyzing the size and fluorescence intensity of the main tumor at the injection site, before and after drug treatment. Using this model, the author showed that leukemia stem cell-like aldehyde dehydrogenase-positive (ALDH+) K562 cells exhibited higher tumorigenic potential and different response to drug

treatment when compared to ALDH-negative cells. These data, in keeping with previous reports showing that hematopoietic cell populations with high ALDH activity demonstrate distinct clonogenic function *in vitro* and exhibit leukemic stem cells properties [113], indicate that imaging-based xenotransplant screening in zebrafish may accelerate the phenotype-driven discovery of anti-leukemic stem cell agents.

Human leukemia cell xenotransplantation in zebrafish has been be used also for the identification of novel therapeutic agents and for the optimization of drug candidate hit compounds in AML. Treatment of human leukemia HL60 cells grafted in zebrafish embryos proved the anti-tumor activity of micheliolide, a natural product that acts as an irreversible pyruvate kinase M2 activator. In a recent work, Yang et al. [114] tested a series of pyrazolo-[3,4-d]pyrimidine derivatives in order to discover new compounds with antiangiogenic activity and endowed with high potency against FMS-like tyrosine kinase 3-driven human AML MV4-11 cells. Among the 33 compounds tested, one derivative was identified as multi-kinase inhibitor characterized by a potent antitumor and antiangiogenic activity in zebrafish-based assays and in preliminary murine tumor xenograft models.

Personalized cancer therapy approaches have been developed that employ the xenotransplantation of patient-derived tumors in mice to test specific targeted therapies [115]. However, costs, technical complexity, and the time it takes to complete these studies, which is not always compatible with patient-directed interventions, call for alternative models able to provide information suitable for individual patient treatment decisions. In a proof-of-concept report, Bentley and colleagues [95] described for the first time the development of a zebrafish xenotransplantation platform for personalized therapeutic interventions in T-ALL. To this aim, zebrafish embryos were injected with different primary patient-derived T-ALL bone marrow samples and specific drug responses was quantitatively determined. One leukemic sample showed a dramatic response to the treatment with the γ-secretase inhibitor compound E, pointing to the presence of a targetable mutation in the NOTCH pathway in this patient. Accordingly, DNA sequencing identified a rare NOTCH1 mutation in these cells, indicating that tumor cell xenotransplantation in zebrafish may help to define the molecular underpinnings of an individual patient, thus providing a novel time-efficient and cost-effective approach for tailoring leukemia therapy. This hypothesis is supported by recent findings showing that the zebrafish xenograft model permits the growth of CD138+ plasma cells derived from multiple myeloma (MM) patients when injected into the perivitelline space of Casper zebrafish embryos [116]. These cells can be followed *in vivo* for 4 days and their growth is inhibited by known anti-MM agents. Notably, both patient–derived bone marrow MM cells and MM cell lines migrate to the caudal hematopoietic niche following intracardiac injection in zebrafish embryos. Homed cells are characterized by a gene expression profile distinct from that displayed by cells arresting outside this niche, including a significant enrichment for genes involved in IL6 signaling, cell adhesion, and angiogenesis [117].

Together, such studies provide compelling evidence that human blood tumor cell grafting in zebrafish embryos may represent a useful tool for drug discovery and a predictive platform to define patient-specific therapies. In addition, they suggest that the injection of tumor cells in different anatomical districts of zebrafish embryos may represent a model suitable for the study of the biology of hematopoietic tumors, including metastatic dissemination and cell homing to the hematopoietic niche.

6. Conclusions

So far, zebrafish research has provided a great contribution in understanding the cellular mechanisms involved in developmental and pathological hematopoiesis. Discoveries such as HSCs arising from the hemogenic endothelium, the promoting properties of PGE2 and the cellular interaction in the perivascular niche highlighted the importance of the model in unravelling the complex processes required in hematopoietic development. Moreover, the insights gained from the zebrafish model of human blood disorders allowed identifying new molecular pathways involved in malignant transformation of hematopoietic cells. The study of adult hematopoiesis is also possible in zebrafish thanks to the standardization of an increasing number of techniques. Recently the development of artificial site-specific nucleases such as ZFNs and TALENs has enabled the investigation of loss-of-function phenotypes for genes of interest [118]. Programmable nucleases have also been recognized as promising tools to knock-in genetic elements and to allow in-frame insertions, the goal of which can be to precisely introduce a desired mutation, for example one identified in patients [119]. The most common strategy for in frame knock-in is to trigger homologous recombination (HR) by the presence of a donor DNA molecule with the desired sequence flanked by homologous arms to both sides of the break in the locus of interest. The CRISPR/Cas9 genome editing system has emerged as the most complex straightforward and simple way to induce targeted DNA double strand breaks in virtually every organism, and thus to activate cell repair mechanisms and enabling knock-in [120]. It has been demonstrated that the CRISPR/Cas9 system is highly efficient and specific in generating heritable mutations and knock-in alleles in zebrafish with negligible off-target effects. The use of this technique will continue the investigation and ongoing research within the zebrafish field continuous to discover more genes and pathway involved in leukemia. Ma et al. suggested the use of the CRISPR/Cas9 system for the generation of a zebrafish model of CML by inducing a double break in *bcr* and *abl1* gene [121], leading to the generation of the fusion gene that is responsible for the disease. Finally, large-scale chemical screens contributed to the discovery of novel compounds capable of interfering with a disease phenotype with potential therapeutic implications. Overall, zebrafish research on hematopoiesis demonstrated the power of this model in studying a variety of hematopoietic phenotypes using advanced assays and provided novel tools for personalized medicine. Ultimately, understanding the molecular events that cause neoplastic transformation and those underlying key stages of disease progression like dissemination and engraftment should improve our ability to treat these diseases at each and every point in their complex evolution.

6.1. Practice points

- Zebrafish provides new insights into normal and malignant hematopoiesis
- Transgenic leukemia models allowed the identification of new molecular targets involved in leukemogenesis
- High-throughput chemical screens enable to test newly synthesized therapeutic compounds in *in vivo* model.

6.2. Research agenda

- Development of cross reactive antibody for hematopoietic lineage analysis
- Optimization of techniques for primary cell engraftment
- Improving depth of understanding of hematopoietic niche physiology
- Modelling leukemia disorders by CRISPR/Cas9 technique.

Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
hpf	hour post-fertilization
dpf	day post-fertilization
ENU	N-ethyl-N-nitrosourea

GFP	green fluorescence protein
HSCs	hematopoietic stem cell
MDS	myelodysplastic syndrome
MDP	myeloproliferative disorder
PLM	posterior lateral mesoderm
KM	Kidney marrow

Transparency document

The Transparency document associated with this article can be found, in online version.

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Conflicts of interest

The authors declare no conflicts of interest.

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