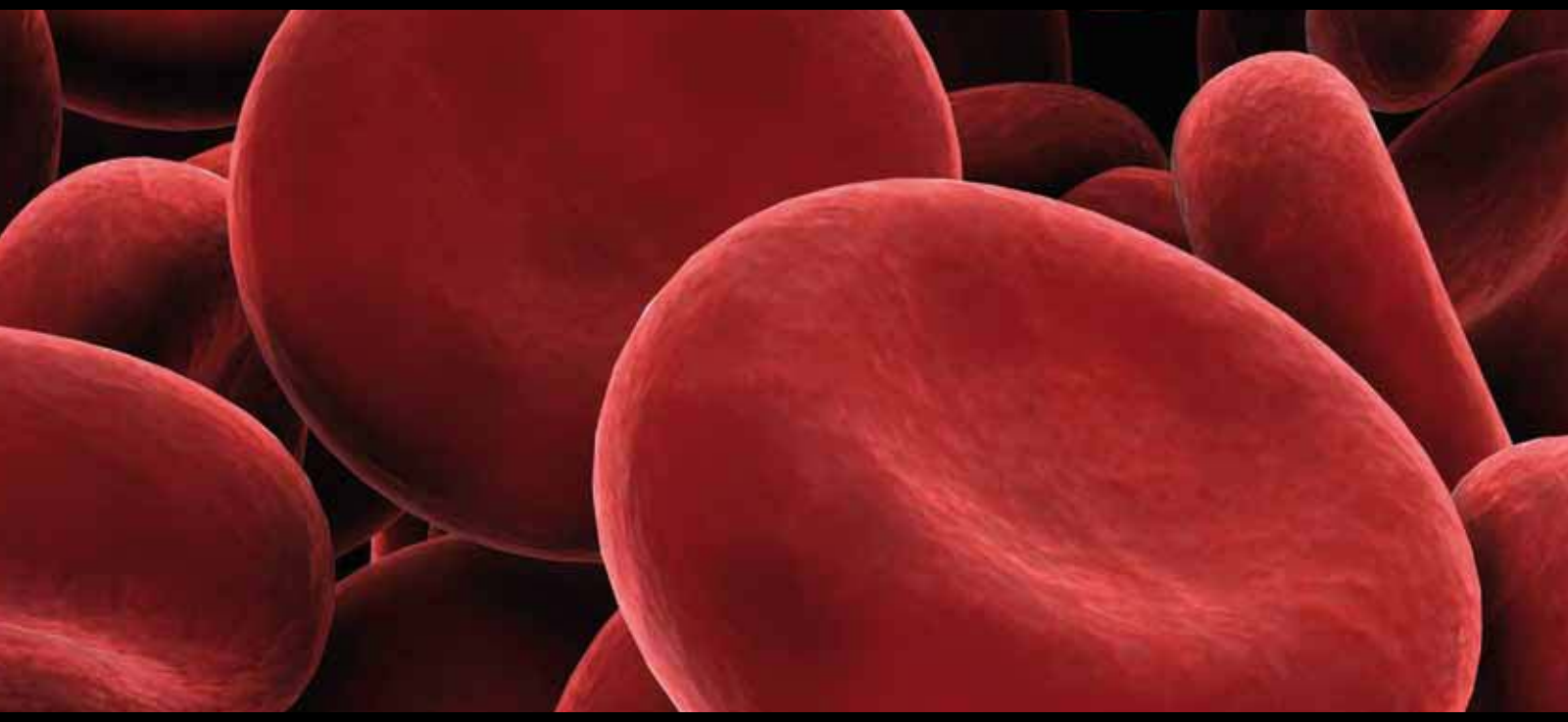
Advances in Hematology

The Zebrafish as a Tool to Study Hematopoiesis, Human Blood Diseases, and Immune Function

Guest Editors: Jason Berman, Elspeth Payne, and Christopher Hall



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Editorial Board

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Editorial

The Zebrafish as a Tool to Study Hematopoiesis, Human Blood Diseases, and Immune Function

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Over the last decade, the zebrafish has cemented itself as a unique model system for providing new insights into the regulatory factors required for vertebrate hematopoiesis. In particular, the ease of genetic manipulation together with the transparency of embryos facilitating high resolution imaging has enabled the fate mapping of a host of blood cell lineages. Most notably, this has included the detailed evaluation of the origin and emergence of hematopoietic stem cells. Genetic conservation between zebrafish and mammals and the construction of well-annotated detailed genomic databases have permitted the use of a number of forward and reverse genetic approaches to study a variety of benign and malignant human blood disorders in this organism. These studies have revealed new molecular players underlying human phenotypes as well as providing platforms both for genetic screens to identify novel interacting partners as well as chemical modifier screens to reveal compounds that may represent new therapeutic strategies. Conserved hematopoietic cell biology extends across the innate and adaptive immune systems, fueling a recent growth of research focused on exploiting the advantages of the zebrafish system to examine vertebrate host-pathogen interactions and the contributions of individual cell subtypes to innate and adaptive immune responses.

This special issue highlights some of the most recent and profound contributions provided by the zebrafish model system to understand hematopoiesis, hematopoietic malignancies, and the vertebrate immune system. As a volume, it highlights the tremendous accomplishments achieved in these diverse areas of hematology using the zebrafish model to date and sets the stage for continued advancement in all spheres of hematology, oncology, and immunology using this highly genetically conserved, easily manipulated, and clearly visualized remarkable organism.

In the paper entitled "*Novel insights into the genetic controls of primitive and definitive hematopoiesis from zebrafish models*," R. Sood and P. Liu review the anatomic sites and developmental waves of primitive and definitive hematopoiesis and emphasize the conservation of critical transcription factors and other genes that regulate these processes. They highlight some of their own recent work in this field in which they utilize a zebrafish *runx1* mutant to identify novel insights into the role of *runx1* in definitive hematopoiesis and identify a hypomorphic allele of *gata1* that provides the opportunity to more precisely attribute the contribution of this transcription factor to various stages of erythroid development.

In the report entitled "*Myelopoiesis and myeloid leukaemogenesis in the zebrafish,*" A. M. Forrester et al. highlight the conservation of myeloid gene regulation in zebrafish and describe the recent advances in this field. A number of studies and approaches are reviewed that have shed light on vertebrate neutrophil, monocyte, eosinophil, and mast cell development and provide a suite of *in vivo* tools to examine the perturbations associated with premalignant and malignant myeloid disease.

Myeloid cells are key players in the innate immune system, an area of increasing investigation using the zebrafish model. A. H. Meijer et al. provide an overview of this area in their paper entitled "*Pathogen recognition and activation*

of the innate immune response in zebrafish." Conservation of toll-like receptors, nucleotide-binding oligomerization domain (NOD)-like receptors, and other key members of the innate immune response are discussed and examined in the context of a number of bacterial pathogens. Novel immune-type receptors (NITRs) and functional orthologues in zebrafish of mammalian NK cell receptors are characterized in the paper by J. Yoder's group entitled "*Development and characterization of anti-Nitr9 antibodies*." C. Wittmann et al. outline the critical role of hydrogen peroxide as a mediator of inflammatory responses in the zebrafish in their paper entitled "*Hydrogen peroxide in inflammation:messenger, guide, and assassin*." Neutrophil behaviour in response to wounds is dissected in more detail in two papers entitled "*Neutrophil reverse migration becomes transparent with zebrafish*" by T. W Starnes and A. Huttenlocher's group and "*Drift-diffusion analysis of neutrophil migration during inflammation resolution in a zebrafish model*" by S. A. Renshaw et al. Huttenlocher's group takes advantage of a neutrophil-specific Lyn oxidation mutant to demonstrate that this Src family kinase is a critical link between hydrogen peroxide produced at the site of a wound and neutrophil chemoattraction. The imaging capabilities of the zebrafish and photoconversion techniques are subsequently exploited by both groups to reveal the process of neutrophil reverse migration for the first time. The purpose of this phenomenon and ultimate fate of these reversely travelling cells remain to be determined. However, the zebrafish is likely to serve a key role in further elucidating the factors underlying this process.

Platelet development and hemostasis in the zebrafish is next addressed in two papers entitled "*Zebrafish thrombocytes: functions and origins*" by P. Jagadeeswaran et al. and "*Characterization of zebrafish von Willebrand factor reveals conservation of domain structure, multimerization, and intracellular storage*" by J. A. Shavit et al. These reports set the stage for the zebrafish to provide new insights into platelet biology and model human bleeding disorders.

This special issue also includes a number of papers highlighting the utility of the zebrafish as a tool in dissecting oncogenic pathways in leukemia pathogenesis, identifying novel therapies, and improving stem cell transplantation. F. E. Moore and D. M. Langenau summarize the transgenic models of leukemia that have been developed by their laboratory and others in their paper entitled "*Through the looking glass: visualizing leukemia growth, migration, and engraftment using fluorescent transgenic zebrafish*." They present the opportunities provided by the transparency of zebrafish embryos and fluorescent labeling to study leukemia cell engraftment, homing, and frequency of leukemia propagating cells. These transgenic leukemia models provide a platform both for further genetic interrogation and high throughput drug screening. In their paper entitled "*Genomic amplification of an endogenous retrovirus in zebrafish T-cell malignancies*," J. K. Frazer et al. utilize array comparative genomic hybridization (aCGH) on the genomes of three zebrafish T-cell leukemia transgenic lines to identify a novel oncogenic retrovirus. Y. Zhang and J. R. Joanna Yeh describe the process for conducting chemical screens in zebrafish embryos in their paper entitled "*In vivo chemical screening for modulators of hematopoiesis and hematological disease*" and highlight the tremendous advantages and opportunities inherent in this approach. In particular, they describe the identification of the prostaglandin pathway and COX proteins in two separate screens: as positive regulators of hematopoietic stem cell development and as targets for inhibition in AML1-ETO driven myeloid disease. Finally, J. L. O. de Jong and L. I. O. Zon have contributed, "*Histocompatibility and hematopoietic transplantation in the zebrafish*," whereby they extend the zebrafish model to studies of matched allogeneic stem cell transplantation, with potential to quantify engraftment and model graft versus host disease.

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Research Article

Development and Characterization of Anti-Nitr9 Antibodies

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The novel immune-type receptors (NITRs), which have been described in numerous bony fish species, are encoded by multigene families of inhibitory and activating receptors and are predicted to be functional orthologs to the mammalian natural killer cell receptors (NKRs). Within the zebrafish NITR family, *nitr9* is the only gene predicted to encode an activating receptor. However, alternative RNA splicing generates three distinct *nitr9* transcripts, each of which encodes a different isoform. Although *nitr9* transcripts have been detected in zebrafish lymphocytes, the specific hematopoietic lineage(s) that expresses Nitr9 remains to be determined. In an effort to better understand the role of NITRs in zebrafish immunity, anti-Nitr9 monoclonal antibodies were generated and evaluated for the ability to recognize the three Nitr9 isoforms. The application of these antibodies to flow cytometry should prove to be useful for identifying the specific lymphocyte lineages that express Nitr9 and may permit the isolation of Nitr9-expressing cells that can be directly assessed for cytotoxic (e.g., NK) function.

1. Introduction

Mammalian natural killer (NK) cells are large, granular lymphocytes of the innate immune system that express several cell surface receptors to regulate cytotoxic function through a complex network of signaling pathways. NK cell receptors include both activating and inhibitory forms that are proficient in distinguishing neoplastic or virally infected cells from normal host cells [1, 2]. The regulation of NK cell cytotoxicity is dependent on the integration of signals from activating and inhibitory receptors [3]. Although it is postulated that NK cell receptors arose early in vertebrate phylogeny, functional data are based primarily on studies of mammalian NK cell receptors [4].

In order to appreciate the origins and evolution of NK cell receptors and their function, it is critical to define equivalent receptor forms in nonmammalian species. The bony fish represent one of the earliest vertebrate lineages with a functional innate and adaptive immune response that closely parallels that of humans and other mammals [5]. A large multigene family of recently and rapidly evolving inhibitory and activating novel immune-type receptors (NITRs) that share structural and functional characteristics with mammalian NK cell receptors has been identified in

multiple fish species [6, 7]. Complete analyses of the NITR gene clusters at the sequence level only have been performed with the zebrafish and medaka genomes [8–11]. Although transcripts of various catfish NITRs have been detected in NK-like, T, B, and macrophage cell lines [12], transcripts of all zebrafish NITRs are detectable in the lymphoid, but not the myeloid, lineage [13]. Of the 39 NITR genes that have been identified within the zebrafish genome, *nitr9* is the only NITR gene that is predicted to encode an activating receptor [10, 11, 14]. Three alternatively spliced transcripts of *nitr9* have been characterized: Nitr9-long (Nitr9L), Nitr9-short (Nitr9S), and Nitr9-supershort (Nitr9SS), which differ in their extracellular domains [13, 14]. Nitr9L is the most similar to other NITRs in that it possesses two extracellular Ig domains: one of the variable (V) type and one of the intermediate (I) type [6]. Nitr9S arises through cryptic splice donor and acceptor sites within the exon encoding the V domain. Nitr9SS lacks the entire V domain exon. The transmembrane domain of all Nitr9 isoforms possesses a positively charged residue: this feature permits Nitr9L to associate with and signal through the adaptor protein Dap12 [14]. Based on protein structures, Nitr9S and Nitr9SS also are expected to signal via Dap12; however, this has not been verified experimentally.

Although *nitr9* transcripts have been detected in zebrafish lymphocytes, the identification and recovery of Nitr9-expressing cells has not been possible. Herein we describe the derivation of two anti-Nitr9 monoclonal antibodies, demonstrate their utility to recognize recombinant Nitr9 by indirect immunofluorescence, flow cytometry, and Western blot analyses, and subsequently identify all three Nitr9 isoforms in zebrafish tissues by Western blot analyses. These antibodies should prove useful for: (1) evaluating Nitr9 protein levels within tissues by Western blot, (2) evaluating the distribution of Nitr9 expressing cells within tissues by indirect immunofluorescence, (3) defining the specific hematopoietic lineage(s) that express Nitr9 by flow cytometry, and (4) purifying Nitr9 expressing cells by fluorescence-activated cell sorting (FACS) for functional characterization.

2. Materials and Methods

2.1. Zebrafish. All experiments involving live zebrafish (*Danio rerio*) were performed in accordance with relevant institutional and national guidelines and regulations and were approved by the North Carolina State University Institutional Animal Care and Use Committee. Adult zebrafish (EkkWill Waterlife Resources, Ruskin, FL) were maintained and sacrificed as described [15].

2.2. Reverse Transcriptase-PCR. Total RNA from dissected zebrafish tissues (2 μg) was reverse transcribed (SuperScript III Reverse Transcriptase, Life Technologies, Carlsbad, CA), and cDNAs were subjected to thermal cycling with gene-specific primers (Table 1) and Titanium *Taq* DNA polymerase (Clontech, Mountain View, CA). The number of PCR cycles used for detecting nitr9 and β-actin (both annealing at 65∘C) was 40 and 25, respectively.

Lymphoid and myeloid cell populations were purified from the kidney of multiple zebrafish and pooled as described [16]. Total RNA from isolated cells (1 μg) was reverse transcribed (SuperScript III Reverse Transcriptase). cDNAs from tissues and isolated cells were subjected to quantitative PCR (Q-PCR) with TaqMan primers and probes (Life Technologies, Carlsbad, CA) (Table 1). Q-PCR was performed on a single-color MyiQ real-time PCR detection system (Bio-Rad, Hercules, CA) using the protocol: 50∘C for 2 min, 95∘C for 10 min, followed by 55 cycles at 95∘C for 15 s and at 60∘C for 1 min. The threshold cycle (C_T) value was calculated by the iQ5 Optical System Software (Bio-Rad). Relative transcript levels of nitr9 were normalized to β-actin and calculated using the $2^{-\Delta\Delta C_T}$ method [17]. All reactions were carried out as technical triplicates.

2.3. Antibody Development and Purification. The coding sequence of the Nitr9 I domain (nucleotides 298–623 of GenBank NM_001005576.1) was amplified by PCR (Table 1) and cloned into pETBlue-1 (EMD Millipore, Billerica, MA), and *E. coli* Tuner cells (EMD Millipore) were transformed employing a standard procedure. Cells were induced, and the Nitr9 I domain was recovered from inclusion bodies.

Swiss Webster mice were immunized with the Nitr9 I domain expressed in *E. coli* and splenocytes were fused with P3X63Ag8.653 cells (CRL-1580, ATCC, Manassas, VA). Approximately 3,000 individual hybridoma supernatants were screened by an enzyme-linked immunosorbent assay (ELISA) against the denatured recombinant Nitr9 I domain (Immunology Core Facility, University of North Carolina, Chapel Hill). The most strongly reactive ∼100 supernatants in turn were screened by parallel Western blot analyses and indirect immunofluorescence. Two single clones, 19.1.1 (herein referred to as anti-Nitr9^{19}) and 90.10.5 (herein referred to as anti-Nitr9^{90}), were selected for additional characterization based on their ability to recognize recombinant Nitr9. Antibody isotypes were determined (IsoStrips: Roche; Indianapolis, IN) to be IgG2b, κ light chain (90.10.5), and IgG2a, κ light chain (19.1.1). Antibodies were purified via protein A agarose columns (Upstate Cell Signaling Solutions; Lake Placid, NY).

2.4. Plasmids and Cell Culture. Nitr9 expression cassettes (without epitope tags) were constructed with *pcDNA3* (Life Technologies). Epitope (FLAG)-tagged Nitr9 (FLAG-Nitr9) expression cassettes were constructed with the *pLF* plasmid which incorporates an amino-terminal leader sequence and FLAG epitope [14]. The coding sequences of zebrafish *nitr9L, nitr9S,* and *nitr9SS* were amplified by PCR and cloned into *pcDNA3* or *pLF.* Nitr9 and FLAG-Nitr9 cassettes were then shuttled into *pIRES2-EGFP* (Clontech) generating: *pNitr9L/EGFP*, *pNitr9S/EGFP, pNitr9SS/EGFP, pFLAG-Nitr9L/EGFP*, *pFLAG-Nitr9S/EGFP,* and *pFLAG-Nitr9SS/EGFP* plasmids (Figure 1). Primer sequences that were used in cloning steps are included in Table 1. Plasmids were transfected into human HEK293T cells using Fugene 6 (Roche) according to the manufacturer's instructions and were harvested 48 hr after transfection.

Table 1: Oligonucleotide primer sequences.

Purpose	Primer sequence
Reverse transcriptase—PCR: *nitr9*	GGATTTTTGGACTTTTCTGTC TCCACATGCGGTAACTGTAC
Reverse transcriptase—PCR: *β-actin*	GGTATGGAATCTTGCGGTATCCAC ATGGGCCAGACTCATCGTACTCCT
TaqMan Q-PCR: *nitr9* (probe = CAAGGTTTGGAAAAGCAC)	GTCAAAGGGACAAGGCTGATAGTT GTTCAAAACAGTGCATGTAAGACTCA
TaqMan Q-PCR: *β-actin* (probe = CCCATGCCATCCTGC)	CCATCTATGAGGGTTACGCTCTTC AGGATCTTCATCAGGTAGTCTGTCA
Amplify *nitr9* I domain for bacterial expression construct	ATGGAAAAGCACACTGTAGTA[a] **TTA**TTTAGAGCCATTCCTGTCC[b]
Amplify *nitr9L* for FLAG-tagged expression cassette	CACCCAAATGCACCACCTGTGTTTGTTAAAC[c] gactgcggccgcTTACTGCTGGTTAGAAAC[d]
Amplify *nitr9S* for FLAG-tagged expression cassette	CACCCAAATGCACCACCTGTG[c] gactgcggccgcTTACTGCTGGTTAGAAAC[d]
Amplify *nitr9SS* for FLAG-tagged expression cassette	CATGATTTAATTCCATCCCA[c] gactgcggccgcTTACTGCTGGTTAGAAAC[d]
Amplify wild type *nitr9L*, *nitr9S* and *nitr9SS* for expression cassettes	gatcggatccgacATGATCAACTTTTGGATTT[e] gatcgaattcTTACTGCTGGTTAGAAACCGAG[f]

[a]An artificial start codon is underlined.
[b]An artificial stop codon is bold.
[c]These primers are designed for blunt PCR cloning into the *Eco*RV site of pLF.
[d]Overhang (5′) sequences are in lower case text and include a *Not* I site for cloning into pLF.
[e]Overhang (5′) sequences are in lower case text and include a *Bam*HI site for cloning into pcDNA3.
[f]Overhang (3′) sequences are in lower case text and include an *Eco*RI site for cloning into pcDNA3.

2.5. Indirect Immunofluorescence. HEK293T cells were transfected in four well chamber slides (Thermo Fisher Scientific, Rochester, NY). Transfected cells were washed in phosphate buffered saline (PBS), fixed with 3% paraformaldehyde for 20 min and treated with 50 mM NH_4Cl, PBS for 5 minutes. Cells were then permeabilized with 1.0% Triton-X-100 in PBS for 5 min, rinsed and blocked with 1% BSA in PBS for 5 min. Permeabilized cells were incubated with the anti-Nitr9^{19}, anti-Nitr9^{90}, or anti-FLAG antibody for 1 hr, rinsed with PBS, incubated with a phycoerythrin (PE) anti-mouse IgG antibody and DAPI (1 : 1000) for 1 hr, and washed with PBS. Chambers were removed from the slides, and coverslips were mounted using immunomount (Thermo Shandon, Pittsburgh, PA). Cells were photographed at 40x magnification using a Leica DM5000 microscope.

2.6. Flow Cytometry. Transfected HEK293T cells were incubated with the anti-Nitr9^{19}, anti-Nitr9^{90}, or anti-FLAG monoclonal antibody for 1 hr, washed in PBS, and incubated for 30 min with an allophycocyanin- (APC-) conjugated anti-mouse IgG secondary antibody. Labeled cells were washed and then fixed with 3% paraformaldehyde and subjected to flow cytometric analysis (BD FACSCalibur, BD Biosciences, San Jose, CA).

2.7. Western Analyses. Transfected HEK293T cells were washed with PBS and lysed with mammalian protein extraction reagent (M-PER, Pierce, Rockford, IL). Kidney, spleen, intestine, and gills were removed from sacrificed adult zebrafish and collected directly into tissue protein extraction reagent (T-PER, Pierce) supplemented with protease inhibitors (Pierce) and homogenized. Lysates were centrifuged to remove nuclei, and cell debris and protein concentrations were determined (BCA Protein Assay, Pierce). Proteins were resolved on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes for Western analyses. Membranes were washed in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated in blocking buffer (100 mM boric acid, 25 mM Na-Borate, 75 mM NaCl, 5% goat serum, and 5% dry milk powder) for 1 hr. Membranes were incubated overnight with primary antibodies in blocking buffer at 4°C. Primary antibodies include anti-Nitr9^{90}, anti-FLAG (M2) mouse monoclonal antibody (Sigma-Aldrich, St. Louis, MO), anti-GFP mouse monoclonal antibody (Roche), and anti-GAPDH rabbit polyclonal antibody (AnaSpec, Fremont, CA). Membranes were washed in TBST, followed by incubation with blocking buffer and either horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Roche) or horseradish

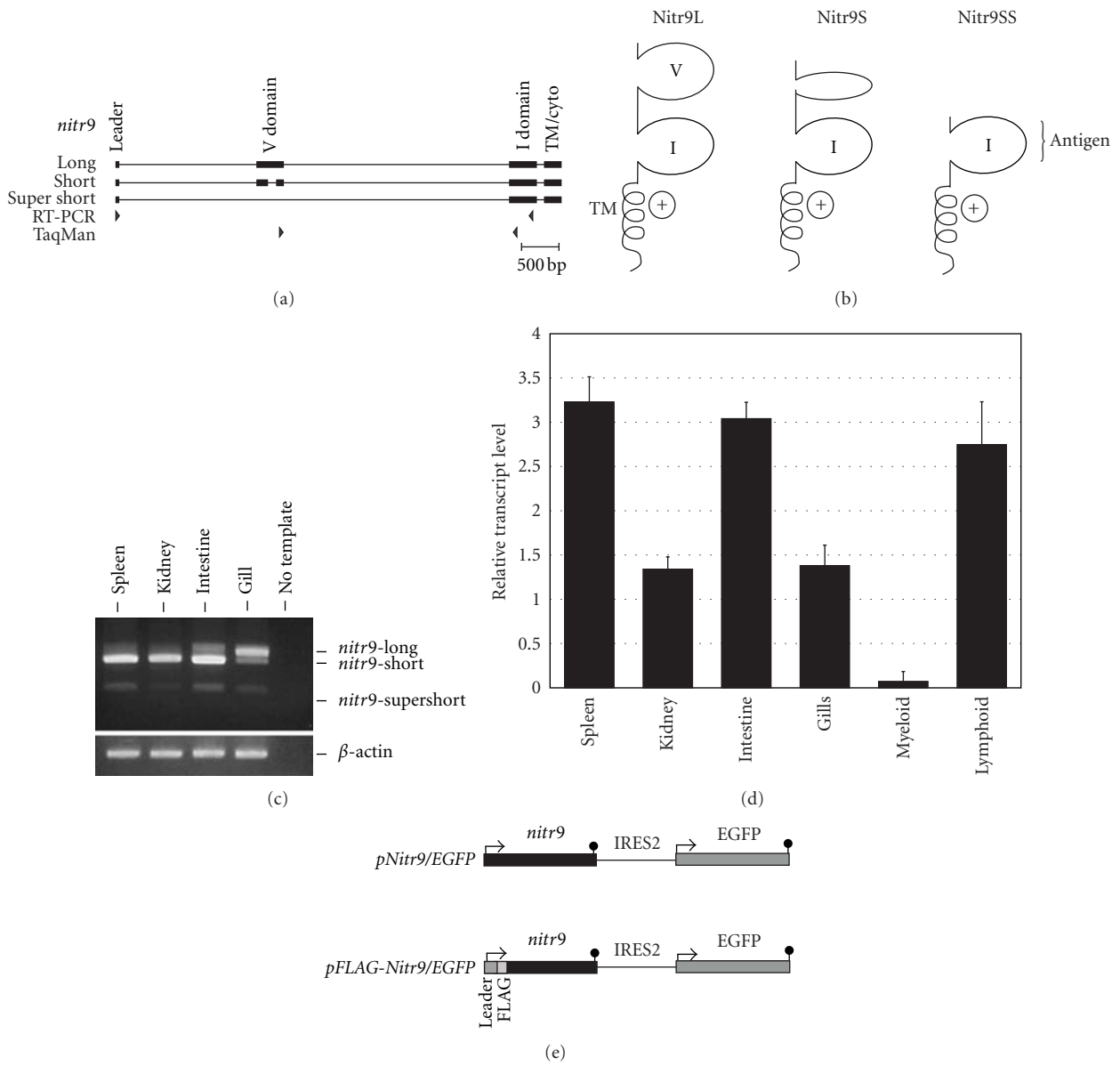

Figure 1: Transcriptional variation and expression of Nitr9. (a) Exon organization of the *nitr9* gene depicting the three transcript variants. Primer positions for PCR are indicated below. (b) The predicted Nitr9 protein isoforms encoded by the three *nitr9* transcripts. Transmembrane (TM) and immunoglobulin domains (of the variable (V) and intermediate (I) types) of Nitr9 are indicated. The I domain of Nitr9 was used as the antigen for antibody production. The positive charge within the TM domain of Nitr9 is represented by a plus sign. (c) RT-PCR with primers whose positions are depicted in (a) detects transcripts of all three *nitr9* isoforms. (d) Quantitative RT-PCR with *nitr9* primers (Table 1), whose positions are depicted in (a), and a TaqMan probe that spans an exon-exon boundary reveal relative levels of *nitr9L/S* transcripts in different tissues. (e) Schematic representation of the recombinant Nitr9 expression constructs used in this paper. Constructs include an internal ribosomal entry sequence (IRES2) permitting the expression of two proteins from a single transcript.

peroxidase-conjugated anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After washing with TBST, the Lumi-Light^{PLUS} western blotting substrate and detection system (Roche) was used to visualize reactivity.

2.8. Endoglycosidase Treatment. Cleared lysates (20 μg) from transfected cells were incubated with N-Glycosidase F (PNGase F, New England Biolabs, Ipswich, MA) for 1 hr at 37°C. Cleared lysates (25 μg) from zebrafish tissues were precipitated with OrgoSOL buffer (G-Biosciences, St. Louis, MO) and resuspended in PNGase buffer for treatment with PNGase F.

3. Results and Discussion

3.1. Nitr9 Isoforms. The genomic organization and predicted protein structures of Nitr9L, Nitr9S, and Nitr9SS are shown in Figures 1(a) and 1(b). All three isoforms are predicted to

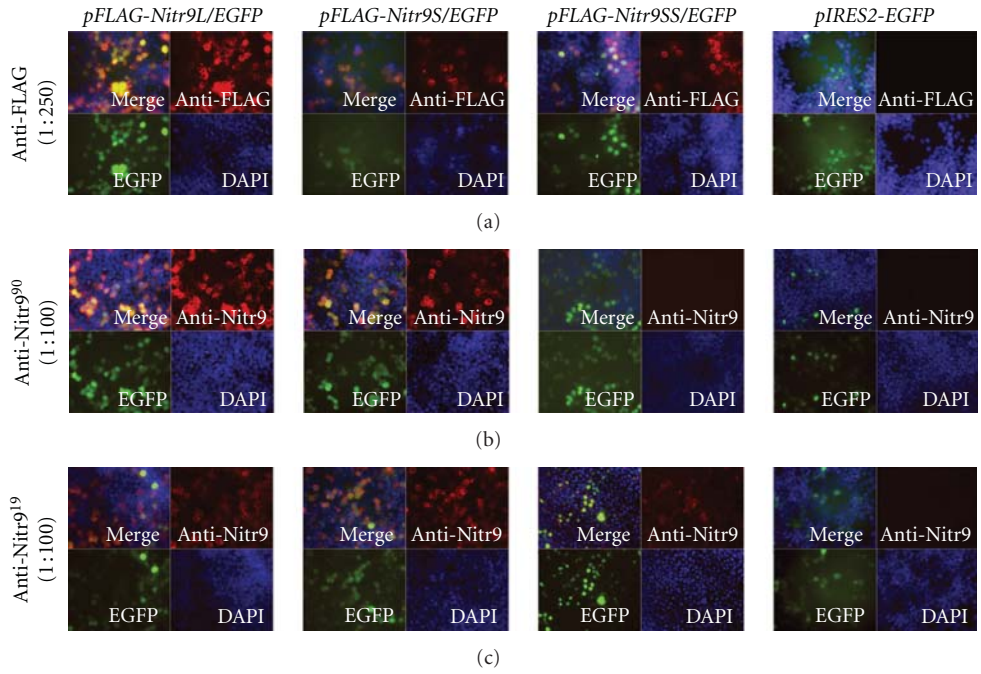

Figure 2: Detection of FLAG-tagged isoforms of Nitr9 from transfected cells by indirect immunofluorescence. HEK293T cells were transfected with plasmids encoding FLAG-tagged Nitr9 isoforms and EGFP as indicated on top of the panels. FLAG-tagged Nitr9 proteins were detected with (a) an anti-FLAG antibody, (b) anti-Nitr9^{90} or (c) anti-Nitr9^{19}, and a PE conjugated secondary antibody (red). Transfected cells can be identified by EGFP expression (green). DAPI labels the nuclei of all cells (blue). The pIRES2-EGFP parental plasmid was included as a negative control.

encode type I transmembrane cell surface receptors that possess a positively charged residue within the transmembrane domain. The *nitr9S* isoform is expressed at higher levels in the zebrafish spleen, kidney, and intestine than the *nitr9L* and *nitr9SS* isoforms, whereas, *nitr9L* transcripts are the most abundant isoform expressed in gills. Transcripts of *nitr9SS* are detected in all four tissues at reduced levels relative to the other isoforms (Figure 1(c)). Q-PCR (Table 1) was employed to determine the combined relative levels of *nitr9L* and *nitr9S* transcripts in these same tissues as well as in purified lymphoid and myeloid cells (the TaqMan primer/probe set employed in this paper does not detect *nitr9SS* transcripts). The combined relative expression level of *nitr9L* and *nitr9S* transcripts is consistently higher in intestine than in kidney and gill (Figure 1(d)). However, the relative expression level of *nitr9L* and *nitr9S* in spleen varied between biological replicates, ranging from levels matching those in intestine to lower levels as observed in kidney and gill. As reported previously, *nitr9* transcripts are present at much higher levels in zebrafish lymphocytes as compared to myeloid cells [13]. In order to generate monoclonal antibodies that could detect all three Nitr9 isoforms, mice were immunized with a bacterially expressed Nitr9 I domain (see Figure 1(b)), and hybridomas were screened for the production of antibodies that recognize recombinant Nitr9 by ELISA, Western blot and indirect immunofluorescence. Two clones, 19.1.1 (herein referred to as anti-Nitr9^{19}) and 90.10.5 (herein referred to as anti-Nitr9^{90}), were selected for further evaluation.

3.2. Detection of Nitr9 Isoforms in Transfected Cells by Indirect Immunofluorescence. In order to determine if anti-Nitr9^{19} and anti-Nitr9^{90} could detect all three isoforms of Nitr9 by indirect immunofluorescence, HEK293T cells were transfected with plasmids that coexpress EGFP and either a FLAG-tagged or endogenous isoform of Nitr9; in this way, any cell expressing Nitr9 also expresses EGFP (Figure 1(e)). To ensure that the recombinant Nitr9 proteins could be detected by immunofluorescence, an anti-FLAG antibody was used to detect all three FLAG-tagged isoforms of Nitr9 in transfected cells (Figure 2(a)). It was then shown that both anti-Nitr9^{19} and anti-Nitr9^{90} recognize FLAG-Nitr9L and FLAG-Nitr9S by immunofluorescence, but either fail to bind (anti-Nitr9^{90}) or bind less effectively (anti-Nitr9^{19}) to FLAG-Nitr9SS (Figures 2(b) and 2(c)). In contrast, both anti-Nitr9^{19} and anti-Nitr9^{90} effectively recognize all three isoforms of endogenous Nitr9 when expressed in transfected cells albeit with an apparent higher background labeling of cells with anti-Nitr9^{19} (Figure 3). It is possible that the FLAG-tag disrupts folding of Nitr9SS or sterically interferes with antibody recognition of the I domain of FLAG-Nitr9SS; this also was observed with Western analyses (discussed below).

3.3. Detection of Nitr9 Isoforms in Transfected Cells by Flow Cytometry. In order to determine if anti-Nitr9^{19} and anti-Nitr9^{90} could detect all three isoforms of Nitr9 by flow cytometry, HEK293T cells were transfected with plasmids encoding an endogenous or FLAG-tagged isoform of Nitr9

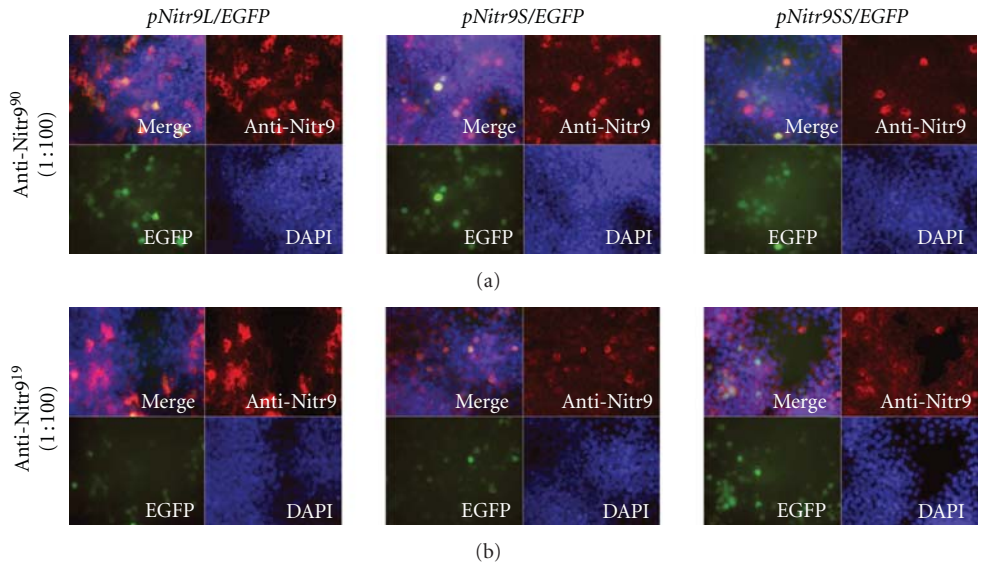

Figure 3: Detection of endogenous isoforms of Nitr9 from transfected cells by indirect immunofluorescence. HEK293T cells were transfected with plasmids encoding endogenous isoforms of Nitr9 and EGFP as indicated on top of the panels. Nitr9 proteins were detected with (a) anti-Nitr9^{90} or (b) anti-Nitr9^{19} and a PE conjugated secondary antibody (red). Transfected cells can be identified by EGFP expression (green). DAPI labels the nuclei of all cells (blue).

and EGFP (Figure 1(e)). Flow cytometry was performed using the anti-FLAG, anti-Nitr9^{19}, and anti-Nitr9^{90} antibodies to detect Nitr9 expressing cells. The percentage of double positive FLAG-Nitr9L expressing cells (i.e., EGFP$^+$ and Nitr9$^+$) was similar (55–63% of EGFP$^+$ cells) when the anti-FLAG or the anti-Nitr9 antibodies were employed (Figure 4(a)). Both anti-Nitr9 antibodies recognize transfected cells expressing the endogenous isoform of Nitr9L with a similar efficiency (61–73% of EGFP$^+$ cells) (Figure 4(b)).

The anti-FLAG monoclonal antibody failed to bind FLAG-Nitr9S and the anti-Nitr9^{19} antibody failed to detect the Nitr9S- or FLAG-Nitr9S-expressing cells (2–9% of EGFP$^+$ cells) (Figures 4(c) and 4(d)). Although the anti-Nitr9^{90} antibody detects FLAG-Nitr9S (31% of EGFP$^+$ cells), it does not recognize endogenous Nitr9S (~5% of EGFP$^+$ cells). Although the Nitr9S and FLAG-Nitr9S proteins are produced by transfected cells (see Figures 2 and 3 and Western blot results below) they may not be expressed effectively on the cell surface. To determine if cell surface expression of Nitr9S requires coexpression of the signaling adaptor protein Dap12, cells were cotransfected with plasmids encoding Nitr9S and zebrafish Dap12. No increase was observed in cell surface labeling by the anti-Nitr9 antibodies (data not shown).

The anti-FLAG and anti-Nitr9^{19} antibodies effectively bound FLAG-Nitr9SS (57% and 31% of EGFP$^+$ cells, resp.). The anti-Nitr9^{90} antibody failed to bind FLAG-Nitr9SS, possibly due to steric hindrance by the FLAG tag (Figure 4(e)) since both anti-Nitr9 antibodies were effective at recognizing Nitr9SS (65–75% of EGFP$^+$ cells; Figure 4(f)).

3.4. Anti-Nitr9^{90} Binds All Three Isoforms of Nitr9 in Western Analyses. In order to evaluate the ability of the anti-Nitr9^{90} antibody to detect the three isoforms of Nitr9 in Western analyses, HEK293T cells were transfected with plasmids encoding endogenous and FLAG-tagged isoforms of Nitr9 (Figure 1(e)). Cell lysates were subjected to Western blot analyses using the anti-Nitr9^{90} antibody. All three isoforms of the endogenous Nitr9 as well as the FLAG-tagged Nitr9L and Nitr9S proteins were detected. A binding pattern equivalent to that seen with the anti-FLAG monoclonal antibody positive control is apparent (Figure 5(a)). However, anti-Nitr9^{90} failed to bind the FLAG-tagged Nitr9SS. As mentioned above, this may be a result of the FLAG-tag blocking access to the specific epitope recognized by this antibody.

Two proteins bands were detected by anti-Nitr9^{90} in both endogenous and FLAG-tagged Nitr9L and Nitr9S transfections that were also bound by the anti-FLAG antibody. Both observed Nitr9L proteins migrated at a higher molecular weight than the predicted size of Nitr9L (34 kD), and one of the observed Nitr9S proteins was larger than the predicted size of Nitr9S (30 kD). The differences are consistent with differential glycosylation (see below). Based on the chemiluminescence exposure times required for detecting the different isoforms of Nitr9, anti-Nitr9^{90} appears to exhibit a higher affinity for Nitr9L as compared to Nitr9S and Nitr9SS. In parallel experiments, the anti-Nitr9^{19} antibody did not bind endogenous Nitr9S and Nitr9SS proteins (data not shown) and was not characterized further in the Western blot analyses.

3.5. Nitr9 Glycosylation in Transfected Cells. Nitr9L, Nitr9S and Nitr9SS possess three (NMSC, NDSR, and NGSK), two (NMSC and NGSK), and one (NGSK) candidate N-linked glycosylation sites, respectively. Treatment of lysates from Nitr9 transfected cells with endoglycosidase (PNGase F) results in the detection of only a single protein of

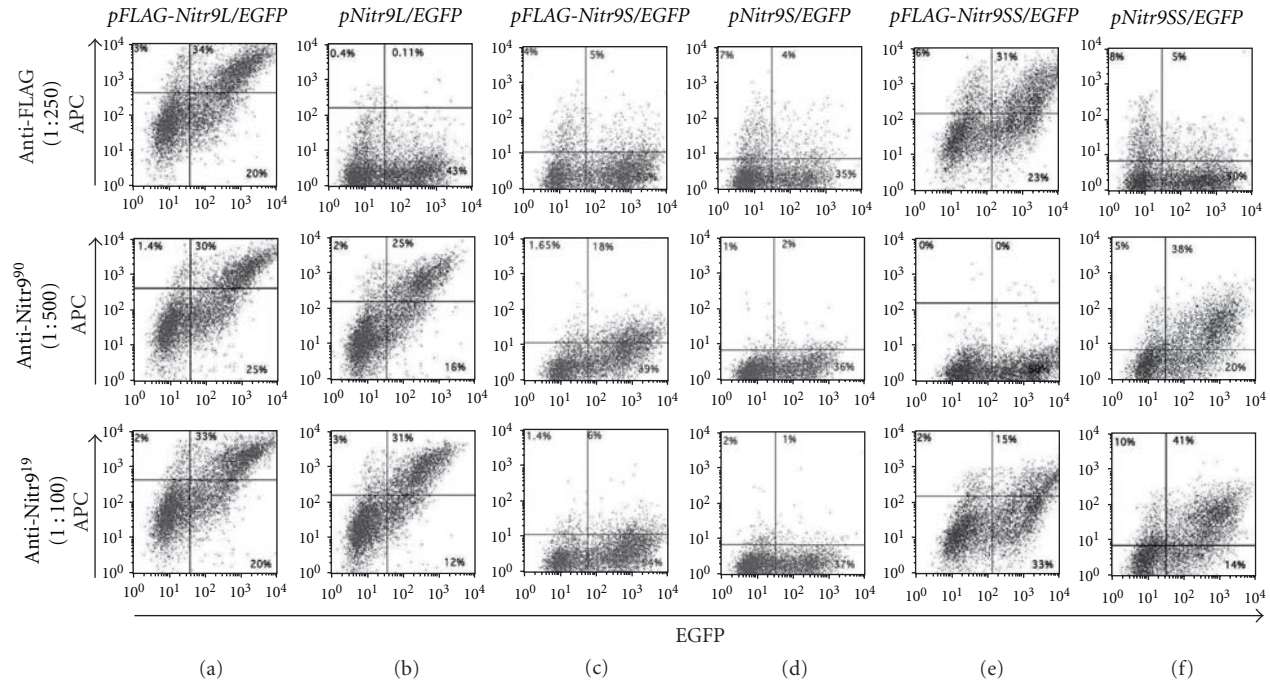

Figure 4: Detection of Nitr9 isoforms by flow cytometry. HEK293T cells were transfected with plasmids encoding FLAG-tagged (a, c, and e) or endogenous (b, d, and f) isoforms of Nitr9 and EGFP as indicated above the panels. Cells were labeled with an anti-FLAG antibody (top row), anti-Nitr9^{90} (middle row) or anti-Nitr9^{19} (bottom row), and an APC conjugated secondary antibody. Flow cytometric analyses were employed to detect EGFP positive (*X* axis) and APC positive (*Y* axis) cells. Isotype-matched antibodies were evaluated as controls for both anti-Nitr9 antibodies and displayed no labeling of transfected cells (data not shown).

the expected size for both Nitr9L and Nitr9S (Figure 5(b)). Both sets of results are consistent with *in vivo* glycosylation. The observed size of Nitr9SS in transfected cells does not appear to be altered by endoglycosidase treatment, with the limitations of detection, suggesting that it may not be glycosylated.

3.6. Nitr9 Proteins Are Differentially Expressed in Different Tissues of Zebrafish. In order to determine if the anti-Nitr9^{90} antibody can recognize endogenous Nitr9, lysates from adult zebrafish tissues were treated with endoglycosidase and subjected to Western blot analyses (Figure 5(c)). Nitr9L and Nitr9S were detected at varying levels in the spleen, kidney, gills, and intestine. Nitr9SS was detected only in the spleen, although faint bands also have been observed in intestine (data not shown). A nonspecific band of approximately 28 kD is detected in zebrafish tissues as well as in HEK293T cells when the anti-Nitr9^{90} antibody is used with large total protein loads (e.g., 25 μg lysate; Figure 5(c)).

4. Conclusions

Three different transcript variants from *nitr9,* the single putative activating NITR gene in zebrafish, and their corresponding protein isoforms have been identified and characterized. The utility of the anti-Nitr9^{19} and anti-Nitr9^{90} monoclonal antibodies for detecting recombinant Nitr9 was demonstrated by indirect immunofluorescence, flow cytometry, and Western blot analyses. The antibodies exhibit profound differences in recognizing the three different Nitr9 isoforms. When employed for indirect immunofluorescence, both anti-Nitr9 antibodies bound efficiently and specifically to cells-expressing all three Nitr9 isoforms. Both anti-Nitr9 antibodies are effective for detecting cell surface expression of Nitr9L and Nitr9SS by flow cytometry. The anti-Nitr9^{90} antibody recognized all three Nitr9 isoforms by Western blot analyses, although a higher affinity for Nitr9L is noted. When using anti-Nitr9^{90} in Western blot analyses with high levels of protein, a nonspecific band was identified. Although the identity of this protein remains unknown, it may represent a well-conserved member of the Ig superfamily.

Marked differences in the relative levels of Nitr9 transcripts and protein isoforms are apparent. Although the PCR analyses (Figure 1(c)) suggest that *nitr9S* may be the predominant mRNA isoform in spleen, kidney, and intestine, Western analyses demonstrate that Nitr9L is the predominant protein isoform expressed in kidney. This discrepancy may reflect differing transcript and protein stability in different tissues or the preferred reactivity of the antibody with Nitr9L (Figure 5(a)).

The monoclonal antibodies described here should be useful for further evaluation of Nitr9 protein levels in zebrafish tissues by Western blot analyses and identifying Nitr9 expressing cells in tissue sections by indirect immunofluorescence. Efforts are underway to purify Nitr9-expressing zebrafish cells employing FACS in order to characterize their morphology and cytotoxic properties.

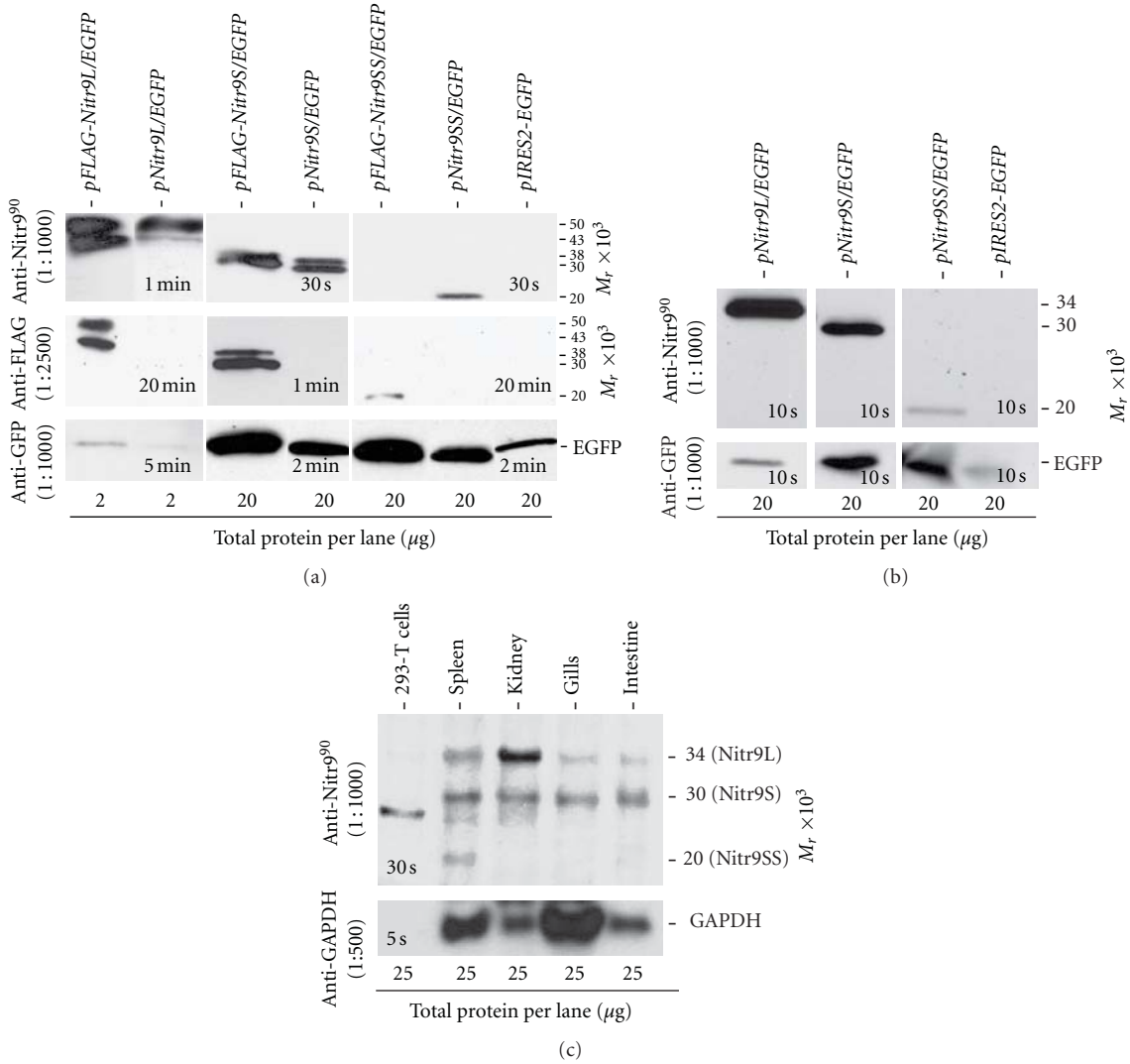

Figure 5: Detection of Nitr9 protein by Western analyses. (a) Western blot analyses of total protein lysates from HEK293T cells transiently transfected with plasmids expressing a Nitr9 isoform and EGFP. Plasmids encode either an endogenous isoform of Nitr9 or a FLAG-tagged Nitr9 as indicated above each lane. The primary antibodies utilized are shown on the left, and the molecular weights of identified bands are shown on the right. The anti-FLAG antibody serves as a positive control for Nitr9 detection, and the anti-GFP antibody indicates transfection efficiency of each plasmid. Note the total protein loaded (bottom) for the Nitr9L isoform is ten times less than that for Nitr9S and Nitr9SS plasmids. Exposure times for chemiluminescence detection are indicated in each panel. (b) Nitr9L and Nitr9S are glycosylated. Western blot analyses of endoglycosidase-treated total protein lysates from HEK293T cells that were transfected with plasmids encoding endogenous Nitr9 isoforms. The anti-Nitr9^{90} antibody recognizes all three Nitr9 isoforms at the predicted size (right). (c) Detection of Nitr9 protein from zebrafish tissues. Western blot analyses of 25 μg of endoglycosidase-treated total protein from zebrafish tissues and HEK293T cells. Note that a nonspecific band (~28 kD) is detected in HEK293T cells as well as in zebrafish kidney and spleen, with high protein loads. Bottom panel indicates loading control using an anti-GAPDH polyclonal antibody.

These antibodies may also prove to be useful for activating (crosslinking) or blocking Nitr9 function in both cell culture and *ex-vivo* functional assays as well as in dissecting isoform-specific functions of NITRs.

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Research Article

Characterization of Zebrafish von Willebrand Factor Reveals Conservation of Domain Structure, Multimerization, and Intracellular Storage

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von Willebrand disease (VWD) is the most common inherited human bleeding disorder and is caused by quantitative or qualitative defects in von Willebrand factor (VWF). VWF is a secreted glycoprotein that circulates as large multimers. While reduced VWF is associated with bleeding, elevations in overall level or multimer size are implicated in thrombosis. The zebrafish is a powerful genetic model in which the hemostatic system is well conserved with mammals. The ability of this organism to generate thousands of offspring and its optical transparency make it unique and complementary to mammalian models of hemostasis. Previously, partial clones of zebrafish *vwf* have been identified, and some functional conservation has been demonstrated. In this paper we clone the complete zebrafish *vwf* cDNA and show that there is conservation of domain structure. Recombinant zebrafish Vwf forms large multimers and pseudo-Weibel-Palade bodies (WPBs) in cell culture. Larval expression is in the pharyngeal arches, yolk sac, and intestinal epithelium. These results provide a foundation for continued study of zebrafish Vwf that may further our understanding of the mechanisms of VWD.

1. Introduction

Vertebrates possess a complex closed circulatory system that requires balanced coordination of various factors that serve to maintain blood flow as well as prevent exsanguination when the system is breached. This is known as hemostasis and consists of a complex array of cellular elements, as well as a network of proteins known as the coagulation cascade. The latter have been highly conserved at the genomic level throughout vertebrate evolution, including mammals, birds, reptiles, and fish [1–3].

One of the central components of coagulation is von Willebrand factor (VWF), deficiencies of which are the basis for the bleeding disorder von Willebrand disease (VWD). The mammalian *VWF* gene consists of 52 exons, and the largest, exon 28, contains several functional domains that are frequently mutated in VWD [4]. VWF is a 260 kDa (kilodalton) secreted glycoprotein that assembles into multimers of over 10,000 kDa [5]. At sites of injury, high molecular weight VWF multimers bind to receptors in the vascular subendothelium and tether platelets to form the primary hemostatic plug [6]. Much of our knowledge of VWF function is derived from characterization of mutations in humans and various mammalian model organisms, including mouse, dog, horse, cat, pig, and rabbit [7, 8]. However, relatively little information is available in other vertebrate models, such as the teleost *Danio rerio* (zebrafish). Teleost fish possess highly conserved orthologs of nearly all blood coagulation factors [1, 3] and have been shown to develop thrombosis in response to a laser-induced injury

[9]. Zebrafish embryonic development is external, rapid, and transparent, greatly simplifying phenotypic screening. Circulation begins approximately 24 hours after fertilization, and vascular development has been well characterized [10]. Forward genetic screens with chemical mutagenesis have been performed to study cardiogenesis, vasculogenesis, and angiogenesis [11–14].

Recently exon 28 was cloned from zebrafish, and conservation of several VWF functions was demonstrated [15], and *in silico* assembly of full length zebrafish *vwf* has also been described [16]. We now report cloning and characterization of the full length zebrafish *vwf* cDNA. Zebrafish Vwf demonstrates conservation of primary human VWF domain structure, as well as the ability to form pseudo-Weibel-Palade bodies (WPBs) and large multimers in cell culture. Unlike mammalian species, at the stages examined it does not appear to be expressed widely in developing endothelium.

2. Material and Methods

2.1. Cloning of Full Length Zebrafish vwf cDNA. Total mRNA was prepared from a single adult zebrafish using TRIzol (Invitrogen, Carlsbad, California). Total cDNA was synthesized with Superscript III reverse transcriptase after priming with random hexamers (Invitrogen). The *vwf* cDNA was assembled in four overlapping PCR amplified fragments using genomic sequence from Zv6 as a template to design primers (Table 1). Unique restriction sites contained in the overlapping sequences were used to sequentially assemble each of the four PCR products into the vector pCR4-TOPO (Invitrogen). The 5′ and 3′ UTRs (untranslated regions) were amplified by RACE (rapid amplification of cDNA ends, Ambion) with ends that overlapped unique restriction sites in the assembled clone. The external RACE primers were designed with restriction sites for the unique 5′ and 3′ vector sites, NotI and SpeI, respectively.

2.2. Multispecies Alignments. Non-zebrafish VWF amino acid sequences were downloaded from the UCSC Genome Browser, http://genome.ucsc.edu/ [17], aligned using ClustalW2, http://www.ebi.ac.uk/Tools/msa/clustalw2/ [18, 19], with output display through BOXSHADE 3.21, http://www.ch.embnet.org/software/BOX_form.html. Domain comparisons were performed using two sequence protein BLAST (Basic Local Alignment Search Tool) with the default settings through the National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/.

2.3. Plasmid Cloning of vwf cDNA. The assembled *vwf* cDNA was cloned into pcDNA3.1/V5-HISA (Invitrogen), which has an 8 amino acid linker, producing pzVwf/V5-HISA. Since expression of tagged human VWF has been shown to be more robust with an 18–20 amino acid linker (R. Montgomery and S. Haberichter, unpublished observations), we amplified this linker from a human VWF/Myc-HIS construct (pVWF/Myc-HIS, linker sequence in Table 1) and cloned it into the 3′ XhoI-PmeI sites (derived from pcDNA3.1/V5-HISA) of pzVwf/V5-HISA, producing pzVwf/Myc-HIS. The human pVWF-EGFP plasmid contains the same linker sequence. p*fli*-zVwf-EGFP was constructed by inserting the *vwf* cDNA into Tol2-*fli*-EGFP [20] in frame with *egfp*.

2.4. Immunofluorescence Analysis. HEK293T cells were maintained in DMEM (Sigma; St Louis, MO) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma). Cells were grown on cover slips until they reached 50–80% confluence, followed by transfection using FuGENE (Roche, Penzberg, Germany) as per manufacturer's instructions. The transfected cover slips were washed in phosphate buffered saline (PBS) and fixed in 10% formalin at room temperature for 25 minutes, followed by fixation/permeabilization at 4°C for 10 minutes in 100% ice cold methanol. After rehydration in PBS for 5 minutes, the cells were incubated with mouse anti-Myc (Santa Cruz Biotechnology, Santa Cruz, California) and rabbit anti-calnexin (Novus Biologicals, Littleton, Colorado) antibodies at dilutions of 1 : 100 and 1 : 500, respectively, at 4°C overnight. Cells were then washed three times in PBS (5 minutes each) and incubated with goat anti-mouse antibody coupled to Alexa Fluor 488 and goat anti-rabbit antibody coupled to Alexa Fluor 594, both at 1 : 200 dilutions for 60 minutes at room temperature. After an additional three washes in PBS, the cover slips were mounted with Prolong Antifade Gold (Invitrogen) and viewed on an inverted Olympus (Melville, New York) confocal microscope. Processing was completed with Olympus FluoView version 5.0.

2.5. Vwf Multimer Analysis. HEK293T (human embryonic kidney) cells were cultured and transfected with pzVwf/V5-HISA or an untagged full length human VWF expressing plasmid (pCineoVWF), as previously described [21]. Conditioned medium from pzVwf/V5-HISA transfected cells was purified over nickel columns per manufacturer's instructions (GE Healthcare Life Sciences, Uppsala, Sweden). Supernatants were analyzed by electrophoresis through a 0.8% (w/v) HGT(P) agarose (FMC Bioproducts, Rockland, Maine) stacking gel and a 1.5% (w/v) HGT(P) agarose running gel containing 0.1% sodium dodecyl sulfate for 16 hours at 40 volts using the Laemmli buffer system and western blotting as previously described [21]. Primary antibodies were a 1 : 5 mixture of anti-V5 antibody (Invitrogen) and anti-HIS antibody (AbD Serotec, Oxford, United Kingdom) or a mixture of monoclonal anti-human VWF antibodies Avw1, 5, and 15 [22].

2.6. Maintenance of Zebrafish Lines and Production of Embryos. Adult zebrafish (AB, TL, EK) were maintained and bred according to standard methods [23]. Embryos collected immediately after fertilization were maintained at 28.5°C and treated with 1-phenyl-2-thiourea (PTU) at 6–8 hpf (hours post fertilization) until fixation in order to prevent pigment formation. At specific time points, embryos were dechorionated or euthanized with tricaine, fixed using 4% paraformaldehyde in PBS overnight at 4°C, and stored at −20°C in methanol up to one month [24].

2.7. RNA Isolation and cDNA Synthesis for RT-PCR of Embryos and Larvae. Total RNA was extracted from at least three

Table 1: List of primers and sequences.

Reference number	Sequence	Description
92	AGTCGGCAGCACATACACAC	*vwf* cloning, assembly of fragment 1 (EcoRI-BstBI)
93	ATCCGGACAGGTCAGTTCAC	*vwf* cloning, assembly of fragment 1 (EcoRI-BstBI)
94	CCTGCAGCTTAAACCCAAAG	*vwf* cloning, assembly of fragment 2 (BstBI-AvaI)
95	AAAGCTTCATCGTCCAGCTC	*vwf* cloning, assembly of fragment 2 (BstBI-AvaI)
96	CTGTTGACGGCAAGTGCTAA	*vwf* cloning, assembly of fragment 3 (AvaI-SbfI)
97	TCTCCTGATGCTGGACACAC	*vwf* cloning, assembly of fragment 3 (AvaI-SbfI)
98	GACGGCAGTGTAACGACAGA	*vwf* cloning, assembly of fragment 4 (SbfI-ApaLI)
99	CCTGCAAGAGAGCCGATAAC	*vwf* cloning, assembly of fragment 4 (SbfI-ApaLI)
116	TGCGTGCTGAATCAAACTGT	*vwf* cloning, 3′ RACE (ApaLI-SpeI), SpeI vector derived
128	AGTCGCCAGGGAATTCATAA	*vwf* cloning, 5′ RACE (NotI-EcoRI), NotI vector derived
130	TTTGATTGACATTTTTATTTATTGTAGTTTA	*vwf* cloning, amplification of 3′ UTR
543	gatttaggtgacactatagCGACATGCAAGTGCAGAAGT	424 bp *vwf* riboprobe (exon 28) with SP6 promoter overhang
544	taatacgactcactatagggGCTGGGTTTTGCTGTAGGAG	424 bp *vwf* riboprobe (exon 28) with T7 promoter overhang
545	gatttaggtgacactatagGGAGTTATCGGCTCTCTTGC	441 bp *vwf* riboprobe (exons 47–52) with SP6 promoter overhang
546	taatacgactcactatagggACACAGACTTGCTGCCACAC	441 bp *vwf* riboprobe (exons 47–52) with T7 promoter overhang
	CTCGAGAGAATTCCACCACACTGGACTAGTGGATC CGAGCTCGGTACCAAGCTTGGGCCCGAACAAAAAC TCATCTCAGAAGAGGATCTGAATAGCGCCGTCGAC CATCATCATCATCATCATTGAGTTTAAAC	XhoI-PmeI human VWF linker sequence (includes Myc/HIS tag)

biological replicates per experimental condition using TRIzol RNA isolation reagent (Invitrogen) according to the manufacturer's instructions. RNA (1 μg) was reverse-transcribed using random hexamers and SuperScript III reverse transcriptase (Invitrogen). First-strand cDNA aliquots from each sample served as templates in PCR reactions using primers for *vwf*.

2.8. In Situ Hybridization. *In situ* hybridization was performed essentially as described with a few modifications [24]. Full length *vwf* cDNA in pCR4-TOPO was linearized with NotI and SpeI (antisense and sense transcripts, respectively) and transcribed *in vitro* using T3 and T7 (Ambion, Austin, Texas), respectively, with digoxigenin labeled nucleotides followed by alkaline hydrolysis per manufacturer's instructions (Roche). Alternatively, 424 and 441 bp fragments were amplified from full length cDNA using primers with SP6 or T7 overhangs (Table 1) and transcribed *in vitro* with digoxigenin labeled nucleotides. Prior to hybridization, riboprobes were heated to 80°C for 3–5 minutes and chilled immediately on ice for at least 5 minutes. Stained embryos were photographed using a Leica MXFLIII stereofluorescent microscope with an Olympus DP-70 digital camera. Embedding was in JB-4 resin as described [25], followed by sectioning at 4–6 μm using a Leica RM2265 ultramicrotome. Imaging of sections was with an Olympus BX-51 upright light microscope and Olympus DP-70 high-resolution digital camera.

3. Results

3.1. Cloning and Characterization of Zebrafish vwf cDNA. According to genomic sequence, the zebrafish *vwf* locus is located on chromosome 18 just downstream of *cd9*, maintaining conservation of synteny with mammalian species [15]. The full length *vwf* cDNA was assembled by RT-PCR of four overlapping fragments from total adult zebrafish cDNA, followed by RACE to complete the 5′ and 3′ UTRs (Section 2). The full length sequence is one amino acid shorter than human VWF with 46% overall identity (Table 2). Alignment of zebrafish Vwf to human VWF using BLAST shows clear delineation of all known domains (Figure 1(a)) with varying degrees of conservation (Table 2). The least conserved are the A1 and A2 domains, which encompass the entirety of exon 28 (Table 2). As in mammals, the *vwf* locus consists of 52 exons, but only spans 81 kb (kilobases), as opposed to 176 kb and 134 kb in the human and murine genomes, respectively. Previous iterations of the zebrafish genome (prior to Zv7) predicted that exon 28 was split into two exons [17]. Both sequence data from this report and previous work [15, 16] demonstrate clearly that the intervening sequence is actually exonic.

Other key features of human VWF are identifiable with varying degrees of conservation. The propeptide cleavage site, Arg-Ser, is highly conserved across all species examined except for medaka, and is a part of the extended RX(R/K)R motif (Figure 1(b)) [26]. The putative ADAMTS13 cleavage site in the A2 domain, Phe-Leu, is discernible due to mammalian orthology of flanking residues and is conserved across all fish species (Figure 1(c)). However, the presumed Phe-Leu cleavage site is only somewhat similar to the highly conserved mammalian and avian Tyr-Met cleavage sequence (Figure 1(c)). More importantly, there is conservation of a leucine orthologous to human Leu^{1603} (Figure 1(c)), which has been shown to be critical for ADAMTS13-mediated proteolysis of VWF [27].

Table 2: Human/zebrafish Vwf domain conservation.

Domain	Identities (%)	Positives (%)	Human length	zebrafish length
D1	51	70	352	351
D2	64	79	360	359
D′	51	71	90	88
D3	56	69	376	370
A1	36	57	220	233
A2	28	56	193	193
A3	42	58	202	207
D4	39	54	372	382
B1	58	73	35	34
B2	52	64	26	30
B3	67	83	25	25
C1	50	58	116	107
C2	48	63	119	117
CK	42	64	90	91
Total	46	62	2813	2812

Alignment of human and zebrafish amino acid sequences using BLAST (http://blast.ncbi.nlm.nih.gov/). Percentage identity represents exact amino acid matches, while positives indicate conserved substitutions. Domain length is in amino acids.

A number of disulfide bonds are required for dimerization and multimerization of human VWF [6]. These are mediated by cysteines at positions 1099, 1142, and several in the C-terminal cystine knot (CK), at 2771, 2773, and 2811, all of which are conserved in zebrafish Vwf. In fact, nearly all cysteine residues are completely conserved, with the exception of Cys^{1669} and Cys^{1670}, located at the C-terminus of the A2 domain [16] and absent in all fish species examined. There was one cysteine present solely in medaka, four residues N-terminal to the propeptide cleavage site, but its absence in other species makes its significance unclear. There is a cysteine in zebrafish Vwf at position 4, which is not conserved in mammalian species, although genomic sequence information for the other teleost species is absent in this region.

3.2. Expression of Vwf in Mammalian Cell Culture. In order to determine if zebrafish Vwf can multimerize, we expressed V5/HIS tagged *vwf* cDNA in HEK293T cells. A ladder of high molecular weight multimers was detected using a mixture of anti-V5 and anti-HIS antibodies (Figure 2). This included high molecular weight multimers similar in size to human VWF (Figure 2).

The zebrafish *vwf* cDNA was cloned into an expression vector in frame with a Myc-HIS tag using the same linker as a human *VWF* cDNA construct. The latter, when transfected into HEK293T cells, is known to form pseudo-WPBs

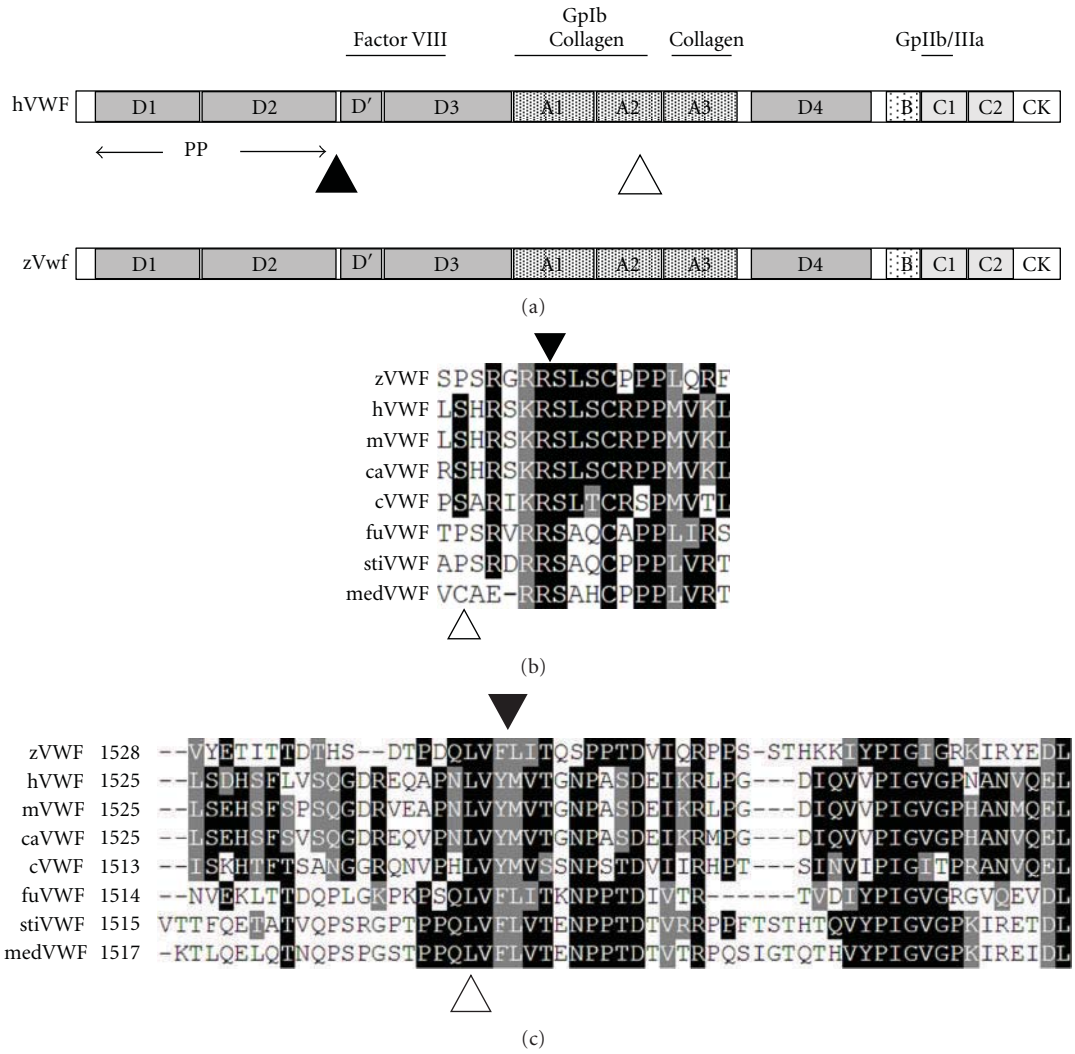

Figure 1: Domain organization of human VWF and multispecies alignment of the VWF propeptide and ADAMTS13 cleavage sites and flanking sequences. Sequence alignment was performed using ClustalW2 followed by output using BOXSHADE (Section 2). (a) Domain organization of human VWF. Upper notations indicate known protein-protein interaction domains (Gp: glycoprotein). The solid triangle indicates the propeptide (PP) cleavage site, and the open triangle indicates the ADAMTS13 cleavage site. "B" indicates domains B1–B3. (b) Alignment of sequences surrounding the Arg-Ser (RS, indicated by the solid triangle) human propeptide cleavage site demonstrates a high degree of conservation. Note the extended RX(R/K)R motif present in all species except for medaka. The open triangle indicates the presence of an unconserved cysteine in medaka Vwf. (c) Alignment at the human ADAMTS13 cleavage site (YM, indicated by the solid triangle) and flanking sequences demonstrates conservation of the Tyr-Met residues in mammalian and avian species, but a Phe-Leu putative site in teleost fish. The invariant Leu (human residue 1603) is indicated by a white triangle. z: zebrafish; h: human; m: mouse; ca: canine; c: chicken; fu: fugu; st: stickleback; med: medaka.

[28, 29]. These structures are produced after VWF has been processed into high molecular weight multimers in the Golgi apparatus. Using an anti-Myc antibody we were able to identify elongated structures consistent with pseudo-WPBs in zebrafish *vwf* transfected cells (Figures 3(d) and 3(g)). These were morphologically similar to those found in human *VWF* transfected cells (Figure 3(a)). Staining with an anti-calnexin antibody to localize endoplasmic reticulum (ER, Figures 3(b), 3(e), and 3(h)) demonstrated no overlap between the structures (Figures 3(c), 3(f), and 3(i)), as expected for WPBs and pseudo-WPBs [28, 29].

3.3. Developmental Patterns of vwf Expression. RT-PCR of whole embryos up to 96 hpf demonstrated increasing levels of *vwf* expression, with the most intense expression at 96 hpf (Figure 4(f)). Whole-mount *in situ* hybridization was used to localize expression from the middle of gastrulation (8 hpf) to 120 hpf. Expression of *vwf* is weakly detectable throughout the embryo at 8 hpf (Figure 4(a)). Stronger expression is observed in 12-hour embryos as a more diffuse pattern throughout the embryo (Figure 4(b)). At 48 hours there is diffuse expression cranially, which extends caudally (Figure 4(c)). At 96–120 hours, strong expression is present

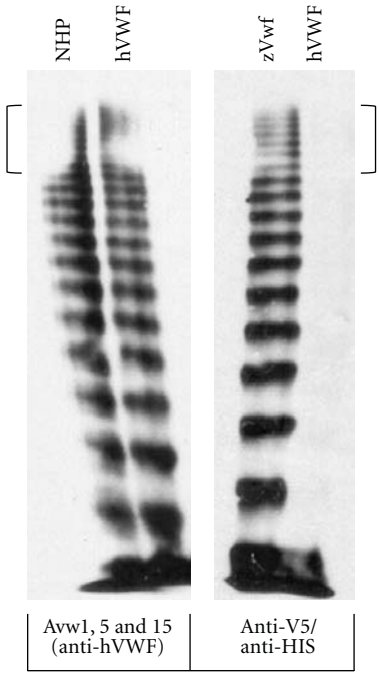

Figure 2: Multimerization of zebrafish Vwf in mammalian cell culture demonstrates high molecular weight multimers similar to human VWF. HEK293T cells were transfected with pzVwf/V5-HISA, expressing V5-HIS tagged zebrafish Vwf (zVwf), or pCi-neoVWF, expressing untagged human VWF (hVWF). Normal human plasma (NHP) and zebrafish and human supernatants were separated by agarose gel electrophoresis, transferred by western blotting, and detected with either a pool of monoclonal anti-hVWF antibodies (Avw1, 5, 15, left panel) or a mixture of anti-V5 and anti-HIS antibodies (for tagged zVwf, right panel). The anti-V5/HIS combination detects zVwf with a multimer pattern, including high molecular weight multimers, indistinguishable from that typically observed for human VWF (brackets indicate high molecular weight multimers for both zebrafish and human VWF).

in the pharyngeal arches, intestinal epithelium, and inner layer of the yolk sac (Figures 4(e), 4(g), and 4(h)).

4. Discussion

VWD is due to quantitative or qualitative deficiency of VWF and has been described in several mammals, including human, horse, cat, pig, rabbit, and dog [7, 8]. Identification and characterization of the human *VWF* cDNA [30–33] enabled the eventual identification of many of these pathogenic mutations as well as partial or full length sequence information in numerous mammalian species [34]. The zebrafish genome project [35] assisted in the identification of much of the *vwf* cDNA [15, 16], but this did not include the complete 5′ and 3′ UTRS. We have now completed cloning and characterization of the full length zebrafish *vwf* cDNA.

We found that *vwf* displays widespread expression in early embryonic development and then becomes more restricted at the larval stage. Mammalian *VWF* is widely expressed in vascular endothelial cell beds of the adult mouse [36], and VWF protein is an established clinical pathologic marker of human vasculature [37]. However, it has not been examined in the developing vertebrate. We hypothesized that there would be widespread expression of zebrafish *vwf* in developing vasculature, but instead found an early broad and then later restricted pattern. A previous study in zebrafish identified Vwf protein expression within the vasculature at the larval stage, although the source was not determined [15]. Therefore one possible explanation for the discrepancy with our results is that larval intravascular Vwf is not produced in endothelial cells but rather comes from the yolk sac or pharyngeal arches. Alternatively, endothelial *vwf* mRNA expression might not be present until later in development.

The expression seen in early embryonic development may possibly reflect maternally derived transcripts [38], while later expression is clearly of embryonic/larval origin. There is no prior evidence for a role of VWF in gastrulation, although the expression in the pharyngeal arches is intriguing. These structures develop into gills [39], the organs responsible for oxygen exchange in fish. The highest levels of mammalian *Vwf* mRNA expression have been identified in the lung [36], suggesting the possibility of an evolutionary conserved role of VWF in these structures.

In order to produce functional VWF activity, high molecular weight multimers are assembled in the trans-Golgi, packaged into WPBs, and secreted. This is followed by circulation in the blood and tethering of platelets to sites of vessel injury, forming the primary platelet plug [6]. It has been previously shown that zebrafish thrombocytes will aggregate in a Vwf-dependent fashion and that morpholino-mediated knockdown results in increased bleeding times and hemorrhage [15]. In this paper we have demonstrated that zebrafish Vwf has the ability to multimerize and form pseudo-WPBs in mammalian cell culture. Taken together, these data suggest that the basic mechanisms of zebrafish Vwf function appear to be conserved.

Previous studies have shown evidence for the presence of the Vwf receptor, GpIb, on thrombocytes in zebrafish and chicken [40, 41]. If thrombocytes bind Vwf as platelets do in mammals, one might expect a high degree of conservation of the Vwf A1 domain, which encodes the GpIb-binding site. The A2 domain, which encodes the Adamts13 cleavage site, is required for the production of properly sized Vwf multimers. When cleavage is reduced, vascular occlusion can occur, while when enhanced, bleeding results [42]. However, there are notable differences between mammalian and non-mammalian vertebrate systems. Despite the overall amino acid similarity and conservation of synteny of Vwf, the A1 and A2 domains display the largest degree of divergence when compared to humans. It is tempting to speculate that the A1 domain has evolved a relatively increased or decreased ability to bind thrombocytes in compensation for the latter's lesser or greater role in the initiation of primary hemostasis. Shear forces required to expose the A1 and A2 domains are likely to be different in zebrafish compared to mammals. Despite their functional similarities, nucleated thrombocytes are clearly different from anucleate platelets, suggesting the possibility that the two function quite differently. Studies of avian thrombocytes, which are also nucleated, have led to the

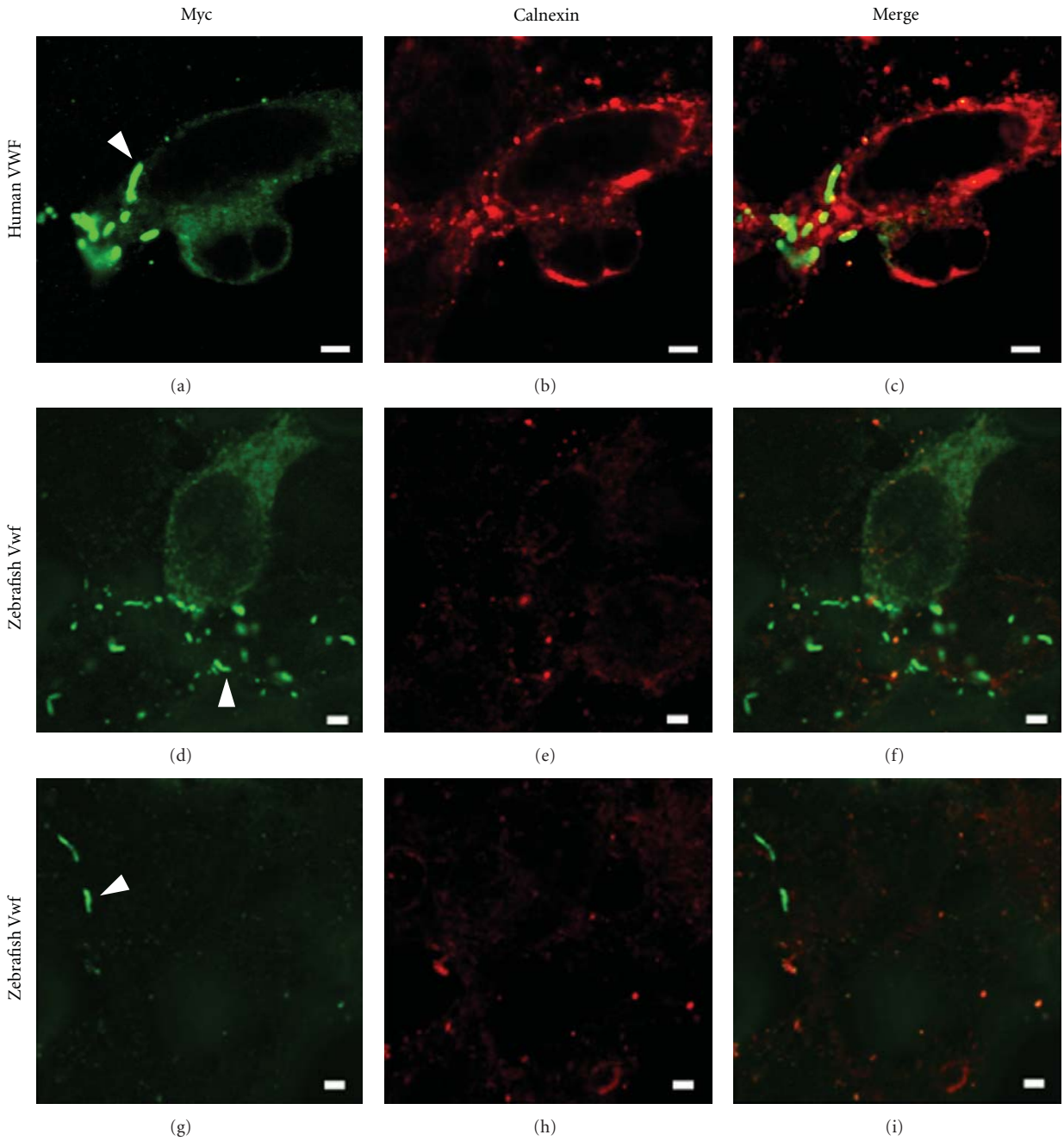

Figure 3: Zebrafish Vwf forms pseudo-Weibel-Palade bodies (pseudo-WPBs) in mammalian cell culture. pVWF/Myc-HIS (human VWF, (a–c)) or pzVwf/Myc-HIS (zebrafish Vwf, (d–i)) plasmids were transfected into HEK293T cells. Anti-Myc antibody conjugated to Alexa Fluor 488 (green channel, (a, d, g)) was used for detection and anti-calnexin antibody conjugated to Alexa Fluor 594 (red channel, (b, e, h)) labeled endoplasmic reticulum (ER). Both constructs demonstrate formation of elongated Myc positive and ER negative structures (absence of yellow signal in the merged panels, (c, f, i)) characteristic of pseudo-WPBs (examples are indicated in (a, d), and (g) by arrowheads). Scale bars, 2.5 μm.

hypothesis that human cardiovascular disease may be related to the existence of platelet rather than thrombocyte-initiated primary hemostasis [41]. Further understanding of the role of thrombocytes and Vwf in zebrafish and avian hemostasis may have potential implications for the treatment of bleeding and thrombotic disorders.

Acknowledgments

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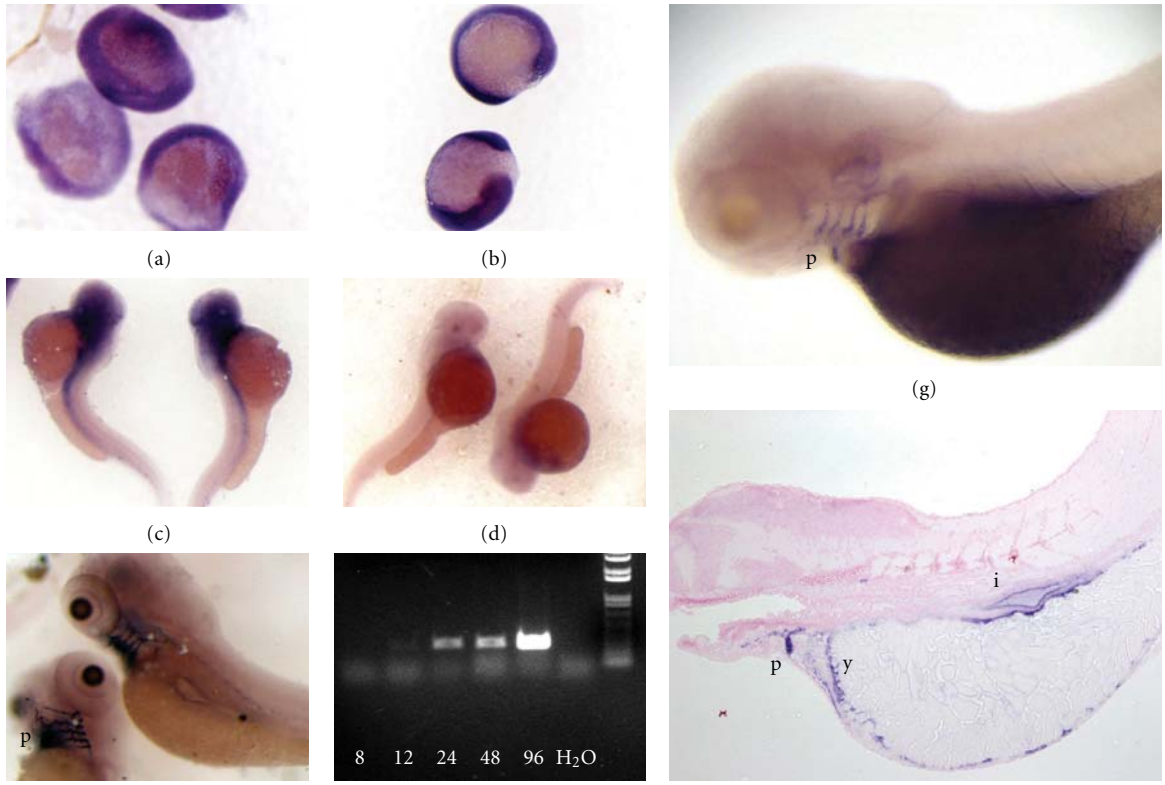

Figure 4: Developmental expression of *vwf* mRNA. Wild type zebrafish offspring were isolated from 8 to 120 hpf, fixed, and *in situ* hybridization was performed (Section 2). (a) Examination at 8 hpf demonstrates weak expression throughout the entire embryo, and staining was completely absent from a sense control. (b) Diffuse expression continues at 12 hpf (staining was completely absent from a sense control), followed by more restricted expression cranially with a stripe that extends caudally at 48 hpf (c). Figure 4(d) is a sense probe as negative control at 48 hpf. (e) 96 hpf shows strong expression in the pharyngeal arches. (f) RT-PCR of cDNA isolated from whole zebrafish embryos and larvae from 8–96 hpf. (g, h) Analysis at 120 hpf shows continued expression in the pharyngeal arches, as well as inner yolk sac layer and intestinal epithelium. Experiments in (a–e) used full length *vwf* riboprobes. Results in (g, h) are representative of hybridization with exon 28 and exon 47–52 riboprobes (Section 2, Table 1). Abbreviations: p: pharyngeal arches; y: inner layer of yolk sac; i: intestinal epithelium.

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Research Article

Drift-Diffusion Analysis of Neutrophil Migration during Inflammation Resolution in a Zebrafish Model

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Neutrophils must be removed from inflammatory sites for inflammation to resolve. Recent work in zebrafish has shown neutrophils can migrate away from inflammatory sites, as well as die in situ. The signals regulating the process of reverse migration are of considerable interest, but remain unknown. We wished to study the behaviour of neutrophils during reverse migration, to see whether they moved away from inflamed sites in a directed fashion in the same way as they are recruited or whether the inherent random component of their migration was enough to account for this behaviour. Using neutrophil-driven photoconvertible Kaede protein in transgenic zebrafish larvae, we were able to specifically label neutrophils at an inflammatory site generated by tailfin transection. The locations of these neutrophils over time were observed and fitted using regression methods with two separate models: pure-diffusion and drift-diffusion equations. While a model hypothesis test (the F-test) suggested that the datapoints could be fitted by the drift-diffusion model, implying a fugetaxis process, dynamic simulation of the models suggested that migration of neutrophils away from a wound is better described by a zero-drift, "diffusion" process. This has implications for understanding the mechanisms of reverse migration and, by extension, neutrophil retention at inflammatory sites.

1. Introduction

The fate of neutrophils following completion of the inflammatory programme is of critical importance for the outcome of episodes of acute inflammation and can determine whether there is prompt healing of a wound or the development of chronic inflammation and tissue injury. Neutrophils recruited to sites of inflammation may leave the site or die *in situ* [1]. The most widely accepted mechanism of neutrophil disposal is the programmed cell death or apoptosis, of the neutrophil followed by macrophage uptake and clearance (reviewed in [2]). Recently, other routes have been proposed; neutrophils may move away from the inflamed site into the bloodstream ("reverse transmigration" [3]), by migration through other tissues ("retrograde chemotaxis" or "reverse migration" [4–6]), or be lost into the inflammatory exudate [7, 8]. Current understanding of the process of reverse migration is reviewed elsewhere [9]. The uncertainty as to the *in vivo* fates of individual cells relates in part to the difficulty in following individual cells during inflammation resolution *in vivo*. The transgenic zebrafish model is emerging as a key model for the study of vertebrate immunity [10] and allows direct imaging and tracking of individual cells, and of populations of cells allowing their fate to be determined *in vivo*. Using a transgenic system, in which neutrophils express the fluorescent protein Kaede, notable for its ability to change fluorescence characteristics on exposure to light, we have assessed the fates of inflammatory neutrophils as

inflammation resolves. Although others have used a similar system to label immune cell populations responding to much smaller stimuli [6], there has been no detailed study of the migratory patterns of neutrophils during inflammation resolution following tail transection.

Using dynamic modelling techniques based on the drift-diffusion equation, we tested the competing hypotheses that neutrophils were directed away from the wound region by proresolution agents produced locally or that they cease responding to existing chemokine gradients and redistribute as a feature of stochastic migratory behaviours.

2. Methods

2.1. Reagents, Zebrafish Lines and Maintenance. All reagents were from Sigma-Aldrich (Poole, UK) unless otherwise stated. Zebrafish were maintained according to standard protocols [11]. The *Tg(lyz: Gal4)i252* [12] and *Tg(UAS: Kaede)s1999t* [13] lines are described elsewhere.

2.2. Microscopy, Photoconversion, and Image Processing. For confocal microscopy, a Perkin Elmer Ultra *VIEW* VoX ERS 6FR Laser Confocal Imaging System (Perkin Elmer INC, USA) with an inverted Olympus IX81 microscope, equipped with six diode laser lines and a Yokogawa CSU-X1 spinning disk, was used to capture images on a 14-bit Hamamatsu C9100-50 Electron Multiplying-Charged Couple Device (EM-CCD) peltier-cooled camera (Hamamatsu Photonics Inc.), through an appropriate filter. For fluorescence microscopy, a Nikon Eclipse TE2000-U Inverted Compound Fluorescence Microscope (Nikon UK Ltd) was used with a Hamamatsu 1394 ORCA-ERA (Hamamatsu Photonics Inc.). Images were captured using Volocity build 5.3.2. A Perkin Elmer Ultra *VIEW* PhotoKinesis device, attached to the microscope described before, was used to photoconvert the Kaede protein using a 405 nm laser line. The device was calibrated using a glass microscope slide (Menzel-Gläzer) covered with fluorescent highlighter ink (Stabilo Boss) as a photobleachable substrate (according to manufacturers instructions). Photoconversion was performed using 40% laser energy for 120 cycles of the 405 nm laser line. The embryos were then released from the agarose gel and transferred to fresh E3. The petri dishes containing the embryos were wrapped in tinfoil to prevent background photoconversion. At the timepoints indicated, embryos were again mounted and widefield fluorescence Z-stacks taken. Neutrophil segmentation was performed in Volocity based on fluorescence intensity, size, and "separate touching objects" feature. The XY position of each fluorescent cell at each timepoint was determined.

2.3. Dynamic Modelling of Neutrophil Behaviour. Neutrophil centroid coordinates in time were exported into Matlab (MathWorks, MA), for analysis. To describe quantitatively the population dynamics of neutrophils, drift-diffusion and pure-diffusion variants of the simple random walk model were used ([14] see Supplementary Material for full details available online at doi: 10.1155/2012/792163). Using parameters identified in these models, the behavior of each model was tested by simulation using a Monte Carlo procedure and the distribution of simulated cell populations compared to the observed data.

3. Results and Discussion

3.1. Characterising the Process of Reverse Migration In Vivo. Reverse migration, either into the circulation or back into tissues, has been described in the zebrafish model [4–6, 15]. In order to define the fates of inflammatory neutrophils, we photoconverted neutrophils in the immediate vicinity of the wound edge (approximately 80 microns) (Figure 1(a)) at defined periods after initiation of inflammation by tailfin transection. Time-lapse videomicroscopy was then performed on a compound fluorescent microscope, and the position of individual cells tracked in Volocity. Kaede protein and its photoconverted form remained stable and detectable well beyond the duration of these experiments (data not shown).

In over 500 hours of observation, no photoconverted neutrophil was ever seen to have left the fish from the wound, to have entered the circulation, or to have migrated via the circulation into a distant site. Neutrophils were seen to migrate away from the site of injury from around 8 hours after injury (Figure 1(b)). Photoconverted neutrophils can be seen to migrate away from the site of injury over the 16-hour time-lapse (Supplementary Movie 1 available online at doi:10.1155/2012/792163). At 4 hpi, neutrophils are densely accumulated around the site of injury, but over the duration of the time-lapse a population of neutrophils appears to spread into the surrounding tissue. Plots of the distance of each cell from the wound edge against time reveal a distinct pattern of neutrophil movement: neutrophils appear to be constrained in their behaviour, gradually increasing their mean distance from the wound, at a rate slower than their maximum speed would permit (Figure 1(c)). The differences between these findings and those of other groups [6] have many potential explanations, including the use of different promoters, different wounding protocols, and different labelling systems.

3.2. Neutrophils Continue to Be Recruited after Peak Inflammation. In mammalian inflammation, neutrophil influx ceases early in the inflammatory response, at least in rabbit models of pneumonia [16]. The neutrophil Kaede model allows us to distinguish the behaviour of neutrophils present at the site of inflammation from the behaviour of those cells in the process of being recruited. The montage in Figure 1 shows only the red photoconverted neutrophils. During the time-lapse, images were also taken using filter sets optimised for green fluorescence. The green neutrophils identified were cells that were not present at the site of injury at 4 hpi. The behaviour of these cells shows that neutrophils are still recruited to the site of inflammation at four hours after injury (Figure 2). There are no green neutrophils seen at the site of injury at 4 hpi because all the cells present have been photoconverted. There is an accumulation of green neutrophils at the site of injury from 6 hpi until 14 hpi. Following this, the number of green neutrophils at the site of

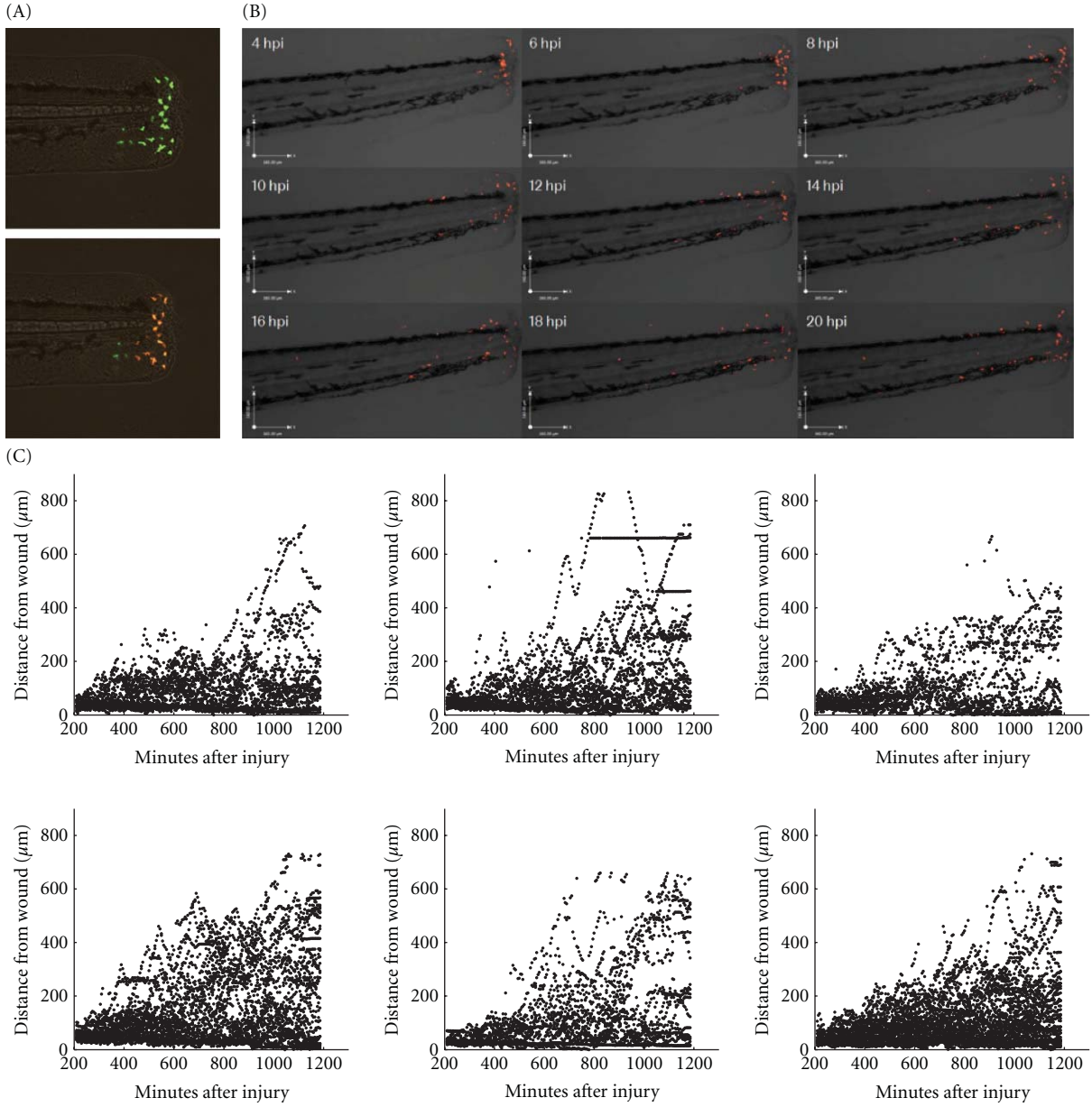


Figure 1: Inflammatory neutrophils exhibit restricted migration away from the site of tissue injury. 3 dpf embryos from transgenic zebrafish expressing Kaede in neutrophils were subjected to tailfin transection under anaesthesia using a sterile scalpel. The embryos were recovered for 4 hours. At four hours after injury the embryo was mounted in 0.5% low melting point agarose for imaging on a Laser Confocal System (Perkin Elmer Inc). The PhotoKinesis device was then used to photoconvert all neutrophils present within the tip of the tailfin. Photoconversion was carried out according to the methods described (120 cycles of 40% 405 nm laser energy), and time-lapse videomicroscopy was performed using a TE2000 fluorescent inverted microscope (Nikon). (a) Composite images of DIC overlaid with the red and green fluorescence channels showing a representative zebrafish tail before (above) and after (below) photoconversion. (b) A montage of DIC images overlaid with the red fluorescence channel at then timepoints indicated after tailfin injury. The redistribution of photoconverted cells can be clearly seen over time. (c) For each neutrophil in six individual fish, the distance from the wound was calculated using algorithms within Volocity and plotted against time.

injury falls. Where individual cells can be seen and followed over time, the pattern of accumulation of neutrophils during inflammation can be accurately determined. This technique has increased sensitivity for detecting continued influx compared to mammalian labelled-cell techniques, and this may explain the differences seen from rabbit pneumonia models where influx is no longer detectable shortly after initiation of the inflammatory episode [17].

3.3. Neutrophils Actively Migrate ("Drift") toward a Wound. Random walk models are often used in biology to describe the movement dynamics of individuals and populations

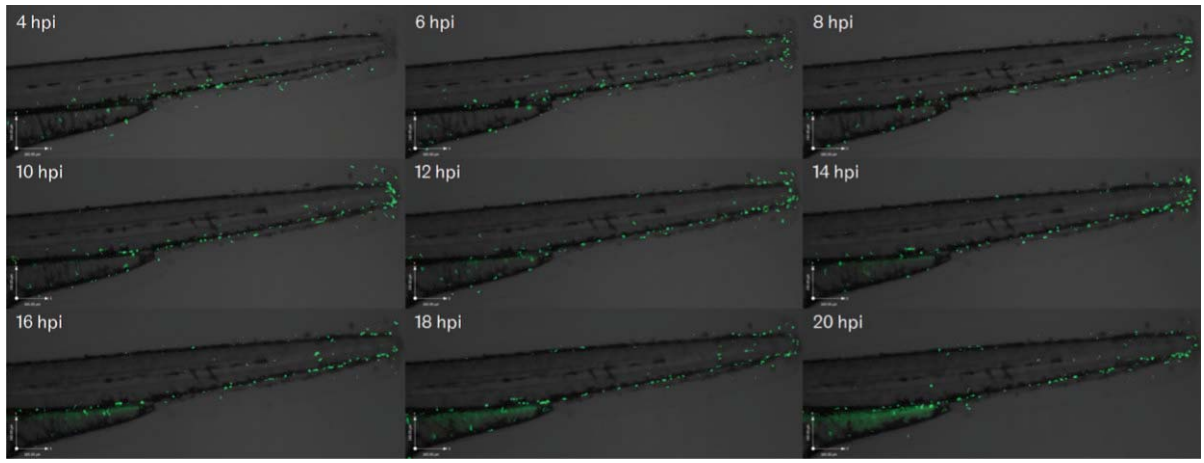


Figure 2: At peak inflammation, new neutrophils are recruited to the site of injury. Photomontage generated from the time-lapse data used in Figure 1(b), and Supplemental Movie 1, imaged using the GFP filterset, showing neutrophil recruitment to the site of injury over the same timespan. Green neutrophils can be seen to accumulate at the site of injury between 6 and 14 hours after injury.

[14, 18] and particularly for cell movement patterns [19–21]. Over short timescales neutrophils exhibit correlated random walk behaviour. However, these local correlations decay over time. The time between our data observations is greater than typical neutrophil persistence times [22] and thus we are able to ignore these local correlations and apply a simple random walk model [18]. To identify any global directional bias apparent in the movement of neutrophils, the simple random walk model was applied to aggregate data. The contribution of active recruitment (chemotaxis) of neutrophils and its reverse (fugetaxis) were examined by establishing the positions of all neutrophils at 4 hours following tail fin transection and modelling their behaviour using a drift-diffusion equation. Non-photoconverted neutrophils were examined to determine the behaviour of neutrophils not at the wound site at the time of photoconversion. Fitting the drift-diffusion equation to the dataset treats the neutrophils as point objects and asks whether they are behaving like simple particles redistributing stochastically ("diffusion") or whether there is an element of active movement towards or away from a chemical gradient (chemotaxis or fugetaxis). The equation (full description in supplemental data) generates a value for the drift co-efficient, for which non-zero values reflect an active rather than purely random migration. The drift was estimated from the linear relationship between time and mean cell distance from the wound (Figure 3). For 6 independent experiments, the coefficient estimates ranged in value from 0.11 to 0.95 μm/min (Table 1). As expected, in all cases cell populations demonstrated active drift toward the wound, consistent with migration directed by a chemotactic process.

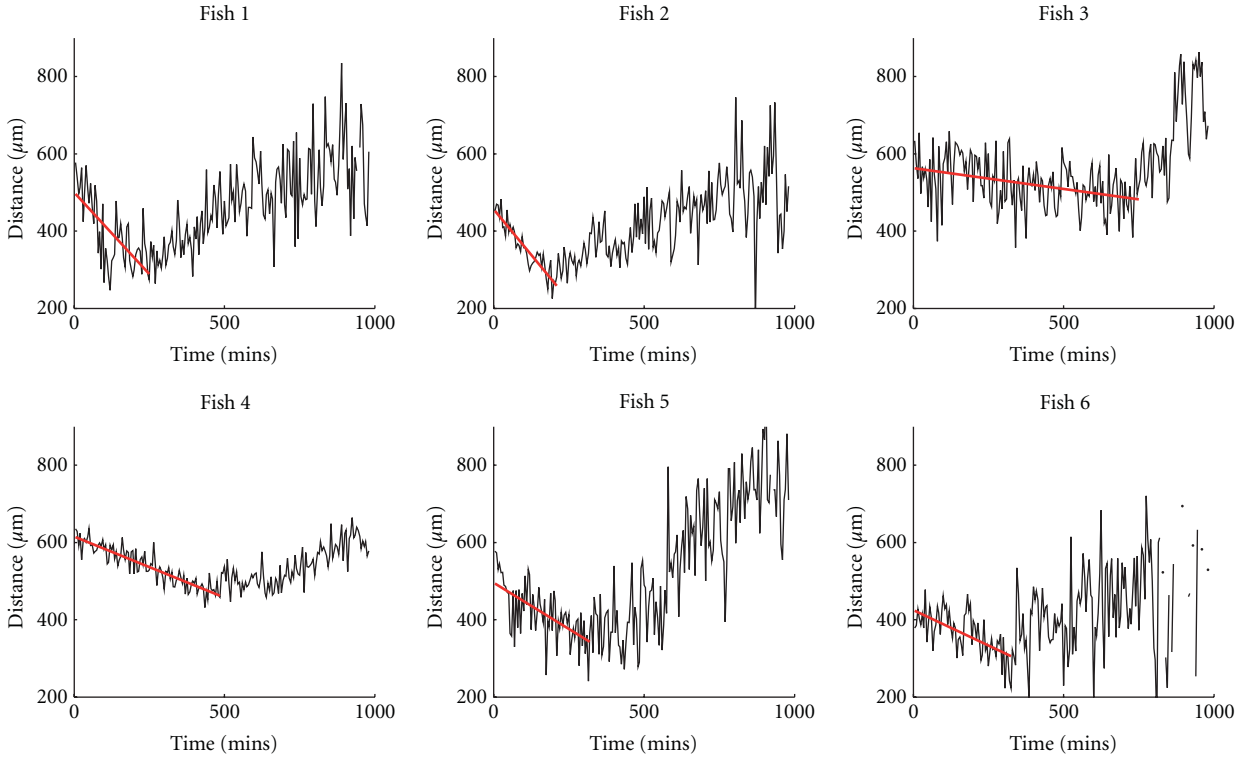
3.4. Migration of Neutrophils away from a Wound Is Better Described by a Zero-Drift, "Diffusion" Process. The same analysis was performed for photoconverted cells present at the site of the wound at the time of photoconversion, 4 hours following the tailfin transection (Table 2). Drift-diffusion and pure-diffusion model fits are compared in Figure 4.

Table 1: Estimated drift coefficients for the model of drift-diffusion describing cell migration toward the wound.

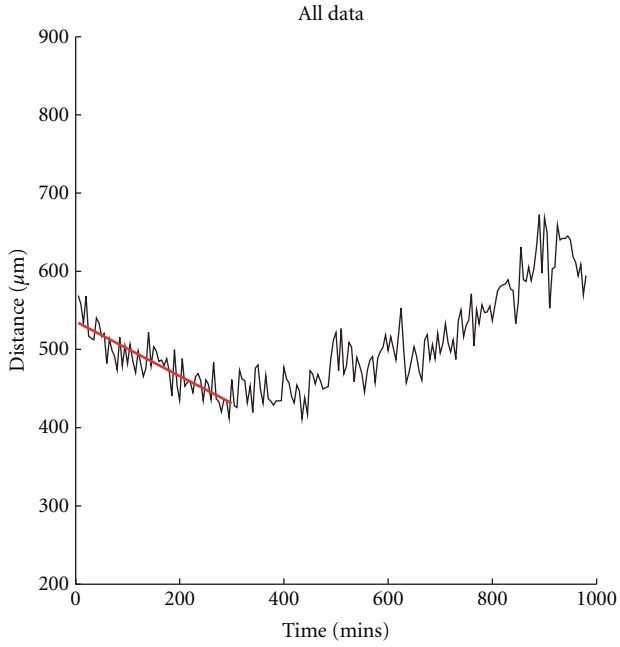
Dataset	Drift coefficient (std dev.)
(1)	−0.85 (0.13)
(2)	−0.95 (0.06)
(3)	−0.11 (0.02)
(4)	−0.32 (0.02)
(5)	−0.48 (0.08)
(6)	−0.37 (0.06)
All data	−0.35 (0.03)

Mathematical testing of the fit of the two models suggested that the drift-diffusion model fitted better with the data, but we were alert to the possibility that drift-diffusion models might appear superior due to the better ability of quadratic fits to model real, noisy data than simple linear fits. Using modeled data comparing the predicted distributions of neutrophils over time by applying drift-diffusion versus pure diffusion models gave a dramatic result: the cell population mode of the drift-diffusion model moved away from the wound over time (Figure 5, red line), in contrast to the observed data, where the mode remained close to the wound (Figure 5, yellow bars). The pure-diffusion model accurately captured this qualitative behavior, more accurately reflecting the observed distribution of neutrophils over time (Figure 5, blue line), suggesting that stochastic redistribution might best describe the pattern of neutrophil behavior during inflammation resolution.

For the larger wounds used in these studies, our data support a stochastic redistribution of neutrophils during inflammation resolution. However, to definitively prove this will require more advanced modelling techniques. For smaller wounds, different principles may apply. Previous studies have suggested that neutrophils leaving the wound follow the same dynamics as those arriving, having the same velocity

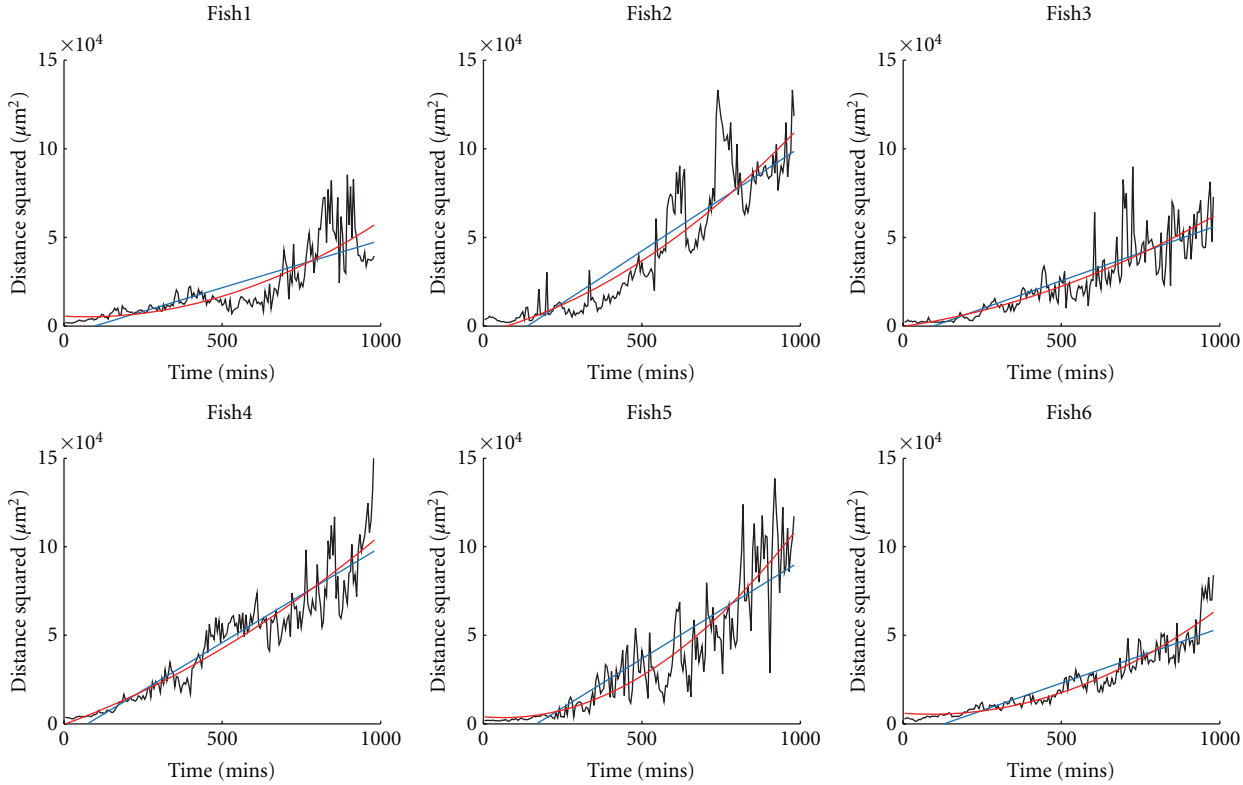


(a)



(b)

Figure 3: Nonphotoconverted neutrophils actively migrate into the wound region. (a) Variation over time of mean cell distance from the wound for the nonphotoconverted (green) neutrophils, observed in each subject 1–6 (black line). Overlaid on each graph is the prediction of mean distance obtained from the linear model used to characterise the initial drift (red line). The time is measured from the start of observations which commenced 4 hours after injury. The cell count in subject 6 (bottom right) was low and sometimes zero near the end of the dataset, which explains the missing sections. (b) Data and model combined over all subjects.



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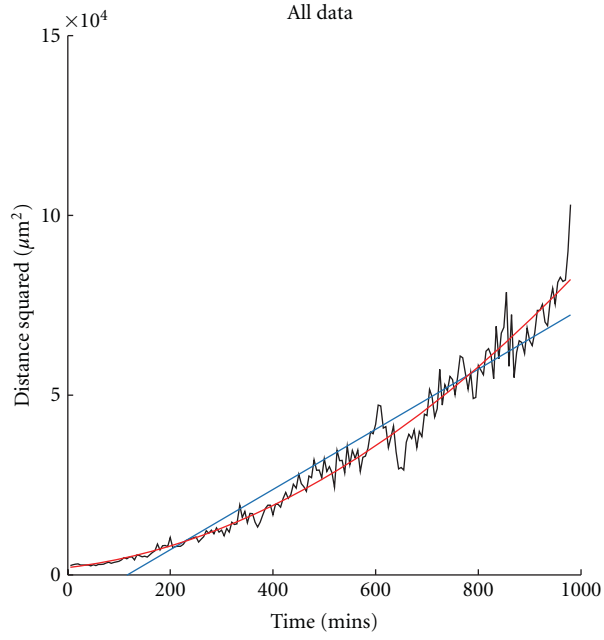

(b)

Figure 4: Inflammatory neutrophil behaviour can be fitted by pure-diffusion and drift-diffusion models. (a) Plots of mean squared cell distance from the wound against time for the photoconverted (red) neutrophils for datasets 1–6. Also shown on each plot are the fits for the linear model corresponding to pure-diffusion with zero drift (blue line) and for the drift-diffusion model (red line). (b) Data and models combined over all subjects.

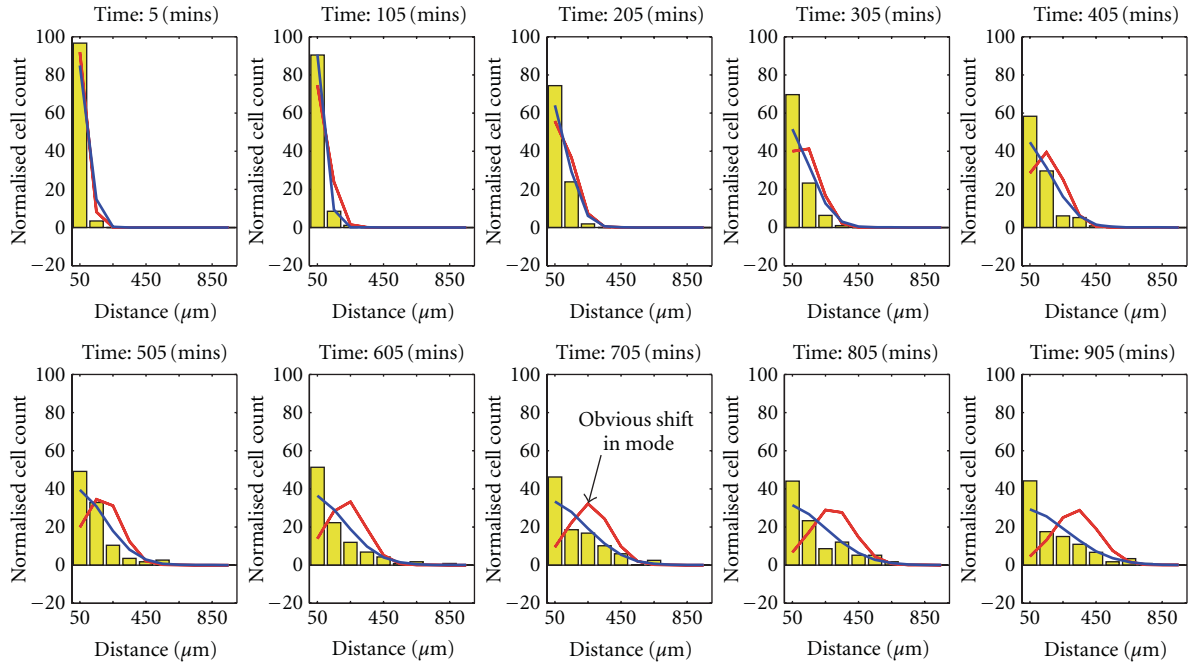

Figure 5: Simulation reveals a pure-diffusion model to be a better fit to the real data. Both the drift-diffusion model (red line) corresponding to drift (0.26 μm/min) and diffusion (8.0 μm/min) and the pure-diffusion model (blue line) corresponding to diffusion (41.8 μm/min) were simulated 1000 times. The simulations were used to produce a distribution for the spatially binned data of each model. The mean values of cell distribution over space are shown by the red and blue lines, respectively (in terms of distance from the wound). Overlaid on these is a corresponding histogram representation (yellow) of the real data (combined over all fish). The histogram bins have width 100 μm and are centered at 50 μm to 950 μm from the wound. The pure-diffusion model shows a correct qualitative prediction of cell distribution whereas the drift-diffusion model predicts that the population mode moves away from the wound over time, in contrast to the observed data.

Table 2: Estimated coefficients for the drift-diffusion model and pure-diffusion model of cell migration away from the wound (standard deviation is given in brackets). An F-test value >5 indicates that the drift-diffusion model should be preferred to the pure-diffusion model.

Dataset	Drift-diffusion model		Pure-diffusion model	F-test
	Drift coefficient	Diffusion coefficient	Diffusion coefficient	
(1)	0.25 (0.05)	−4 (10)	27 (2)	38
(2)	0.27 (0.07)	23 (15)	56 (4)	28
(3)	0.19 (0.05)	13 (10)	32 (3)	14
(4)	0.21 (0.05)	32 (11)	54 (3)	14
(5)	0.35 (0.07)	−8(14)	55 (4)	82
(6)	0.27 (0.03)	−7 (6)	31 (2)	145
All data	0.26 (0.02)	8 (3)	41.8 (0.10)	267

and directionality [15]. However, those data rely on preselection of tracks directly leaving the wound, and may give different results to studies considering the whole population of cells.

This approach uses static point data for each neutrophil; an alternative approach would be to investigate the dynamics using individual track data. Such an approach has been applied to proteins in living cells [23, 24] and to *in vivo* melanoma cell tracks [25]. Care is needed when considering cell tracks as a naive approach could misrepresent short-term correlations in track direction as biased migration. In addition, to identify tracks requires faster sampling of observations which must be balanced against total experiment runtime.

Although the pure-diffusion model appears to fit the data well, it consistently underestimates the number of photoconverted cells remaining adjacent to the wound, suggesting some cells are actively retained at the wound site. To completely address this will require the development of systems incorporating multiple models to reflect the dynamic mix of neutrophil behaviours present within a single population.

4. Conclusions

From this analysis, we conclude that the two key neutrophil migratory behaviours regulating neutrophil numbers during the inflammatory response—movement of neutrophils in and out of wounds—are qualitatively different processes. Neutrophils are recruited actively towards the site of injury ("drift"), but as inflammation resolves, their movement away is better modelled by stochastic redistribution ("diffusion"). This has implications for our understanding of how neutrophils might be retained at sites of inflammation in disease states.

Acknowledgments

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Review Article

Novel Insights into the Genetic Controls of Primitive and Definitive Hematopoiesis from Zebrafish Models

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Hematopoiesis is a dynamic process where initiation and maintenance of hematopoietic stem cells, as well as their differentiation into erythroid, myeloid and lymphoid lineages, are tightly regulated by a network of transcription factors. Understanding the genetic controls of hematopoiesis is crucial as perturbations in hematopoiesis lead to diseases such as anemia, thrombocytopenia, or cancers, including leukemias and lymphomas. Animal models, particularly conventional and conditional knockout mice, have played major roles in our understanding of the genetic controls of hematopoiesis. However, knockout mice for most of the hematopoietic transcription factors are embryonic lethal, thus precluding the analysis of their roles during the transition from embryonic to adult hematopoiesis. Zebrafish are an ideal model organism to determine the function of a gene during embryonic-to-adult transition of hematopoiesis since bloodless zebrafish embryos can develop normally into early larval stage by obtaining oxygen through diffusion. In this review, we discuss the current status of the ontogeny and regulation of hematopoiesis in zebrafish. By providing specific examples of zebrafish morphants and mutants, we have highlighted the contributions of the zebrafish model to our overall understanding of the roles of transcription factors in regulation of primitive and definitive hematopoiesis.

1. Zebrafish as a Model for Hematopoiesis

Recently, zebrafish have emerged as a powerful vertebrate model system due to their external fertilization, optically clear embryos, rapid development, availability of tools for manipulations of gene expression during development, and the ability to generate genetic mutants by random (insertional and chemical) and targeted mutagenesis [1–3]. Microinjections of antisense morpholinos, which cause transient knockdown of gene activity, and mRNA allows for analysis of the effects of loss and gain of function of specific genes during development [4]. Whole-mount in situ hybridization (WISH) is a powerful technique to analyze the spatiotemporal expression of genes, and placing genes in regulatory cascades by analysis of genetic mutants and/or embryos injected with morpholinos (commonly termed as morphants) [5, 6].

Specifically for hematopoiesis, zebrafish blood contains cells of all hematopoietic lineages [7–11] and orthologs of most transcription factors involved in mammalian hematopoiesis have been identified indicating evolutionarily conserved pathways of regulation [12–15]. Initial validation of the use of zebrafish for hematopoiesis research came from the forward genetic screens. In 1996, two large-scale chemical mutagenesis screens were performed to identify mutants with a variety of phenotypes [16, 17]. Of these, characterization of 46 mutants with blood phenotypes by allelic complementation suggested roles for at least 26 genes in hematopoiesis [18, 19]. Subsequent efforts by several groups identified the underlying genetic defects in many of these mutants by positional cloning or candidate gene approaches. In addition to identifying the genes previously known to have a role in hematopoiesis (e.g., *gata1*, *sptb*, and, *alas2*), these mutants also uncovered novel genes with roles in hematopoiesis, (e.g., *slc25a37*, *slc40a1*, and *glrx5*) [20–25]. Subsequent forward genetic screens focusing on mutants affecting specific hematopoietic lineages have identified additional conserved pathways of regulation between zebrafish and mammals [26–28].

This led to a surge of activity in zebrafish research laboratories, developing a variety of tools for thorough

analysis of hematopoiesis. Lineage-specific transgenic lines were generated using promoters of a variety of hematopoietic genes driving fluorescent markers (reviewed in [29, 30] and listed in Table 1), allowing for visual observations of hematopoietic lineages in real-time during development. Advances in imaging combined with the ability to perform lineage tracing made it possible to follow the fate of specifically marked cells during development in a live vertebrate animal model [31, 32]. Sorting of hematopoietic cells by fluorescence-activated cell sorting (FACS), *in vitro* culturing using zebrafish-specific cytokines and kidney stromal cells, and the ability to perform transplantation have facilitated characterization of hematopoietic potential of different mutants [33–37].

While forward screens are biased by the phenotype being screened, mutants in any specific gene can be generated using reverse genetic approaches. This has been made possible in zebrafish in the last decade by TILLING (Targeting-Induced Local Lesions IN Genomes) [55, 60], and more recently by targeted mutagenesis using zinc-finger and transcription-activator-like-effector nucleases (i.e. ZFNs and TALENs) [61–64]. Furthermore, effects of gene dosage can be analyzed by injecting suboptimal doses of antisense morpholinos or studying hypomorphic alleles generated by TILLING. In this review, we discuss how the technical advances and genomic tools discussed above went hand-in-hand with the elucidation of genetic controls of hematopoiesis in zebrafish.

2. Ontogeny of Vertebrate Hematopoiesis

In mammals, hematopoiesis occurs in successive but overlapping waves that occur at distinct anatomical locations [65]. Overall, the hematopoietic process is distinguished into primitive and definitive hematopoiesis based on the type of blood cells generated. Primitive hematopoiesis is transient in nature and produces unipotent blood cells that arise directly from the mesoderm. Definitive hematopoiesis produces multipotent blood cells that give rise to multiple different lineages through cellular intermediates and support blood cell development throughout the life of the organism. Here, we have summarized the overall process of mammalian hematopoiesis based on the studies using mouse models.

During embryogenesis, primitive hematopoiesis occurs in two distinct waves in the extraembryonic yolk sac blood islands, producing primitive macrophages and primitive erythrocytes, respectively, thus providing the developing embryos with oxygen and their first line of defense against pathogens [66]. There is some support for the presence of additional lineages, particularly megakaryocytes, during primitive hematopoiesis [67].

Definitive hematopoiesis also occurs in two distinct waves. The first wave of definitive hematopoiesis produces a transient population of cells, termed erythroid-myeloid progenitors (EMPs) in the yolk sac and fetal liver [68, 69]. The second wave of definitive hematopoiesis produces hematopoietic stem cells (HSCs) from the hemogenic endothelium of the embryo that includes the aorta-gonad-mesonephros (AGM) region of the embryo, yolk sac, and placenta [65, 70–72]. HSCs from these sites migrate through circulation to fetal liver to support hematopoiesis during embryogenesis [65, 70, 73]. Recently, Chen and colleagues [74] demonstrated that EMPs and HSCs are derived from two different hemogenic endothelial populations. Unlike HSCs, EMPs lack the potential to give rise to lymphocytes.

The site of adult hematopoiesis, where HSCs undergo differentiation to generate lineage-committed progenitors that give rise to all the mature blood cell types and self-renewal to maintain a constant supply of HSCs, is bone marrow [75]. The prevailing thinking, based on the current data, is that HSCs emerging from the hemogenic endothelial cells in the AGM region of the developing mouse embryo give rise to most (if not all) bone marrow hematopoietic cells [73, 76]. The shifting sites of hematopoiesis are thought to provide specific microenvironment cues required for the specification, and migration of precursors for lineage commitment [77, 78].

Although the overall process of hematopoiesis is well defined, we have just begun to elucidate the exact nature of the molecular controls and lineage relationships using *in vitro* colony assays and animal models, particularly mice and zebrafish. The key questions revolved around the generation, migration, and differentiation of HSCs into lineage-committed progenitors and how these processes are regulated to maintain a critical balance required for proper functioning of the hematopoietic system.

2.1. Primitive Hematopoiesis in Zebrafish. In zebrafish, the first blood cells can be observed in circulation at around 26 hours post fertilization (hpf). However, based on the expression patterns of the genes involved in primitive hematopoiesis, it is clear that the primitive hematopoiesis starts at ~11 hpf in the lateral plate mesoderm (LPM) during somitogenesis. The erythroid precursors are observed as bilateral stripes in the posterior lateral mesoderm (PLM) that fuse along the midline to form the intermediate cell mass (ICM) located in the trunk dorsal to the yolk tube extension by 24 hpf [29, 75, 77, 79–81]. Primitive myeloid progenitors initiate at the anterior lateral mesoderm (ALM) and differentiate into macrophages in the rostral blood island [80, 82]. Thus, primitive hematopoiesis in zebrafish occurs in two waves, producing primitive macrophages and primitive erythrocytes, respectively. In addition, neutrophils and thrombocytes have also been detected during primitive hematopoiesis in zebrafish. However, the origin of neutrophils during primitive hematopoiesis is not clear, as two recent reports presented contradictory data on their origin from either primitive macrophage lineage [83] or primitive erythrocyte lineage [84] using fate-mapping techniques. Thus, primitive blood cells in zebrafish appear to have diverse lineages, similar to the mouse [67]. However, further studies are required to clearly define the lineage relationships between these cell types during primitive hematopoiesis.

2.2. Definitive Hematopoiesis in Zebrafish. The hallmark of definitive hematopoiesis is generation of multipotential HSCs that can undergo self-renewal and differentiation to

Table 1: Lineage-specific mutant and transgenic lines for zebrafish hematopoiesis research.

Lineage	Marker	Mutant lines		Transgenic lines	
		Mutant designation and mutation type	References	Line designation	References
Hemangioblast	*tal1/scl*	t21384, K183X	[38]	PAC-tal1:GFP 5.0tal1:EGFP	[39, 40]
	lmo2	None		lmo2:EGFP lmo2:DsRed	[41]
EMPs	*runx1*	hg1, W84X	[42, 43]	runx1P1:EGFP	[44]
HSCs	*runx1*	hg1, W84X	[42, 43]	runx1P2:EGFP	[44]
	cmyb	t25217, I181N hkz3, truncation in transactivation domain	[45] [46]	cmyb:EGFP	Developed by the Zon lab, used in [47]
	cd41	None		cd41:GFP	[33, 34]
Erythropoiesis	*gata1*	m651 (*vlad tepes*), R339X hg2, T301K	[23] [48]	gata1:GFP gata1:DsRed	[37, 49]
Myelopoiesis: GMPs	*spi1/pu.1*	None		spi1:EGFP zpu.1:EGFP	[50, 51]
Myelopoiesis: Neutrophils, Macrophages, Monocytes	*mpx*	None		mpx:GFP	[52]
	lyz	None		lyz:EGFP lyz:DsRed	[53]
	mpeg1	None		mpeg1:EGFP mpeg1:mCherry	[54]
Lymphopoiesis	*rag1*	t26683, R797X	[55]	rag1:GFP	[56]
	lck	None		lck:EGFP	[57]
	ikzf1/ikaros	t24980, Q360X	[58]	ikzf1:GFP	[59]

produce cells of erythroid, myeloid, and lymphoid lineages. In zebrafish HSCs can be identified by their expression of *runx1* and *cmyb* as early as 26 hpf in the ventral wall of the dorsal aorta and hence this region of the embryo is referred to as the AGM [13, 29]. Two recent studies have unequivocally demonstrated the origin of HSCs from the hemogenic endothelium lining the ventral wall of the dorsal aorta using time lapse imaging and lineage tracing in double transgenic lines marking HSCs and endothelial cells with different fluorescent markers [47, 85]. A novel process of cell transition, termed endothelial hematopoietic transition (EHT), appeared to be involved in the production of HSCs from hemogenic endothelium [85]. Similar to the mouse, a transient multipotent progenitor population of EMPs supports definitive hematopoiesis during embryogenesis and these EMPs originate in the posterior blood island (PBI) of zebrafish [86].

The sites of adult hematopoiesis in zebrafish are kidney marrow (analogous to the mammalian bone marrow) and thymus (for T cells) [13, 29, 87]. Up until recently, a site analogous to mammalian fetal liver was not recognized in the zebrafish. Therefore, HSCs from AGM were presumed to support embryonic definitive hematopoiesis and migrate to thymus and kidney for adult definitive hematopoiesis. However, two independent studies demonstrated the existence of an intermediate site of hematopoiesis posterior to the yolk tube extension, termed caudal hematopoietic tissue (CHT), using imaging and cell tracing techniques [88, 89]. It was proposed that the function of CHT is analogous to that of the fetal liver in mammals for supporting definitive hematopoiesis during embryogenesis. By tracing the generation and migration of HSCs using cd41:GFP^{low} cells, Kissa and colleagues [90] validated the migratory route of HSCs as being AGM to CHT and then to thymus and pronephros. Recently, Hess and Boehm [91] elegantly imaged the process of thymopoiesis in real time in zebrafish using triple transgenic lines and their data suggested that AGM is a major source of thymus-settling lymphoid progenitors compared to CHT.

Thus, based on the current status of our understanding, definitive hematopoiesis in zebrafish occurs in two waves: first wave produces transient EMPs in the PBI region and second wave produces HSCs in the AGM region that migrate to CHT to support larval definitive hematopoiesis and to thymus and kidney marrow to support adult definitive hematopoiesis. It is not clear if the migration of HSCs from AGM to kidney and thymus is via CHT only or also occurs directly as was previously assumed.

3. Elucidation of Genetic Controls of Hematopoiesis in Zebrafish

Despite the spatial and temporal differences during hematopoiesis between zebrafish and mammals as discussed above, the overall process is highly conserved producing the same effective repertoire of hematopoietic cells. It begins from a cell, termed hemangioblast, that serves as a common

precursor for hematopoiesis and vasculogenesis [92, 93]. A complex network of regulatory signals is involved in the specification and lineage commitment of precursors during primitive and definitive hematopoiesis in mammals. These include homeobox, notch, vegf, and wnt signaling pathways as well as specific transcription factors, such as Tal1 (Scl), Lmo2, Gata1, Cmyb, Runx1, Spi1 (Pu.1), and Ikzf1 (Ikaros), which are shown to function in a hierarchical manner [5, 94–99]. The importance of proper functioning of these transcription factors is evident from the preponderance of mutations and genomic rearrangements disrupting their activity detected in several blood disorders, particularly leukemias and lymphomas [100–106].

Animal models, where level of gene activity can be manipulated, have played a critical role in advancing our understanding of the genetic controls of hematopoiesis. However, knockout mice are embryonic lethal at mid-to-late gestation for *Tal1*, *Lmo2*, *Gata1*, *Sfpi1 (Pu.1)*, *Myb*, and *Runx1*, thus precluding the examination of their roles in later stages of hematopoiesis [107–112]. Conditional knockout is a useful tool to determine the function of these genes later in life; however, it has been difficult to use this technology to study the initiating events of a lineage, especially for the HSCs, since appropriate promoters to drive Cre recombinase expression may not be available. Zebrafish provide an advantage over mouse models due to their ability to survive without blood for several days and are, therefore, a suitable model organism for investigating the effects of loss of function of genes that cause embryonic lethality in mice due to the hematopoietic defects. Here, we discuss the contributions of zebrafish mutants, morphants, and transgenic lines to our understanding of the regulatory cascade controlling the hematopoiesis process (Table 1 lists the lineage-specific transgenic lines and genetic mutants in transcription factors involved in regulation of hematopoiesis). The common theme in the studies reviewed below is utilization of the unique features of zebrafish embryos and available tools for analysis of the disruptions to the gene activity in an effort to understand the overall process.

3.1. Genes Involved at the Hemangioblast Level: tal1 and lmo2. Based on their expression in both hematopoietic and endothelial cells, and the phenotypes of loss of function animal models, the T-cell acute lymphocytic leukemia 1 (*TAL1*) and the LIM domain only 2 (*LMO2*) genes are both believed to function at the hemangioblast level [12, 113]. Both genes were identified from translocations occurring in T-cell acute lymphoblastic leukemia, *TAL1* from translocation t(1;14) and *LMO2* from translocation t(11;14) [102, 104]. TAL1 is a basic helix-loop-helix (bHLH) transcription factor where the bHLH domain is involved in DNA binding as part of a multiprotein complex that includes LMO2 as a bridging protein. LMO2 belongs to the LMO family of zinc-finger proteins that are characterized by 2 LIM domains, each composed of 2 zinc fingers [104]. Knockout mice for *Tal1* and *Lmo2* died *in utero* by embryonic days 9.5–10.5 (E9.5-10.5) due to lack of embryonic erythropoiesis [108, 112]. Thus, their roles during definitive and adult hematopoiesis were investigated by *in vitro* colony assays, chimeric mice, and/or conditional knockout mice [114, 115]. Failure to produce any myeloid colonies *in vitro* from $Tal1^{-/-}$ yolk sac cells indicated a block at the EMP level [108]. Using conditional knockout mice, Hall and colleagues [114, 116] demonstrated that adult hematopoiesis can occur independent of Tal1 function with minor defects in erythropoiesis and megakaryopoiesis. On the other hand, Lmo2 was shown to be absolutely necessary for adult hematopoiesis based on the analysis of chimeric mice derived from $Lmo2^{-/-}$ embryonic stem cells [115].

In zebrafish, *tal1* is expressed in the ALM and PLM from ~11 hpf and in the posterior ICM at 26 hpf, validating its role in primitive hematopoiesis [39, 117, 118]. First direct proof for the exact site of HSC initiation between the dorsal aorta and the posterior cardinal vein being analogous to AGM in zebrafish came from the examination of Tg(tal1-PAC-GFP) embryos by time lapse imaging [40]. Loss-of-function analyses for *tal1* have been performed using morpholinos and a genetic truncation mutation, K183X, which deletes the bHLH domain [38, 119–121]. Homozygous mutant embryos ($tal1^{K183X/K183X}$) exhibited lack of expression of markers of both primitive and definitive lineages and also lacked visible circulation at 26 hpf [38]. These studies not only confirmed the role of Tal1 during primitive hematopoiesis, but also provided direct evidence for the role of Tal1 in the initiation of definitive hematopoiesis. However, mutant embryos died due to pericardial edema and defects in heart morphogenesis and could not be studied for the role of Tal1 in transition of embryonic to adult stages of definitive hematopoiesis.

In zebrafish, *lmo2* expression in the ALM and PLM is detected about 20 minutes after the *tal1* expression and phenotype of *lmo2* morphants is very similar to the *tal1* morphants, supporting their roles as part of the multiprotein complex during hemangioblast development [41, 122]. To date, no genetic mutants have been reported for *lmo2*. Overall, zebrafish studies have confirmed the strict requirements for Tal1 and Lmo2 in initiation of both primitive and definitive hematopoiesis.

3.2. Genes Involved at the HSC Level: runx1 and cmyb. The onset of definitive hematopoiesis in the AGM is marked by the specification of HSCs, which support hematopoiesis throughout the life of a vertebrate. *runx1* and *cmyb* have been used interchangeably as the earliest markers of definitive hematopoiesis due to their expression in the AGM during HSCs specification [12, 123]. However, we have just begun to elucidate their precise roles in HSCs specification, migration to the sites of larval and adult hematopoiesis, and differentiation into erythroid, myeloid, and lymphoid lineages.

RUNX1 belongs to a family of genes (3 members in mammals and 4 in zebrafish) that encode for the alpha subunits of a heterodimeric complex that binds DNA through the highly conserved runt domain. A single gene, *CBFB*, encodes for the beta subunit, which does not bind to DNA by itself but increases the affinity of alpha subunits to bind to DNA after heterodimerization through their runt domains [124]. Promoters of many hematopoietic genes, for example, *SPI1* and *GATA1*, contain RUNX1 DNA binding sites [125–127]. *RUNX1* was first identified in the t(8;21) translocation frequently observed in acute myeloid leukemias and its

dimerization partner, *CBFB,* is also frequently involved in genomic rearrangements associated with leukemia [100, 128, 129]. Furthermore, mutations affecting the level of RUNX1 activity leading to loss of function, dominant negative gain of function, and/or overexpression are associated with other blood disorders such as familial platelet disorder with predisposition to acute myeloid leukemia and myelodysplastic syndrome, suggesting that the process of hematopoiesis is very sensitive to the level of RUNX1 activity [130–132].

Studies using knockout mouse models demonstrated that Runx1 is essential for the initiation of HSCs generation during definitive hematopoiesis as the mutant mice failed to develop fetal liver hematopoiesis and died *in utero* at E12.5 [111]. Conditional knockout mice were able to develop all lineages but showed defects in megakaryocyte maturation and differentiation of B and T cells [133, 134]. Recent elegant fate mapping experiments in mouse embryos by Chen and colleagues demonstrated that Runx1 is required for the emergence of HSCs from the hemogenic endothelium [135]. Taken together, these data suggest a strict requirement of Runx1 in the generation of HSCs to initiate definitive hematopoiesis and in further differentiation of certain lineages but not for the maintenance of HSCs if they are already produced (reviewed in [73]).

Zebrafish *runx1* was identified based on its high similarity to the human *RUNX1* in the runt homology domain [123, 136]. Since then, several studies have validated the critical requirement of Runx1 in the initiation of definitive hematopoiesis by morpholinos and characterization of a variety of hematopoietic mutants [95, 97, 136, 137]. As these studies were performed prior to the recognition of CHT being the site of embryonic definitive hematopoiesis, they did not address Runx1 requirements in specification of EMPs and their transient nature precluded analysis of Runx1 requirements in adult hematopoiesis. None of the hematopoietic mutants from forward genetic screens mapped to the *runx1* locus.

Therefore, our group performed TILLING to identify a truncation mutation, W84X, in the runt domain of *runx1* [42, 43]. Homozygous mutant embryos displayed a complete lack of cells expressing markers of HSCs, definitive erythroid, myeloid, and lymphoid lineages in the CHT and thymus between 3–5 dpf [42, 43]. However, utilizing Tg(cd41:GFP) transgenic zebrafish, we were able to demonstrate that cd41^{+} cells were formed in the *runx1*$^{W84X/W84X}$ fish in the AGM and CHT regions and migrate to the pronephros, even though they were negative for other HSC markers such as *cmyb*. Based on the analysis of circulating blood cells, the mutant fish displayed 3 distinct phases: first phase of normal circulating blood cells until around 6–8 dpf (presumably from normal primitive hematopoiesis), second phase of bloodless stage until around 20 dpf leading to death in most larvae (defective larval definitive hematopoiesis), and astonishingly, ~20% of the mutant larvae resumed blood circulation and grew as phenotypically normal adult fish with multilineage adult hematopoiesis [43]. We do not know exactly how these 20% *runx1* mutant larvae were rescued. One possibility is that the cd41^{+} cells observed in these embryos are hematopoiesis-committed or -primed mesoderm cells, which could restart hematopoiesis in permissive conditions, such as compensation by *runx2a, runx2b,* and *runx3* genes or other genetic and/or epigenetic changes. Another scenario is that two waves of definitive hematopoiesis exist, one for larval and the other adult, while Runx1 is only required for the larval stage. For both scenarios, most larvae died due to lack of circulating blood cells resulting from defective larval hematopoiesis. It is interesting to note that alternate *runx1* promoters are used during establishment of EMPs and HSCs (Table 1) as demonstrated recently by Lam and colleagues [44].

Similarly, MYB, a cellular homolog of the V-MYB proto-oncogene, is a critical transcription factor required for definitive hematopoiesis. A number of mouse models, including conventional and conditional knockouts as well as hypomorphic alleles, have been generated for functional analysis of Myb requirements during hematopoiesis, as discussed in a recent review by Greig and colleagues [109]. These studies have highlighted the key difference between Runx1 and Myb requirements during definitive hematopoiesis to be the generation of HSCs. *Myb* knockout mice displayed defects in erythroid and myeloid development and died *in utero* at E15.5, which is much later than the stage when HSCs are generated [109]. Furthermore, $Myb^{-/-}$ ES cells were able to produce T cell progenitors in $Rag1^{-/-}$ chimeric mice [138]. Thus, Myb deficiency causes a block in HSCs differentiation and lineage commitment rather than HSCs specification. Lieu and Reddy [139] demonstrated important contributions of Myb to self-renewal and differentiation of HSCs during adult hematopoiesis.

Recently, two groups reported characterization of loss of function mutants for *cmyb* in zebrafish: (1) allele t25127 with a missense mutation, I181N, affecting DNA binding domain and (2) allele hkz3, a splice site mutation leading to truncation of the transactivation domain. These mutants were identified from forward genetic screens for defects in thymopoiesis and lack of lysozyme C (*lyz*) expression, respectively [45, 46]. Homozygous embryos for either mutation showed lack of definitive hematopoiesis but behaved differently with respect to survival. *cmyb*$^{I181N/I181N}$ mutant embryos displayed severe anemia and became bloodless by 20 dpf. Although the mutants survived for 2-3 months with stunted growth, there were no detectable hematopoietic cells by FACS or histology [45]. This is in contrast to our finding with *runx1*$^{W84X/W84X}$ mutants, thus suggesting differential requirements for *runx1* and *cmyb* activities during larval and adult hematopoiesis. On the other hand, most of the cmyb^{hkz3} mutants (splice site mutation affecting the transactivation domain) died by 10 dpf. The authors did not explain the reason for this difference. We speculate that the husbandry differences between laboratories might be the reason for their differential survival in the absence of blood cells. Using time-lapse imaging of *cmyb*^{hkz3}*/Tg(cd41:GFP)* embryos and lineage tracing, Zhang and colleagues [46] demonstrated an important role for *cmyb* in the migration of HSCs from ventral wall of the dorsal aorta (VDA) to CHT, thereby proposing that migratory defects of HSCs maybe the cause of failure of definitive hematopoiesis in *cmyb* deficient embryos. Thus, zebrafish models of *cmyb* deficiency have

provided novel insights into its role in the migration of HSCs from AGM to CHT during definitive hematopoiesis.

3.3. Genes Involved at the Level of Erythropoiesis, Myelopoiesis, and Lymphopoiesis: gata1, spi1, and ikzf1. Differentiation of HSCs during definitive hematopoiesis into lineage-committed progenitors, which further differentiate into mature blood cells, is mediated by lineage-specific transcription factors [77]. Unlike HSCs, these lineage-committed progenitors lack the potential for self-renewal and thus require a constant supply of HSCs for their production [87, 140]. The first series of lineage-committed multi-potent progenitors are termed common myeloid and common lymphoid progenitors (CMPs and CLPs). In mammals, CMPs further differentiate into megakaryocyte-erythroid progenitors (MEPs) that produce mature erythrocytes and platelets (erythropoiesis), and granulocyte/macrophage progenitors (GMPs) for the generation of mature myeloid cells (myelopoiesis). CLPs produce mature lymphoid lineage cells (lymphopoiesis). However, intermediate multilineage progenitors have not been identified in zebrafish yet, and all lineage relationships are speculative. Here, we have summarized the genetic controls of erythropoiesis, myelopoiesis, and lymphopoiesis in zebrafish.

Erythropoiesis involves differentiation of erythroid-myeloid progenitors into mature erythrocytes and thrombocytes. The master regulator of erythropoiesis is GATA1, a transcription factor belonging to the GATA family (6 members) that contains a conserved DNA binding domain consisting of two zinc fingers [140, 141]. Its consensus DNA binding site, WGATAR, is found in regulatory regions of most erythroid-specific genes [142]. Human mutations in *GATA1* are associated with anemia, thrombocytopenia and acute megakaryoblastic leukemia in Down Syndrome patients [143]. *Gata1* knockout mouse embryos die by E10.5 due to severe defects in erythropoiesis during primitive hematopoiesis, precluding assessment of its role in definitive hematopoiesis without generating conditional knockout mice [107, 144].

The zebrafish *gata1* gene was identified by cross-hybridization with the zinc-finger region of Xenopus *Gata1* [145]. Its expression is consistent with the sites of erythropoiesis during primitive hematopoiesis starting at 5-somite stage [49]. Using positional cloning of one of the bloodless mutants, termed *vlad tepes* or vlt^{m651}, identified in the 1996 large-scale forward screens, our group identified a truncation mutation, R339X, distal to the C-terminal zinc-finger domain in Gata1 [23]. As expected, homozygous mutant embryos displayed defects in primitive erythropoiesis and lacked visible circulating blood cells at the onset of circulation. Evaluation of definitive hematopoiesis by WISH revealed similar defects in erythropoiesis but normal development of myeloid and lymphoid lineages, thus demonstrating the specific role of Gata1 in generation of erythroid progenitor cells not only during primitive but also during definitive hematopoiesis [23, 48].

Myelopoiesis involves differentiation of erythroid-myeloid progenitors into differentiated macrophages/monocytes, mast cells, and granulocytes, including neutrophils and eosinophils [9, 80, 82]. The master regulator of myelopoiesis is *SPI1* (previously known as *PU.1)*, an oncogene originally identified as the site of genomic rearrangements by spleen focus-forming proviral insertion in erythroblastic tumors [103]. SPI1 belongs to the ETS family of transcription factors that bind DNA through a purine rich sequence, termed the PU box [146]. *Sfpi1* knockout mice died around E18 due to multilineage defects, implicating additional roles of *Sfpi1* in erythropoiesis and lymphopoiesis [110]. *In vitro* studies have demonstrated the importance of a negative cross-regulation of Gata1 and Sfpi1 during erythroid and myeloid differentiation from CMPs [140]. Unlike mammals, the sites of erythropoiesis (PLM) and myelopoiesis (ALM) are separate in zebrafish during embryogenesis [50, 51]. However, upregulation of myelopoiesis in *gata1* morphants and ectopic expression of *gata1* in *spi1* morphants proved that similar cross-regulation of these two transcription factors is critical for the proper commitments of erythroid and myeloid lineages in zebrafish [147, 148].

Lymphopoiesis involves differentiation of lymphoid progenitors into mature T and B cells that participate in a functional immune system of the organism [11]. Primary lymphoid organs for T-cell maturation in zebrafish are bilateral thymii, which are marked by expression of *rag1*, *ikzf1* and *lck* starting at ~72 hpf [56, 57, 59]. Pancreas has been suggested as an intermediate site for the production of B cells [149] between 4 dpf to ~3 weeks, at which point B cells become evident in the kidney. However, this remains to be verified, as no good transgenic markers of B cells currently exist to follow their development in real time. The master regulator of lymphopoiesis is the transcription factor IKZF1 (previously known as IKAROS) [150]. IKZF1 contains six zinc-fingers that are involved in DNA binding and protein-protein interactions [151]. By analysis of knockout mice, Wang and colleagues [152] demonstrated differential requirements of Ikzf1 for B- and T-cell differentiation during fetal and adult hematopoiesis. *Ikzf1* null mice displayed complete blockage of differentiation of B cells during both fetal and postnatal stages. On the other hand, they displayed blockage of differentiation of T cells only during the fetal stage. Postnatal T-cell development recovered, albeit with deregulation of CD4 versus CD8 lineage commitment. Overall, their data suggested that *Ikzf1* is essential for lymphopoiesis (both B and T cells) during fetal hematopoiesis, but it is dispensable for adult T cell development. Similar to the knockout mice, zebrafish with a truncation mutation, Q360X, in *ikzf1* ($ikzf1^{t24980}$), which removes the C-terminal two zinc fingers essential for protein-protein interactions, are adult viable [58]. Mutant fish displayed complete lack of lymphopoiesis during larval stage, and partial recovery after 14 dpf. Although the mutant fish survived and lived up to at least 17 months in nonsterile conditions, they displayed abnormal and inefficient lymphoid development. However, it is interesting to note that similar to our observations of two phases of definitive hematopoiesis in *runx1* mutants, zebrafish lacking Ikzf1 activity potentially demonstrated two phases of lymphoid development. In both cases, the larval phase is gene activity dependent while the adult phase develops to some extent despite the lack of gene activity.

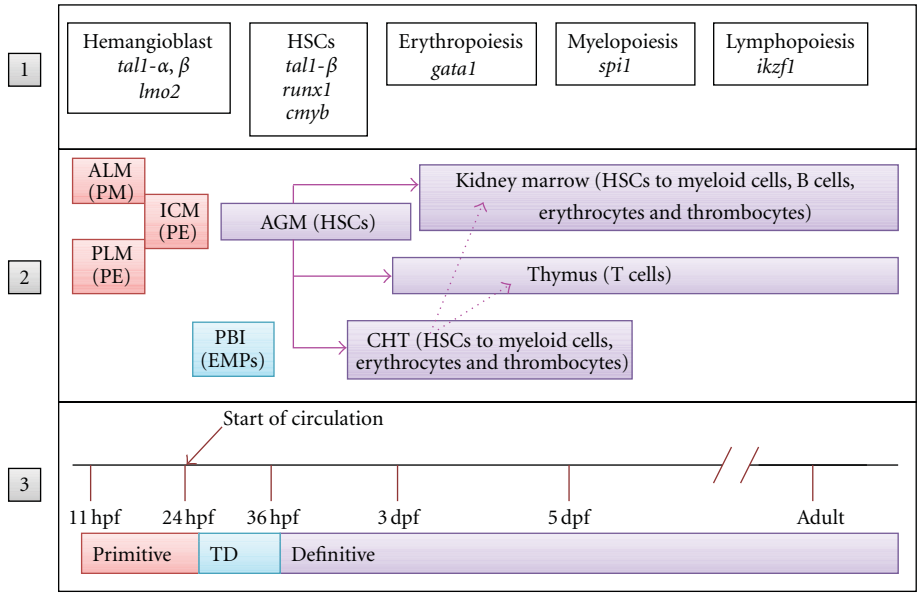

Figure 1: A schematic of overall view of zebrafish hematopoiesis with shifting sites, types of cells produced at each site, and genes involved, shown in 3 tiers as described below. Tier 1: lineage-specific transcription factors that control primitive and definitive hematopoiesis in zebrafish. Tier 2: the sites of action during each stage of hematopoiesis and the types of cells produced at each of the sites. The site boxes are color matched with waves of hematopoiesis and temporally placed according to the developmental stages in Tier 3. Tier 3: the time scale depicting the stage of development in hpf (hours postfertilization) and dpf (days postfertilization) and different waves of hematopoiesis. The abbreviations used are as follows: ALM: anterior lateral mesoderm, PLM: posterior lateral mesoderm, PBI: posterior blood island, AGM: aorta-gonad-mesonephros, CHT: caudal hematopoietic tissue, PM: primitive macrophages, PE: primitive erythrocytes, HSCs: hematopoietic stem cells, TD: transient definitive wave.

4. Different Activity-Levels, Domains, and Isoforms of the Same Transcription Factors Are Required during Different Stages of Hematopoiesis

Recent studies have demonstrated the need to address dosage requirements of transcription factors in the hematopoietic cascade as opposed to a simple on versus off situation [153–156]. In zebrafish, it is relatively easy to manipulate gene dosage by careful tuning of morpholino doses and generation of hypomorphic alleles using TILLING. Therefore, differential requirements for some of the transcription factors either in terms of level of activity or different isoforms have been demonstrated recently in zebrafish, as discussed below.

4.1. Tal1. As discussed previously, Tal1 plays critical roles during both primitive and definitive hematopoiesis. Using different doses of morpholinos to completely or partially abolish Tal1 activity, Juarez and colleagues [120] demonstrated differential requirements of *tal1* expression for erythroid specification and maturation during primitive hematopoiesis. Their work showed that lower activity of Tal1 was sufficient for primitive erythroid specification but not their maturation. Furthermore, by complementation experiments with wild-type and DNA-binding mutant forms of Tal1, they demonstrated differential requirements for the DNA-binding activity of Tal1 during erythroid specification and maturation. Their data suggested different mechanisms of target gene regulation during erythrocyte specification and maturation by Tal1: direct binding to promoters of the target genes involved in erythroid maturation and indirect regulation through other protein complexes for genes involved in erythroid specification.

Further complexity to Tal1 requirements during primitive and definitive hematopoiesis became obvious from the analysis of its two isoforms: the full-length form termed Tal1-α and a shorter form lacking the first 146 amino acids, termed Tal1-β. Using morpholinos to specifically target the α and β forms, Qian and colleagues [157] demonstrated that both forms act redundantly in initiation of primitive hematopoiesis, while only the Tal1-β form is required for the specification of HSCs in the AGM to initiate definitive hematopoiesis. Ren and colleagues [158] examined the requirements of Tal1-α and Tal1-β during angioblast and HSC specification, also demonstrating the requirement for Tal1-β in HSC specification. Thus, zebrafish research has contributed significantly to our understanding of the regulation of different stages of hematopoiesis by Tal1.

4.2. Gata1. Similar to Tal1, Gata1 activity is crucial for erythropoiesis during both primitive and definitive hematopoiesis. Recently, we described a hypomorphic allele of Gata1 due to a missense mutation, T301K, in its C-terminal zinc finger [48]. This mutation reduces DNA binding affinity and diminishes transactivation of target gene expression by Gata1 [48]. The $gata1^{T301K/T301K}$ fish had defective primitive erythropoiesis but normal definitive hematopoiesis. By combining the T301K allele with the

Gata1 null allele of *vlad tepes*, we were able to generate an allelic series with different Gata1 activity levels, listed in the descending order: $gata1^{+/+}$, $gata1^{+/T301K}$, $gata1^{+/vlt}$, $gata1^{T301K/T301K}$, $gata1^{T301K/vlt}$, $gata1^{vlt/vlt}$. Analysis of fish with these genotypes demonstrated that erythropoiesis during primitive hematopoiesis requires higher activity level of Gata1 than erythropoiesis and thrombopoiesis during definitive hematopoiesis [48].

5. Concluding Remarks

Depicted in Figure 1 is a schematic of the overall view of zebrafish hematopoiesis emerging from these studies. It is clear from the above-mentioned studies that zebrafish has played a significant role in our understanding of the genetic controls of hematopoiesis, particularly the dosage-specific requirements during different stages. The viability to adulthood with multi-lineage hematopoiesis in *runx1* knockout zebrafish clearly demonstrated that Runx1 is dispensable for adult hematopoiesis. Similarly, Ikzf1 was found to be dispensable for adult lymphopoiesis. On the other hand, Cmyb was found to be essential for adult hematopoiesis, while dispensable for larval definitive stage. Genetic mutants need to be generated for *spi1* to elucidate its exact role in maintaining proper balance between adult erythropoiesis and myelopoiesis.

Proper functioning of the genetic controls regulating hematopoiesis is crucial for normal development of all the blood lineages. Mutations in critical genes at many of the steps lead to leukemogenesis. Thus, adult viable mutant zebrafish would allow us to understand the process of leukemogenesis. Furthermore, the recent application of next generation sequencing technologies to a variety of leukemia samples have led to the identification of several new genes mutated in leukemias [159, 160]. We anticipate that understanding their roles in normal hematopoiesis using the many advantages of the zebrafish model for hematopoiesis research would aid in therapeutic advances in the coming years.

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Review Article

Myelopoiesis and Myeloid Leukaemogenesis in the Zebrafish

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Over the past ten years, studies using the zebrafish model have contributed to our understanding of vertebrate haematopoiesis, myelopoiesis, and myeloid leukaemogenesis. Novel insights into the conservation of haematopoietic lineages and improvements in our capacity to identify, isolate, and culture such haematopoietic cells continue to enhance our ability to use this simple organism to address disease biology. Coupled with the strengths of the zebrafish embryo to dissect developmental myelopoiesis and the continually expanding repertoire of models of myeloid malignancies, this versatile organism has established its niche as a valuable tool to address key questions in the field of myelopoiesis and myeloid leukaemogenesis. In this paper, we address the recent advances and future directions in the field of myelopoiesis and leukaemogenesis using the zebrafish system.

1. Introduction

The zebrafish is emerging as a powerful model system in which to study haematopoiesis and leukaemogenesis. In addition to the benefits afforded by scale and simplicity of this versatile genetic model system for studying developmental aspects of haematopoiesis, the last decade has seen an explosion of molecular methods and models to facilitate studies informing on haematopoietic disease biology, particularly leukaemogenesis and cancer. At its inception as a cancer model, proliferation and angiogenesis were proposed as phenotypic attributes as readouts relevant to cancer pathogenesis [1]. However, it was the generation of a transgenic zebrafish expressing the *C-myc* oncogene under the control of the *rag2* promoter that went on to develop T-cell acute lymphoblastic leukaemia (ALL), which really revolutionized the view of the scientific world on this small organism as a cancer disease model [2]. In the ensuing 10 years, many models of oncogene induced cancer have been generated in zebrafish along with mutagenesis strategies to identify novel tumour suppressor genes or chromosome instability loci [3–5]. The utility of such models to answer key biological questions continues to grow. In this paper, we focus on developments in the field of myelopoiesis in the zebrafish, cancer models affecting the myeloid lineages, and how these have instructed our knowledge on the biology of these diseases.

2. Zebrafish Myeloid Development

Zebrafish haematopoiesis occurs in two waves in the developing embryo, termed primitive and definitive [6]. In contrast to human and murine haematopoiesis (where primitive haematopoiesis initiates with the development of primitive erythroid cells in the blood islands of the yolk sac), in zebrafish, primitive wave erythroid cells develop from caudal lateral plate mesoderm in bilateral stripes that migrate towards the midline forming a structure termed the intermediate cell mass (ICM). A population of primitive macrophages also emerges from a distinct anatomical location in the anterior lateral plate mesoderm (ALPM) between 12 and 24 hours after fertilization (hpf) [7, 8]. Definitive haematopoiesis initiates at around 24 hpf in the posterior

blood island (PBI), with the emergence of bipotent erythromyeloid progenitors (EMPs). These cells are marked in their undifferentiated state by combined expression of *gata1* and *lmo2* or by expression of *cd41* [6]. These cells have both proliferative and differentiation potential and increase in number, peaking at 30–36 hpf. This wave of haematopoiesis gives rise to further erythrocytes and myeloid cells and recently has been shown to give rise to early mast cells in developing embryos [9]. Multipotent definitive haematopoietic stem cells (HSCs) expressing *cd41*, *c-myb,* and *runx1* arise directly from *kdrl*-expressing haemogenic endothelium in the ventral wall of the aorta starting around 26–28 hpf [10, 11]. These cells then migrate to the caudal haematopoietic tissue (CHT) where they seed and divide giving rise to all lineages of adult blood cells. These cells go on to populate the adult organs of haematopoiesis in the zebrafish, the kidney and the thymus. The precise timing of the move from primitive wave haematopoiesis to definitive wave haematopoiesis has yet to be fully established, but evidence from globin gene expression and mutants with normal primitive wave blood production suggests that the major contribution of haematopoiesis comes from definitive HSC derived cells by around 5 days post fertilization [12–14].

3. Tools for Dissecting Myelopoiesis

Cross-reactive antibodies to zebrafish proteins are lacking, arguably more so in the haematopoietic system than in others. This limitation means that the detailed lineage and differentiation status analysis of haematopoiesis, so elegantly understood in the murine system, is currently challenging to undertake in the zebrafish. Thus a major endeavour in recent years has been the generation of new tools for such analysis in the haematopoietic system. Along with the development of these tools has also come a much broader understanding of myeloid lineage development in zebrafish. The first transgenics developed to mark myeloid cells expressed enhanced green fluorescent protein (eGFP) from the major myeloid transcription factor *pu.1*. Tg(*spi1/pu.1:eGFP*) animals express eGFP in primitive wave myeloid cells but by 2 days postfertilization (dpf), expression of eGFP in myeloid cells is markedly reduced as *pu.1* expression is downregulated [15, 16]. To visualize neutrophil granulocytes later in development, several transgenic lines have been generated by various laboratories. These include the Tg(*lysc:dsRed*) and Tg(*lysc:eGFP*) lines [17] as well as Tg(*mpx:eGFP*) [18, 19] and Tg(*myd88:eGFP*) [20]. While all of these lines label predominantly neutrophil granulocytes, it is notable that the overlap in expression of the endogenous transcripts (by *in situ* hybridization) or protein (by antibody) as well as the reporter gene expression between transgenic lines is not fully concordant, suggesting that subtly different populations are labelled by each transgene depending on the developmental time point of evaluation [17, 21]. Some of these subtleties in gene and protein expression have been addressed. *L-plastin* specifically has in some early studies been suggested to mark monocyte/macrophage lineage cells but there is a clear evidence that this protein is expressed (as in mammals) in all leucocytes [21]. The Tg(*lysc:eGFP*) expresses GFP from 22 hpf, initially in primitive macrophages arising from the ALPM. Expression of eGFP increases and is notable in the CHT (likely labelling and differentiating definitive myeloid cells) and the developing brain and retina (more likely to represent the on-going expression in a proportion of macrophages). To clarify precisely which cells express the eGFP from the Tg(*lysc:eGFP*) transgene, Hall et al. performed anti-GFP staining along with fluorescent *in situ* hybridization for *mpx*, *l-plastin*, and *fms*. Dual staining was observed for eGFP with each of these myeloid transcripts; however, there were some eGFP (lysc) expressing cells that did not express *mpx*, some *fms* expressing cells that did not express eGFP (lysc), and some *l-plastin* expressing cells that did not express eGFP. Thus, the Tg(*lysc:eGFP*) marks primitive macrophages and a majority of developing granulocytes but does not label all *mpx* positive granulocytes or all *fms* expressing macrophages [17]. It is conceivable that these subtleties may in time come to give us more detailed information about subpopulation of myeloid cells, such as their stage of differentiation. More recently transgenic lines using the *mpeg1* or *fms* (*csf1r*) promoter [22, 23] have been used to distinguish macrophage populations from granulocytic myeloid cells, further enhancing studies of innate immune system. However, *fms* reporter animals exhibit expression in neural crest-derived xanthophores as well as macrophages, which may result in some limitations in the use of this system. By contrast, the *mpeg1* promoter appears exclusive to macrophages, but expression in adult fish is maintained only in zebrafish lines generated using direct transgenic approaches, and not detectable in those lines in which *mpeg1* is linked to a *GAL4/UAS* expression system. To further delineate the expression pattern of macrophages and other mononuclear phagocytes in adult zebrafish, a promoter fragment of the MHC class II beta gene, *mhc2dab,* was isolated. By virtue of the combined transgene expression, the Tg(*mhc2dab:eGFP*) transgenic line in combination with Tg(*CD45:dsRed*) (which labels all leukocytes except B cells) has now allowed identification of macrophages and dendritic cells as well as B lymphocytes in adult zebrafish tissues [24].

Several recent studies have also delineated additional granulocytic subpopulations. Zebrafish mast cells can be identified by expression of the *cpa5* transcript, and, like their mammalian counterpart are positive for toluidine blue, express mast cell tryptase and Cd117 at the protein level [25], as well as elements of the Tol-like receptor (TLR) pathway as evidenced by coexpression of *cpa5* and *GFP* in the Tg(*myd88:eGFP*) transgenic line [26]. These cells have also been isolated after fixation by flow cytometry of fast red stained *in situ* hybridization for *cpa5* [27]. The distinction of zebrafish mast cells from zebrafish eosinophils has also been addressed using a BAC-engineered transgenic line expressing GFP from the *gata2* promoter. This study confirmed the presence of and described in detail the characteristics of zebrafish eosinophils. In the Tg(*gata2:eGFP*) line, eosinophils express high levels of eGFP and have high forward and side scatter characteristics by flow cytometry. These cells were also demonstrated to be functionally orthologous to human eosinophils [28]. A summary of transgenic

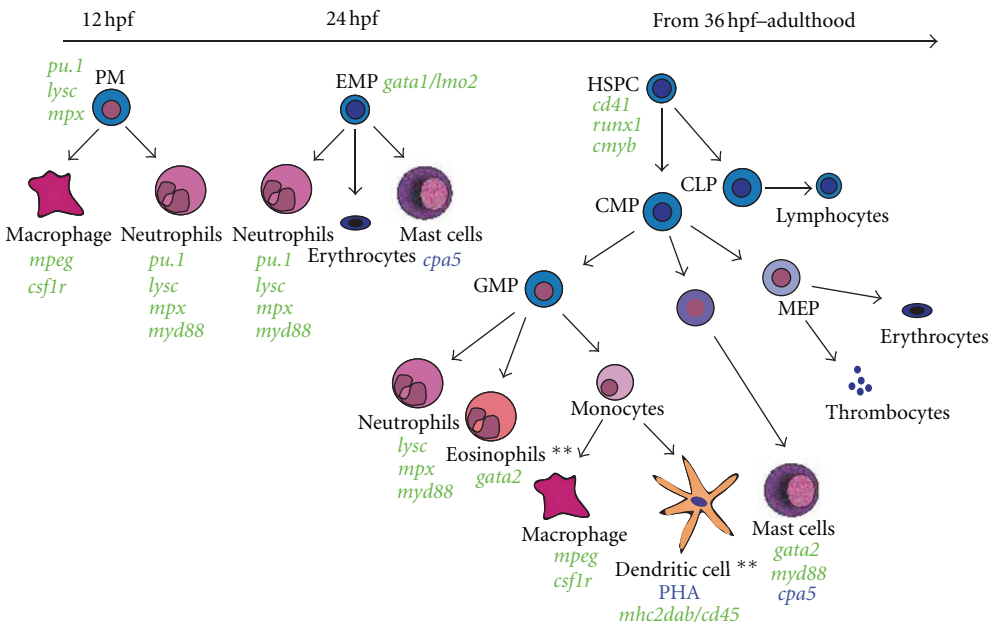

Figure 1: Overview of zebrafish developmental myelopoiesis, key transgenic lines, and lineage identification tools labelling myeloid cell populations during developmental haematopoiesis. (Transgenic lines are shown in green, other specific lineage identifiers are in blue.) PM: primitive myelopoiesis; EMP: erythromyeloid progenitors; HSPCs: haematopoietic stem and progenitor cells; CMP: common myeloid progenitor; CLP: common lymphoid progenitor; MEP: megakaryocyte/erythroid progenitor; GMP: granulocyte/monocyte progenitor; PHA: peanut haemaglutinin. **Denotes lineages only demonstrated in adult zebrafish. Lineage intermediates are shown for clarity but are yet to be isolated as distinct populations in zebrafish.

lines and markers facilitating myeloid populations is shown in Figure 1.

As well as facilitating assessment of the ontogeny and spectrum of zebrafish haematopoietic and immune systems, the utility of this array of transgenic animals extends to a more functional analysis of zebrafish haematopoiesis, which will be particularly useful in zebrafish disease models. Once again utilizing cell sorting by flow cytometry, Stachura et al. have established an assay system in which to assess the clonogenic myeloerythroid capability of subpopulations of haematopoietic cells [29]. This recent study utilized traditional clonogenic techniques, commonly used for mammalian haematopoietic cell analysis in methylcellulose, facilitated by recombinant zebrafish growth factors, erythropoietin and granulocyte colony stimulating factor and serum derived from carp. Such studies are in their infancy in the zebrafish system but should lead the way to further capability to assess clonogenic and lineage potential of individual cells and populations. Critically, this will allow more detailed biological analysis of haematopoietic populations which are currently lacking.

4. Studies of Developmental Myelopoiesis

Many aspects of myelopoiesis have been interrogated using the zebrafish embryo. Foremost, forward genetic screens have been employed to identify novel genes required for primitive or definitive myelopoiesis. The critical role of transcription factors and developmental microenvironment in determining haematopoietic lineage fate choice has also been elegantly addressed using this model, using reverse genetics and transplantation techniques. More recently transient heterologous overexpression of mutated human oncogenes has provided some mechanistic insight into the potential pathogenetic effects of such genes on normal developmental haematopoiesis and malignant transformation. In addition functional studies have also addressed aspects of the innate immune system using the zebrafish (also reviewed elsewhere in this issue of AIH). What follows is a summary of a selection of studies in zebrafish that highlight its diverse and unique capacity to answer a range of biological questions pertaining to myelopoiesis.

4.1. A Myeloid Mutant Identified in a Forward Genetic Screen. Several zebrafish studies have identified novel genes involved in myelopoiesis. Bolli et al. identified the *grechetto* mutant with a mutation within the *cpsf1* gene from an early pressure genetic screen for genes involved in definitive myelopoiesis at 5 dpf. On further investigation, *grechetto* mutants displayed pan-haematopoietic defects, arising from apoptotic cell death of developing haematopoietic stem and progenitor cells (HSPCs). The CPSF1 protein is part of a complex of genes required for processing of the 3′UTR and addition of the poly(A) tail on a subset of pre-mRNAs. CPSF1 specifically recognizes a canonical polyadenylation signal within these pre-mRNAs. Bolli et al., showed that in *grechetto* mutants the transcript encoding the snRNP70 lacked a poly(A) tail [13]. This gene was also identified

from a screen for abnormal HSC production [30] and is of particular note because of its role in normal pre-mRNA splicing. Since publication of this report in zebrafish, both loss of function and gain of function mutations in several genes required for normal splicing have been identified as contributing to the pathogenesis of human myelodysplastic syndromes (MDS) [31, 32].

4.2. Lineage Fate Choice Studies. Studies in zebrafish embryos have also shed light on the lineage fate decisions during developmental haematopoiesis. Elegant studies of Rhodes and Galloway showed the interplay between the major myeloid and erythroid transcription factors *pu.1* and *gata-1*, respectively, in regulating the fate choice between erythropoiesis and myelopoiesis [33, 34]. Building on these studies Monteiro et al. examined the "bloodless" moonshine mutant carrying a truncating mutation in the transcription intermediate factor-1γ (*tif*1γ) gene. While previous studies had demonstrated a requirement for *tif*1γ in maintenance of primitive erythropoiesis [35], definitive haematopoiesis had not been examined. In this study Monteiro et al. showed that HSPCs are specified and emerge normally from the aorta in moonshine mutants. Subsequently *tif*1γ is required for normal erythroid differentiation in the CHT at 4 dpf, while expression of differentiated myeloid markers (*mpx* and *l-plastin*) were expanded in the same region. Moonshine mutants also showed increased levels of *pu.1* and reduced levels of *gata-1* at this time in the CHT suggesting that *tif*1γ may interplay with these transcription factors in the regulation of myeloid versus erythroid fate in progenitor cells derived from definitive HSCs. To determine whether these findings may also be relevant to other stages of haematopoietic development, expression of erythroid and myeloid lineage markers were assessed in moonshine mutants along with *gata-1* and *pu.1* morphants at various time points during developmental haematopoiesis [36]. The authors concluded that *tif*1γ modulates the erythro-myeloid fate choice by regulating the expression of *gata-1* and *pu.1,* and this regulation showed distinct patterns during specific phases of developmental haematopoiesis. This study demonstrated a novel role for *tif*1γ as a regulator of cell fate decisions, and also highlighted the dynamic changes in levels of transcription factors and their interactions that occur during developmental haematopoiesis.

A recent study by Li et al., has also addresses lineage fate decisions between the macrophage versus the granulocytic lineages. In this study the interferon regulatory factor 8 (*irf8*) was identified as a novel regulator of terminal myeloid differentiation downstream of *pu.1*, that promoted the development of the macrophage lineage at the expense of neutrophils during primitive and definitive haematopoiesis [37]. Morpholino knockdown of *irf8* depleted the number of embryonic macrophages and expanded the neutrophil population with the underlying mechanism determined to be a cellular fate switch. There was no definitive evidence for decreased neutrophil apoptosis or increased proliferation to account for increased neutrophil numbers and double-labelling of *l-plastin* and *mpx* or *fms* in *irf8* morphants revealed a predominance of *l-plastin* and *mpx* positive cells. Transgenic overexpression of *irf8* achieved through generation of a Tg(*hsp70:irf8myc*) transgenic line, promoted macrophage development at the expense of neutrophils [37], but could not rescue macrophage development following *pu.1*-morpholino injection. Interestingly, *Irf8*-mutant mice develop a chronic-myelogenous-leukaemia- (CML-) like syndrome with elevated numbers of neutrophils [38, 39]. Taken in this context, this study not only identifies a novel role for *irf8* in normal myelopoiesis, but also highlights mechanisms that could be possibly hijacked during leukaemogenesis.

4.3. Functional Assessment of Human Leukaemia Mutations Using Developmental Myelopoiesis. Novel insights into the biology of haematopoietic malignancies have also been gained using zebrafish models expressing haematopoietic oncogenes as detailed in the subsequent section. However, one recent study has harnessed the developmental myeloid phenotype of a zebrafish mutant to functionally interrogate the effects of human nonsynonymous sequence variants (NSVs) found in human acute myeloid leukaemia (AML). In this study *ddx18* mutant zebrafish were shown to have aberrant myelopoiesis resulting from p53-dependent cell cycle arrest. Sanger sequencing of the *DDX18* gene then identified 4 NSVs in samples from patients with AML. Rescue experiments were then performed using the *ddx18* mutant zebrafish and identified that one of the NSVs appeared to exert a dominant negative effect on developmental myelopoiesis [40]. While this study was based on Sanger sequencing targeting the *DDX18* gene, it paves the way to utilize the zebrafish for other such strategies to interrogate novel NSVs now being identified in the thousands from whole genome and whole exome sequencing efforts, for functional relevance. Furthermore the value of this strategy will become even more powerful as additional models of existing known leukaemic variants and oncogenes become more prevalent, facilitating combined knockdown/overexpression studies using the existing models to test NSVs.

4.4. Heterologous Overexpression Studies. Overexpression and knockdown studies of myeloid oncogenes and tumour suppressor genes, respectively, have also been informative in studies using the zebrafish embryo. The nucleophosmin 1 (*NPM1*) gene encoding the ubiquitous nucleolar phosphoprotein nucleophosmin is lost in over one-third of patients with AML or MDS associated with loss of chromosome 5q [41]. In addition heterozygous gain-of-function mutations in *NPM1* are the most common mutations found in AML accounting for one-third of cases with normal karyotype [42]. Structurally, these mutations result in the generation of a novel nuclear export signal and loss of nucleolar localization signal and thus, in contrast to the normal exclusively nucleolar localization of NPM, mutated NPM is located in the nucleolus, nucleoplasm, and cytoplasm [43]. Furthermore, because NPM contains an oligomerisation domain, NPM mutants relocate at least some of the residual wild-type NPM to the cytoplasm and nucleoplasm. Such NPM mutants

have therefore been named NPMc+ to denote their cytoplasmic localization. Heterologous overexpression of the most common *NPM1* mutation resulting in NPMc+ (NPM mutant A) was undertaken in a study by Bolli et al. Overexpression of NPMc+ resulted in mislocalization of the zebrafish orthologues of *NPM1* (*npm1a* and *npm1b*) to the cytoplasm indicating that human NPM can oligomerize with the zebrafish Npm genes. In addition, primitive myeloid cell numbers were increased, as were *c-myb* expressing cells in the ventral wall of the aorta and *gata1/lmo2* double expressing cells in the CHT. This data suggested that NPMc+ mutant protein led to the expansion of HSPCs as well as developing primitive myeloid and erytho-myeloid progenitor cells [44]. Interestingly, such expansion of myeloid progenitors has also subsequently been demonstrated in a mouse knockin model of NPMc+ mediated leukaemia [45].

4.5. Innate Immune System. Cells of the myeloid lineage form the principle components of the innate immune system and, as such, production and development of such cells are stimulated upon exposure to pathogens. G-CSF/CSF3 and its receptor, CSF3R, have well-established roles in haematopoiesis, directing myeloid differentiation of HSCs and proliferation of progenitors [46]. In particular, CSF3 is strongly expressed in response to microbiological toxins in the blood, such as bacterial lipopolysaccharide (LPS), to promote myelopoiesis (especially granulocytes) and cellular migration towards the infection site [47]. Zebrafish possess a homologous csf3/csf3r signalling axis that functions similarly to its mammalian counterpart [48]. Overexpression of *csf3* mRNA expands embryonic myelopoiesis, but loss of zebrafish *csf3r* blocks myelopoiesis entirely with losses of *fms*-, *lyz*-, and *mpx*-expressing populations. Furthermore, exposing embryos to LPS stimulates *csf3* and *csf3r* expression, and leads to an "emergency" increase in *lyz*-expressing granulocytes in a *csf3r*-dependent manner.

Inducible nitric oxide (iNOS/NOS2) signalling also participates in the inflammatory response to infection. The zebrafish homologue, *nos2a*, appears to be dispensable for normal formation of HSPCs [49]. However, using morpholinos and L-NAME or L-NMMA (pan-NOS pharmacologic inhibitors), Hall et al. determined that Nos2a protein is required downstream of C/ebpβ to expand the HSPC population (as evidenced by increased *c-myb* and *runx1* expression) and promote myeloid differentiation in response to *Salmonella* infection [50]. In this study, zebrafish *nos2a* appears to primarily favour production of neutrophil granulocytes (evidenced by increased *lyz* expression). Hall et al. further confirmed the importance of *csf3r* signalling for "emergency" myelopoiesis during infection, as *csf3r* morphants could not mount a myeloid response upon exposure to *Salmonella*.

5. Lessons from Transgenic Zebrafish Models of Myeloid Malignancies

Aged wild-type zebrafish (24+ months) are susceptible to the development of a spectrum of neoplasms with an incidence rate around 11% [3], however the incidence of haematopoietic malignancies is rare. Studies of transgenic zebrafish, with tissue specific or ubiquitous promoters driving human or murine oncogenes, have however resulted in faithful models of myeloid leukaemias with features of their human disease counterparts. Below is a summary of the existing models of myeloid leukaemia, the novel findings such models have contributed to our understanding of human myeloid malignancies and a critique of existing and emerging technologies within this field.

5.1. K-RAS. Le et al. developed a model of K-RAS-mediated malignant disease by generating a Cre/*lox*-inducible *K-RAS*^{G12D} allele driven by the β-actin promoter. Tg(*β-actin:loxP-eGFP-loxP:K-RAS*^{G12D}) zebrafish crossed to a zebrafish carrying a heat shock promoter (*hsp70*) driving *cre* expression resulted in the development of a myeloproliferative neoplasm (MPN) between 34 to 66 days of life, with increased myelomonocytes and myeloid precursors in kidney marrow, and a significant loss of mature erythrocytes [51]. Notably these malignancies occurred in the absence of any heat shock and were rare in animals that had been exposed to heat shock. Sibling animals exposed to heat shock developed more aggressive, nonhaematopoietic neoplasms such as rhabdomyosarcoma and died as a result of these in early life, suggesting that only low doses of activated K-RAS were necessary to transform haematopoietic cells, or that expression of *cre* from the *hsp70* promoter in the haematopoietic lineage was greater or more leaky than in other tissues.

5.2. MOZ-TIF2. Using the *pu.1* promoter to drive transgene expression in myeloid cells, Tg(*pu.1:MOZ-TIF2-eGFP*) fish were the first to demonstrate overt AML in zebrafish at 14 to 26 months of life, showing an accumulation of immature myelomonocytes in the kidney marrow and a reduction in haematopoietic cells within the spleen [52]. It is notable, however, that both Tg(*β-actin:K-RAS*) and Tg(*pu.1:MOZ-TIF2-EGFP*) fish showed a low penetrance of disease, and their underlying molecular mechanisms remain unexplored.

5.3. Tel-jak2a. A handful of studies have provided more mechanistic insight into oncogenic activity in zebrafish myelopoiesis. In such a study, Tg(*pu.1:FLAG-tel-jak2a*) fish utilized a fusion oncogene created from the zebrafish orthologues of *TEL* and *JAK2*, rather than use of human cDNA [53]. In embryos, *tel-jak2a* expression leads to an accumulation of large myeloid cells in blood smears, induction of the cell cycle, and a gain in cells expressing the myeloid markers *pu.1* and *l-plastin* at 24 hpf. Interestingly, despite a loss of circulating mature erythrocytes by 48 hpf, Tg(*pu.1:FLAG-tel-jak2a*) fish also showed expanded distribution of erythroid markers *gata1* and *βe3-globin* at 24 hpf and 48 hpf. This is in keeping with other studies of Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling having wide-ranging effects on haematopoiesis in zebrafish embryos. For example, mutant *chordin* zebrafish that overexpress *jak2a* also show upregulation of both erythroid

and myeloid genetic markers [54]. This phenotype in *chordin* mutants could be rescued by injection of *jak2a* morpholino or pharmacological treatment with the Jak2 inhibitor, AG490, and phenocopied in wild-type embryos by injection of constitutively active *jak2a* mRNA. This study also suggested that the likely mechanism for the haematopoietic phenotype was hyperphosphorylation of Stat5 because the injection of zebrafish *stat5* mRNA carrying a hyperactive H298R/N714F mutation led to increases in erythroid, myeloid, and B cell numbers [55]. Similar findings were observed in a zebrafish model of the myeloproliferative disease, polycythemia vera (PCV), where erythroid dysregulation by $jak2a^{V581F}$ mRNA could be rescued by injection of *stat5* morpholino [56]. Despite these promising embryonic findings, however, none of the Tg(*pu.1:FLAG-tel-jak2a*) transgenic embryos survived to adulthood [53].

5.4. NUP98-HOXA9. Recently, our group described a myeloid-specific, Cre/*lox*-inducible Tg(*pu.1:NUP98-HOXA9*) fish line that exhibits MPN in 23% of fish between 19 and 23 months of life [57]. Despite evidence of myeloid proliferation and delayed cell maturation in kidney marrow, no animals were identified with overt AML. However, mechanistic insights were gained at the embryonic level. Following DNA-damaging irradiation, Tg(*pu.1:NUP98-HOXA9*) embryos showed increased numbers of cells in G2-M transition compared to controls and absence of a normal apoptotic response, which may result from an upregulation of *bcl2*. Furthermore, embryos showed altered haematopoiesis at 28 hpf, with increased myeloid development marked by *pu.1*, *l-plastin*, and *lysc*, at the expense of erythroid development marked by *gata1*, suggesting that expression of the NUP98-HOXA9 fusion oncoprotein is capable of altering the cell fate and myeloid cell differentiation. These early phenotypes in Tg*(pu.1:NUP98-HOXA9)* embryos highlight a potential mechanism whereby HSPCs carrying this oncogene have increased likelihood of acquiring additional mutations due to their impaired DNA damage response and also carry an aberrant population of less differentiated myeloid cells that may be preferentially targeted and thus may mechanistically account for the predisposition of these fish to develop overt MPNs [57].

5.5. AML1-ETO. Expression of the *AML1-ETO* oncogene, driven by the heat shock protein 70 (*hsp70*) promoter also results in disruption of developmental myelopoiesis in zebrafish embryos [58]. In this study, embryos show the appearance of cells with blast-like morphology, as well as upregulation of *pu.1* and downregulation of *gata1* at 20–22 hpf. Interestingly, there was a differential impact on more mature myeloid lineages, with increased granulocytes marked by *mpx*, but decreased numbers of cells expressing *l-plastin*. The transforming mechanism was identified as a downregulation of *scl,* one of the master transcription factors for embryonic haematopoiesis. All phenotypes were rescued by injecting Tg(*hsp70:AML1-ETO*) embryos with either *scl* mRNA or *pu.1* morpholino.

To date, the Tg(*hsp70:AML1-ETO*) line represents the most successful use of zebrafish to study the molecular biology of myeloid leukaemia. Despite the absence of an overt adult disease phenotype, Tg(*hsp70:AML1-ETO*) embryos have been an instrumental research tool in the identification of genetic and chemical modifiers of myeloid oncogenesis. A subset of human AML cases show deletions on chromosome 9q, which are specifically associated with the t(8;21) translocation yielding *AML1-ETO.* The effects of del(9q) result from the loss of two genes, transducin-like enhancer of split 1 (*TLE1*) and *TLE4*, in the Notch signaling pathway. A reverse genetics approach used morpholino knockdown of the zebrafish *TLE* homolog, *groucho3*, in Tg(*hsp70:AML1-ETO*) embryos to show an acceleration of the haematopoietic phenotype, namely the appearance of blast-like cells, the increase in *mpx* expression, and a loss of circulating erythrocytes [59]. In human AML, the AML1-ETO oncoprotein disrupts epigenetic programming through recruitment of histone deacetylase complexes (HDAC), which can be pharmacologically targeted by HDAC inhibitors such as trichostatin A (TSA). Taking advantage of this phenotype, Yeh and colleagues used the rescue of *gata1* expression by TSA as a proof of principle springboard for a chemical modifier screen with a library of known bioactive compounds [60]. Interestingly, they identified COX2 inhibitors, such as NS-398 and indomethacin, as novel therapeutic agents against AML1-ETO, and subsequently demonstrated the critical importance of COX2-prostaglandin E_2 signalling through the Wnt/β-catenin pathway [61] to the altered haematopoiesis in Tg(*hsp70:AML1-ETO*) fish. This proved to be an important discovery—soon after, this same pathway and therapeutic strategy was identified in a mouse model of *Hoxa9;Meis1*-induced AML [62].

5.6. Technical Challenges and Advances. The reason behind the long latency and low penetrance of overt myeloid leukaemia in zebrafish models of this disease may lie in part with the lack of available myeloid-targeted promoters that are active in early blood cells. Even with the success of the *pu.1* promoter used in several studies, endogenous zebrafish *pu.1* expression is downregulated during terminal myeloid differentiation, and has been found to be active in only ~2% of adult haematopoietic kidney marrow cells [16]. This could account for the low incidence of AML in Tg(*pu.1:MOZ-TIF2-eGFP*) fish and the lack of progression to overt AML in Tg(*pu.1:NUP98-HOXA9*) fish. Targeted promoters have also proven troublesome in other models of fish leukaemia. Sabaawy et al. showed that expression of the oncogene *TEL:AML1* from ubiquitous zebrafish *β-actin* and xenopus elongation factor 1 (*Xef1*) promoters but not early lymphoid targeted fish using the *rag2* promoter could produce pre-B (ALL) [63]. Such lessons suggest that the use of promoters that are active earlier in zebrafish blood development may prove more robust at driving leukaemic transformation. However, the use of ubiquitous promoters carry the caveat of off-target effects, as seen in Tg(*β-actin:K-RASG12D*) fish where MPN was one of a spectrum of disease phenotypes, including rhabdomyosarcoma, intestinal

hyperplasia, and malignant peripheral nerve sheath tumours [51].

Potency of the oncogenic signal is another hurdle to successfully modelling leukaemia in fish. For example, Tg(*pu.1:FLAG-tel-jak2a*) fish as well as the early models of Tg(*rag2:eGFP-Myc*) fish [2] display such severe abnormalities that animals do not survive to breeding age, and so embryos must be reinjected for every study. Cre/*lox*-inducible strategies can be helpful to establish germline transmission of the oncogene, but historically the most reliable method to control Cre activity was to use the *hsp70* promoter, which is known to have leaky expression [51, 57]. This in turn has also suggested that oncogene dosage is likely to have a direct impact on the penetrance and type of malignancies induced as described above for the Tg(*β-actin:loxP-eGFP-loxP:K-RASG12D*) [51]. Direct use of the *hsp70* promoter to drive oncogene expression has proven fruitful in the study of *AML1-ETO*, but the absence of an adult phenotype may reflect the transience of promoter activity following heat-shock activation. Tamoxifen-inducible Cre recombinase (Cre-ERT2) may allow tighter temporal control of transgene expression [64] and can dramatically improve the leaky expression in Tg(*hsp70:Cre*) animals [65]. Hans et al. show that, even at temperature ranges of 37–42°C, recombination events can be blocked completely in Tg(*hsp70:Cre-ERT2*) animals if tamoxifen is not applied following heat shock.

Other intriguing developments include the generation of zebrafish with mosaic expression of oncogenic transgenes [66, 67] allowing more detailed analysis of the effect on oncoprotein expression in individual cells. In mice, the use of lineage-restricted myeloid promoters, for example, *CathepsinG* [68, 69], *Mrp8* [69, 70], has not limited the success of oncogenic transformation and, in fact, committed myeloid progenitor cells have been identified as the leukaemia-initiating cell (LIC) in many karyotypes of AML [69–73]. In the zebrafish, the use of more lineage-restricted myeloid promoters (i.e., *lysc*, *mpx*, *mpeg*, *fms*) have flourished in the field of leukocyte trafficking [17, 22, 23, 74] so these may ultimately provide alternative tools for future fish models of myeloid leukaemogenesis.

Finally, given that overt AML has been achieved in only one zebrafish model to date suggests that the acquisition of mutations within collaborating proto-oncogenes and/or inactivation of tumour suppressor genes may occur less readily in the short life expectancy of the zebrafish. Alternatively, the acquisition of disease promoting cooperating mutations may be masked by increased genetic redundancy that has resulted from the additional round of gene duplication undergone in the teleost genome. However, the zebrafish is well suited to test specific interactions between collaborating oncogenes due to its high fecundity and thus capacity to generate large number of animals with a range of genotypes, as recently demonstrated in neuroblastoma by Zhu et al. [75]. Transgenic fish harbouring multiple oncogenes have also been a successful strategy for modulating the incidence of zebrafish ALL [76]. Thus future strategies to assess the contribution of collaborating mutations could be targeted at overexpression/knockdown strategies of two, three, or four genes.

Until recently, stable gene knockout studies of tumour suppressor genes have been difficult to achieve in most zebrafish laboratories. While the clinical relevance of such models is apparent from mutant alleles derived from targeting induced local lesions in Genomes (TILLING), such as p53 mutant animals [77–79], targeted, heritable gene knockdown in zebrafish has been a major challenge for the community over the past decade. The last few years have seen a major sea change with the snowballing of technical advances in this regard. Initial reports of zinc finger nuclease- (ZFN-) induced cleavage and repair resulting in gene knockouts from two groups [80, 81] followed shortly by the publication of the oligomerized pool engineering (OPEN) system for *in vitro* identification and validation of potential gene targeting zinc fingers by Keith Joung's laboratory [82, 83] have highlighted the potential to harness this technology even in smaller laboratories. Less than 2 years later, the same groups had further refined their *in vitro* and in silico systems to allow accuracy in identification of target sites using bioinformatics alone [84]. Most recently, evidence has shown that transcriptional activator-like nucleases (TALENs), engineered from DNA binding proteins of the Xanthomonas bacteria function even more faithfully in the zebrafish system to target the enzymatic cleavage component of the FOK1 endonuclease to within a few bases of the desired double stranded DNA break [85, 86]. Of course we continue to avidly anticipate the optimization of homologous recombination methodologies to finally permit conditional knockin models of disease.

6. Using the Zebrafish as a Xenograft Model for Myeloid Leukaemia

Overall, compared to the lymphoid tumours, models of myeloid leukaemia are relatively less penetrant with leukaemia rates ranging from 25% [51] to <1% [52]. The generation of novel promoters may facilitate more faithful models of human myeloid disease in zebrafish. In particular, dissection of the zebrafish *runx1* promoters has provided new insights into the regulation of this gene in zebrafish but may also prove to be a better driver of oncogene-induced malignant myeloid disease [87]. One potential complimentary strategy is the recent interest in developing methodologies for xenotransplantation of human or mouse cancer cells into zebrafish and applying this approach to myeloid disease [88]. Tissue culture assays and animal models have been instrumental in determining key molecular pathway in cancer and novel drug development. However, *in vitro* assays lack the critical context of the tumour microenvironment, while mouse xenografts are cost-prohibitive and require extensive engraftment time. By contrast, the use of zebrafish facilitates scalability, where large numbers of rapidly developing, externally fertilized transparent embryos can be used to screen compounds in a high-throughput manner. By using embryos at 48 hpf, xenograft rejection is minimized, by virtue of their lack of an adaptive immune system during the first week of life [89].

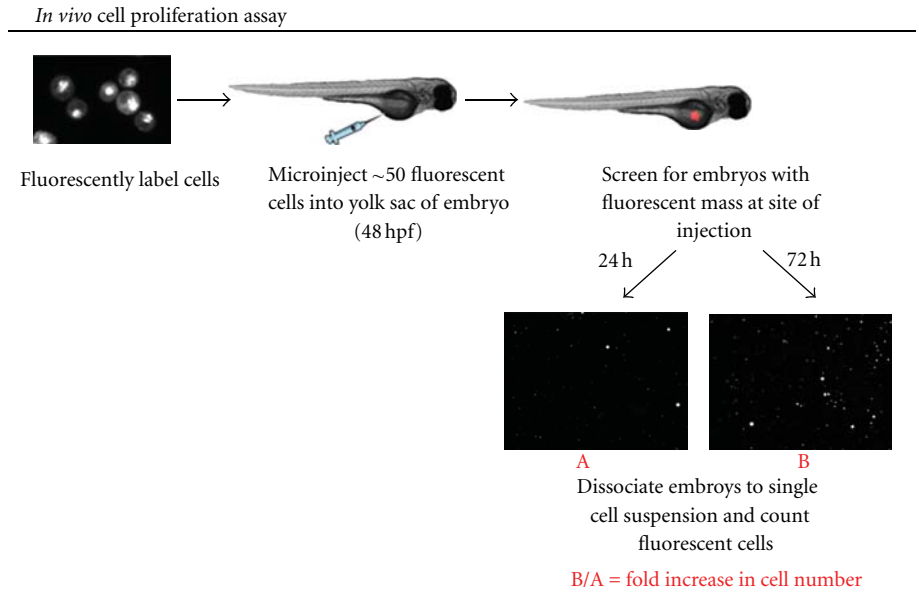

Figure 2: Schematic of *in vivo* cell proliferation assay in xenotransplanted zebrafish embryos. Human leukemia cells are fluorescently labelled with a cell tracking dye. Approximately 25–50 fluorescently labelled cells are microinjected into the yolk sac of 48 hpf casper embryos. Embryos are screened using fluorescent microscopy for the presence of a fluorescent mass at the site of injection. Positive embryos are divided into two groups; one of which is maintained at 35C for 24 h, and the other group is maintained for until the time point of interest with or without drug exposure. At the end of each time point embryos are enzymatically dissociated to a single cell suspension and the number of fluorescent cells in the suspension is counted using a semiautomated macro in Image J (NIH, Bethesda, MD). The number of fluorescent cells present at the later time point divided by the number of fluorescent cells present at 24 h represents the fold increase in cell number. Adapted from Corkery et al. [90].

A number of anatomic sites in the embryo have been trialled for xenografting, but the yolk sac is generally considered the ideal anatomic location and has been used in the leukaemia xenotransplantation studies to date [90, 91]. Incubation of xenografted embryos at 35°C enables growth of injected human cell lines in a fully constituted, 3D, *in vivo* microenvironment, without compromising zebrafish embryogenesis [89, 90, 92]. Two groups, including ours, have exploited xenotransplantation for the study of myeloid leukaemias [90, 93]. Both groups demonstrated successful engraftment and proliferation of CM-DiI fluorescently labelled K562 erythroleukemia and NB4 acute promyelocytic leukaemia (APL) cell lines following yolk sac injection in 48 hpf zebrafish embryos. Moreover, response to targeted therapy with imatinib mesylate in K562 cells harbouring the BCR-ABL1 oncoprotein or with all-*trans* retinoic acid (ATRA), a targeted inhibitor of the PML-RARα oncoprotein found in NB4 cells was observed with the addition of these compounds to the water of xenografted embryos. Pruvot et al. observed a reduction in the number of xenografted K562 cells upon exposure to imatinib and a dose-dependent teratogenic effect and death of NB4 cell xenografted embryos treated with ATRA. Our group have developed a robust *ex vivo* cell proliferation assay to quantify cell numbers over time following xenotransplantation (Figure 2) and demonstrated that xenografted K562 cells specifically responded to imatinib, resulting in decreased cell numbers but no embryonic toxicity. Similar results were obtained with ATRA for xenografted NB4 cells. Importantly, when therapeutic agents were swapped and applied against the opposite cell type, leukaemia cells continued to proliferate demonstrating that human cancer cells can be specifically targeted in a zebrafish xenotransplantation model. These studies open the door for using the zebrafish xenotransplantation platform to rapidly assess the efficacy of novel compounds on the proliferation of human leukaemia cells *in vivo*. Xenotransplantation could also enable screens of currently available anticancer agents for off-label, *in vivo* activity against human leukaemia cells. More recently, as has been demonstrated for some gastrointestinal tumours [94], we have undertaken studies using primary leukaemia patient-derived bone marrow (Tugce Balci, Dale Corkery, Graham Dellaire and Jason Berman, unpublished results). We have seen similar robust engraftment, proliferation, and circulation of primary leukaemia samples and confirmed this process to be an active process, requiring functional living cells, as fixed control cells remained in the yolk. Other groups have further demonstrated differential engraftment of human leukemia subpopulations, with engraftment of CD34+ putative leukaemia stem cells but not from CD34− cells, indicating that zebrafish models may reflect the biology of disease in a similar way as mouse models and enable studies on tumorigenicity and tumour stem cells [93, 95, 96]. In parallel, with other tools, such as the development of syngeneic fish lines (CG1) [76] and the *casper* mutant fish line that permanently maintains transparency into adulthood [97], xenotransplantation will enable the zebrafish to explore questions of leukemia initiating cell frequency, clonogenicity, and the ability to serially transplant

disease. Given the complexity of genetic lesions that can present in AML and the heterogeneity of treatment response inherent in this disease, xenotransplantation models could ultimately be used in real-time analysis of primary patient biopsies as an informative diagnostic tool to predict effective therapeutic regimens and/or inform subsequent preclinical murine studies of promising novel agents, ultimately leading to Phase I clinical trials.

7. Conclusions and Future Studies

The zebrafish embryo has contributed significantly to our understanding of the developmental biology of haematopoiesis and myelopoiesis over the past decade. The exponential rise in our ability to dissect the biology of myeloid cells in this small vertebrate will no doubt fuel further insights and broaden the scope for current models of myeloid leukaemias. The advent of TALENs and zinc finger nucleases as well as the zebrafish mutation project at the Sanger Centre (http://www.sanger.ac.uk/Projects/D_rerio/zmp/) promises to deliver us knockouts for all genes in the zebrafish genome that will greatly enhance future studies, particularly of tumour suppressor genes in myeloid disease.

The forward genetic screens that identified so many novel mediators of haematopoiesis in the late 90's [98, 99] including identification of a novel human disease gene [100] have been somewhat out of vogue in recent years. However, completion of the sequencing of the zebrafish genome alongside rapidly reducing costs and improving technology for deep sequencing methodologies are likely to enhance our ability to map such mutations, even in more complex genetic backgrounds. Thus genetic modifier screens of phenotypes observed in myeloid malignancies or development may prove fruitful in the future.

One of the greatest promises for the future of the zebrafish model is its ability to make a significant contribution to the field of myeloid leukaemogenesis by identifying novel therapeutic compounds through chemical screens targeting developmental or early larval phenotypes. The ability to undertake larger scale screening projects even within the environment of academia is becoming more accessible across the zebrafish community and is being enhanced by the application of this platform to xenogeneic cells as well as recent advances in automated image acquisition and analysis capabilities [101]. The growing recognition and acceptance of the zebrafish for studying myeloid biology will enable it to secure a place among other model systems including mouse and cell culture, as a component in a pipeline of preclinical tools to better interrogate molecular pathways and rapidly identify novel therapies with conserved effects across organisms likely to impact outcome for patients with myeloid diseases.

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Review Article

Neutrophil Reverse Migration Becomes Transparent with Zebrafish

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The precise control of neutrophil-mediated inflammation is critical for both host defense and the prevention of immunopathology. In vivo imaging studies in zebrafish, and more recently in mice, have made the novel observation that neutrophils leave a site of inflammation through a process called neutrophil reverse migration. The application of advanced imaging techniques to the genetically tractable, optically transparent zebrafish larvae was critical for these advances. Still, the mechanisms underlying neutrophil reverse migration and its effects on the resolution or priming of immune responses remain unclear. Here, we review the current knowledge of neutrophil reverse migration, its potential roles in host immunity, and the live imaging tools that make zebrafish a valuable model for increasing our knowledge of neutrophil behavior in vivo.

1. Introduction

"Certain of the lower animals, transparent enough to be observed alive, clearly show in their midst a host of small cells with moving extensions. In these animals the smallest lesion brings an accumulation of these elements at the point of damage. In small transparent larvae, it can easily be shown that the moving cells, reunited at the damage point do often close over foreign bodies [1]." Ilya Mechnikov, one of the fathers of immunology, spoke these words at his Nobel Prize lecture in 1908. More than one hundred years after his seminal studies using transparent starfish larvae to illuminate a role for phagocytosis in immunity, we are again exploiting the power of transparent larvae for research on the immune system. Studies of neutrophils in both humans and mammalian model systems have brought great advances in our knowledge of their functions; however, zebrafish, a small tropical fish with transparent larvae, have demonstrated that direct observation of neutrophils in live animals can provide important insights that would have otherwise faced significant technical challenges using mice.

Neutrophils are the most abundant leukocytes in both humans and zebrafish, and they are critical for defending the host against microbial infection [2]. In response to wounding, infection, or other inflammatory stimuli, neutrophils are rapidly recruited to perform their well-known effector functions: degranulation, phagocytosis, production of reactive oxygen species (ROS), secretion of proinflammatory cytokines, and extrusion of neutrophil extracellular traps (NETs) [3, 4]. These responses are acknowledged to kill and sequester microorganisms at their site of entry and promote the activation of the adaptive immune system [4]. Until recently, it was thought that neutrophils, which responded to a wound, had a single fate: death [5, 6]. There remains clear evidence for neutrophil apoptosis in the abundance of pus that emanates from infected wounds, and the clearance of dead neutrophils from the site of inflammation has been demonstrated to occur through phagocytosis by macrophages [4–7]. However, studies of neutrophil wound responses using live zebrafish embryos revealed for the first time that neutrophils could leave an extravascular site of inflammation and persist in the host

[8, 9]. This reverse migration process requires two distinct, but related steps: migration of neutrophils away from the inflamed area and reverse transendothelial migration to enter the vascular lumen.

The observation that neutrophils can reverse migrate away from a wound in zebrafish [8] and in mice [10] raises new questions about neutrophil functions. First, the mechanism that neutrophils use to perform reverse migration and the signals that trigger it are entirely unknown. The fate of reverse-migrated neutrophils also remains to be explored. However, recent studies have demonstrated that neutrophils can affect the adaptive immune system and regulate systemic inflammation in previously unappreciated ways, raising intriguing possibilities for the roles of reverse-migrated neutrophils. Because of the conservation of functions between human and zebrafish neutrophils, as well as the many tools available for live imaging and genetic manipulation, zebrafish will certainly continue to be a critical resource for elucidating the mechanisms and functions of neutrophil reverse migration. Here, we review the features of zebrafish and some of the tools that make them particularly well suited to this task. Additionally, we will discuss the current state of the neutrophil reverse migration field and its implications for the regulation immune responses.

2. Zebrafish as a Model for Studies of Immunity

An important feature of any model organism is the ability to infer similarity of function with the species of interest, typically humans. The high conservation of immune cell lineages and effector functions indicates the suitability of zebrafish as a model through which we can better understand the human immune system. Zebrafish have many immune cell lineages in common with humans: neutrophils [8, 11, 12], macrophages [11, 13–15], T cells [16], B cells [17], mast cells [18], eosinophils [11, 19], and basophils [11]. However, the 2–4 days-post-fertilization larvae used for most zebrafish neutrophil research do not have T or B cells [16]. Particularly important for the study of neutrophil reverse migration is the conservation of function within the innate immune system. Like human neutrophils, zebrafish neutrophils are the first responders to inflammatory stimuli, where they are able to phagocytose bacteria and degranulate [15, 20, 21]. Further support for the conservation of neutrophil functions is in the recapitulation of neutrophil phenotypes in zebrafish models of Wiskott-Aldrich syndrome (WAS), warts-hypogammaglobulinemia-infections-myelokathexis (WHIM) syndrome, and leukocyte adhesion deficiency-(LAD-) like syndrome [22–24]. Many other immune effector functions are present in both fish and mammals, and these have been expertly reviewed elsewhere [25–28].

The genetic tractability of zebrafish is another attractive point of this model system, and the tools available for genetic manipulation are rapidly improving. Historically, suppression of gene expression in zebrafish has been performed by morpholino oligonucleotides, nucleic acid analogs that bind pre-mRNA to prevent splicing or the initiation of translation [29]. This method has the disadvantage of being transient and affecting the entire organism. However, more recent developments indicate that shRNA-mediated knockdowns are possible in zebrafish, which should allow the creation of transgenics with tissue-specific knockdowns [30]. While previous knockout technology relied on random mutagenesis and screening, new approaches promise to increase the availability of knockout zebrafish to the community [23, 31]. Zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN) both rely on modular DNA recognition motifs coupled to nucleases to introduce highly localized DNA lesions [32–34]. Tissue specific, inducible gene expression systems, such as those that rely on cre-mediated recombination, are actively being developed and will be critical in assessing the functions of genes whose long-term expression is detrimental [35–37]. The relative ease of transgenesis and the growing complement of tools for genetic manipulation are a very attractive point of the zebrafish model and should drive advances in understanding leukocyte behavior.

Perhaps the single most significant advantage of zebrafish larvae as a model organism is their optical clarity, which allows for noninvasive, live imaging. Furthermore, live zebrafish imaging can be accomplished with commonly available confocal microscopes or fluorescence stereomicroscopes, obviating the need for highly specialized equipment and techniques used for in vivo imaging in mice. The ability to perform live imaging in zebrafish has been enabled by the discovery of cell lineage-specific promoters that can drive expression of fluorescent proteins that label cells or other proteins of interest. Two promoters, myeloperoxidase (mpx) and lysozyme c (lyz), are used to drive neutrophil-specific expression [8, 12, 38, 39]. Recent advances have also allowed tissue-specific expression in macrophages using the macrophage-expressed gene-1 (mpeg1) and colony stimulating factor 1 receptor (csf1r) promoters [14, 15]. These promoters have enabled the creation of stable transgenic lines and the characterization of neutrophil or macrophage responses to wounds, infections, and other inflammatory stimuli.

3. Imaging Tools Used to Advance Zebrafish Research

The use of tissue-specific expression with powerful imaging tools has facilitated the application of a cell biology toolkit to zebrafish inflammation research and increased our fundamental knowledge of neutrophil motility and wound recruitment. The first studies utilizing fluorescent neutrophils in zebrafish larvae demonstrated that neutrophils rapidly respond to mechanical wound-induced stimuli, which raised two fundamental questions: (1) What are the intracellular signals that promote directional migration and (2) What are the signals recruiting neutrophils to wounds? Advances in zebrafish imaging strategies have helped to answer both of these questions.

In order to query the signaling responsible for neutrophil motility and wound responses, Yoo et al. applied

several imaging techniques from cell biology [40]. The GFP-tagged ratiometric probe, pleckstrin homology domain of Akt (PHAKT-EGFP), allowed live imaging of $PI(3,4)P_2$-$PI(3,4,5)P_3$ inside of neutrophils. Additionally, this study made use of photoactivatable Rac, whose activity could be induced in individual neutrophils with the targeted application of 458 nm laser light [41]. Finally, the F-actin probes, Lifeact and utrophin calponin homology domain (UtrCH), allowed simultaneous in vivo imaging of total F-actin and stable F-actin, respectively [42, 43]. Imaging of PHAKT-GFP demonstrated that $PI(3,4)P_2$-$PI(3,4,5)P_3$ accumulated at the leading edge of migrating neutrophils, and inhibition PI(3)K prevented leading edge $PI(3,4,5)P_3$ production, leading edge protrusion, and neutrophil motility. While photoactivation of Rac in normal neutrophils could be used to precisely control motility, photoactivation in PI(3)K inhibited cells could induce protrusions but not motility. Additionally, Rac activation could not induce proper F-actin polarization in PI(3)K-inhibited cells. This suggested a two-tiered model of PI(3)K activity in migrating neutrophils, where PI(3)K was needed for Rac-mediated leading edge protrusion but was also necessary for Rac-independent F-actin polarization [40].

The question of how neutrophils are recruited to wounds has also been partially answered by the application of advanced imaging techniques to zebrafish research. The fluorescent, reversible, genetically encoded, ratiometric probe, HyPer, is able to detect hydrogen peroxide production in vivo [44]. Niethammer et al. used this probe to show that wounding zebrafish fins induced a burst of hydrogen peroxide that was necessary for the early recruitment of neutrophils to wounds [45]. Wound-produced hydrogen peroxide was subsequently demonstrated to attract neutrophils through the oxidation-mediated activation of the src-family kinase, Lyn. The ability to perform a neutrophil-specific rescue with wild-type and oxidation-mutant Lyn, a major benefit of the zebrafish system, was critical to confirming this finding [46]. Overall, these advances have demonstrated the value of coupling fluorescent bioprobes and fluorescent-tagged proteins to live, in vivo studies of neutrophil behavior. The continued innovation of advanced imaging techniques will be critical to future advances in understanding neutrophil behavior.

4. Neutrophils Leave Wounds via Reverse Migration

Prior to the observation of neutrophil reverse migration, the previous paradigm of neutrophil responses, based on mammalian studies, was that neutrophils underwent apoptosis after responding to an inflammatory stimulus [5, 6]. The process of macrophage clearance of apoptotic neutrophils in tissues has been well established [4, 7]. However, a previous study using an experimental rat model of nephritis showed that intravascular neutrophils do not necessarily undergo apoptosis but can leave a site of inflammation through glomerular capillaries, suggesting that alternative mechanisms may mediate resolution of neutrophil-mediated inflammation [47]. In support of this idea, in vivo imaging of zebrafish neutrophils was the first direct demonstration that reverse migration was responsible for clearance of neutrophils from the interstitium of wounded tissues [8]. Indeed, studies of neutrophil reverse migration in zebrafish larvae have found that neutrophil apoptosis at a wound site is a rare event, occurring in less than 3% of the responding neutrophils [48]. Mathias et al. were able to demonstrate this reverse migratory behavior by tracking wound-responsive neutrophils in the transgenic (Tg) zebrafish line, Tg(mpx : GFP), in which GFP is expressed specifically in neutrophils. This study further demonstrated that neutrophils undergoing both forward and reverse migration to a wound had nearly equivalent velocity and directionality, implying that each was a robust, active process [8]. Using zebrafish, other groups have also observed neutrophil reverse migration under similar experimental conditions [38, 48, 49].

While illuminating, technical challenges prevented the exploration of some questions about the fate and individual behavior of neutrophils responding to a wound. The application of the photoconvertible protein, Dendra2, which can be switched from green to red fluorescence with 405 nm light, allowed these questions to be more definitively explored [50]. Dendra2-labeled neutrophils that had reached a wound were photoconverted, permitting detailed tracking of a small number of neutrophils. Thus, it was determined that individual neutrophils often traffic between the wound and the vasculature repeatedly before leaving permanently. The significance of this oscillatory behavior remains unclear, but it may reflect the competition of signals between two endpoints. Transgenic zebrafish with GFP-labeled vasculature, Tg(fli1 : EGFP), demonstrated that some reverse-migrating neutrophils do enter the vasculature, performing a true reverse transendothelial migration (Figures 1(a)–1(c)). While the oscillatory neutrophil migration and reverse transendothelial migration appear to be steps along a common pathway in the movement of neutrophils away from inflamed tissue, it is not yet clear what triggers the progression between these steps. Finally, the fate of neutrophils that responded to wounds was determined by tracking photoconverted neutrophils for two days after wounding, demonstrating two important findings: neutrophils survived for multiple days after wounding, and they were found dispersed throughout the body without obvious tissue preferences (Figure 1(d)) [9]. While these observations of neutrophil reverse migration in zebrafish were intriguing, they still faced criticism that this could be a zebrafish or larva-specific phenomenon and not applicable to mammals.

Around the time of the first observation of neutrophil reverse migration in zebrafish, two groups made observations that suggested the existence of neutrophil reverse migration in mammalian systems. Primary human neutrophils that were cocultured on monolayers of endothelial cells in vitro were observed to transmigrate through the endothelium and subsequently perform reverse transendothelial migration to return to the apical surface [51]. These reverse transmigrated (RT) neutrophils demonstrated decreased adhesion to the endothelial surface

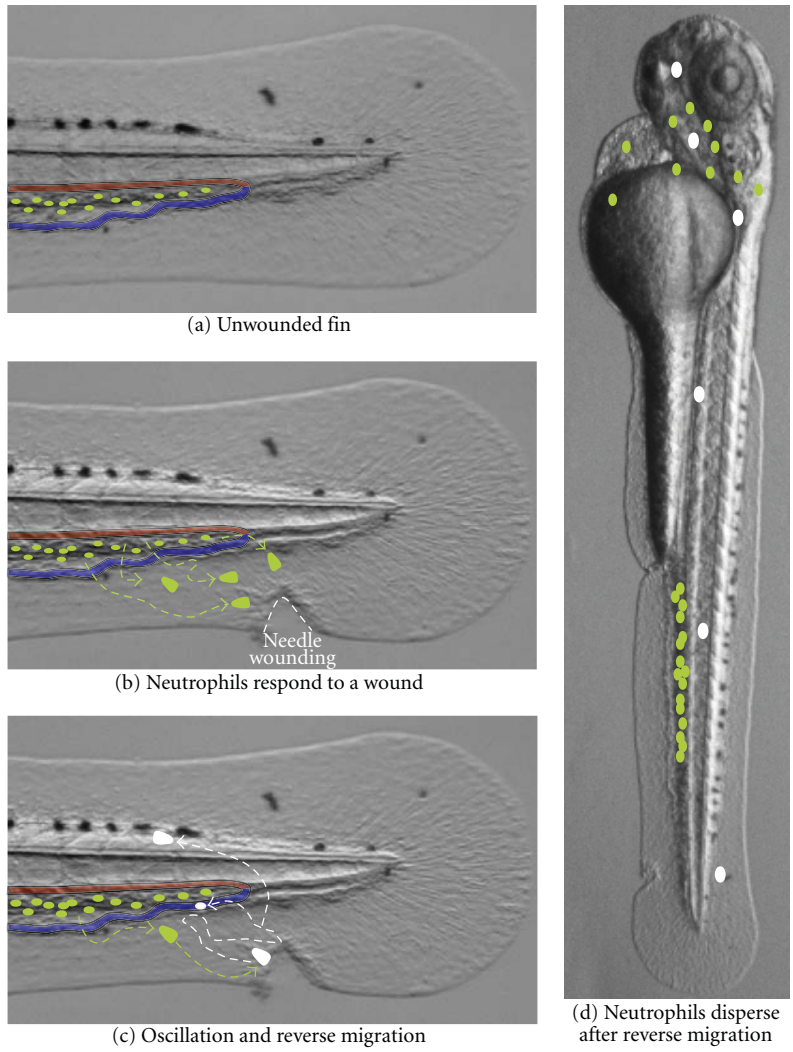

Figure 1: Neutrophil reverse migration in zebrafish larvae. This diagram illustrates the behavior of neutrophils undergoing reverse migration over an image of a 3-day postfertilization zebrafish larva that was PTU-treated to prevent pigmentation. (a) In unwounded larva, neutrophils (green ovals) are present in the caudal hematopoietic tissue (CHT), which is situated between the caudal artery (red shading) and the caudal vein (blue shading). (b) In response to a wound, neutrophils are mobilized from the CHT, and they migrate through the tissue towards a wound. (c) The green to white color change represents the ability to photoconvert individual neutrophils that reach a wound. Neutrophils often migrate between the wound and the vasculature multiple times before finally departing. Neutrophils have been observed performing reverse migration by entering the vasculature and by migrating through tissues in zebrafish larvae. (d) Reverse migrated neutrophils (white) are found dispersed throughout the body without an obvious tissue preference 2 days after leaving wounds.

and decreased tendency to undergo forward transendothelial migration as compared to "fresh" neutrophils. Another interesting finding from this study was a unique surface phenotype for neutrophils that had undergone the reverse migration process. CXCR1, the receptor for the neutrophil chemoattractant IL-8, CD11b (integrin α_M chain), and CD54 (intercellular adhesion molecule-1) along with other cell surface markers were used to distinguish the different neutrophil populations. RT neutrophils were found to be $CD11b^{high}CD54^{high}CXCR1^{low}$, which differentiated them from freshly isolated ($CD11b^{low}CD54^{low}CXCR1^{high}$) neutrophils [51]. Additionally, a study by Maletto et al. found that neutrophils in mice that had been immunized against ova peptide would transport an FITC-labeled ova peptide from the site of footpad injection to local lymph nodes. Extensive flow cytometric and histopathologic analysis demonstrated that the cells bearing ova-FITC were indeed neutrophils. One caveat of this study was that ova-FITC containing neutrophils were only found in lymph nodes in the ipsilateral, but not contralateral leg, to the site of ova administration, implying that lymphatic drainage may have been responsible for their dissemination [52]. While not definitive proof of reverse migration, these findings supported the idea that neutrophils could survive after responding to an inflammatory stimulus and could affect the immune response in a manner spatiotemporally separated from the site of this stimulus. Subsequent in vivo studies in mice have also demonstrated that neutrophils can

directly interact with B cells and T cells in lymphoid tissue, further strengthening the concept of neutrophils existing outside of their conventional roles [10, 53–55].

The most direct support for the observations of reverse migration in zebrafish has come from a recent intravital imaging study using mice. Woodfin et al. used a system in which intrascrotal inflammation, induced by ischemia-reperfusion injury, allowed the monitoring of fluorescently-labeled neutrophils. Approximately 10% of the transendothelial migration events observed with this assay were reverse transendothelial migration. Additionally, it was observed that ischemia-reperfusion injury disrupted the localization of junctional adhesion molecule C (JAM-C) to endothelial junctions and that mice with JAM-$C^{-/-}$ endothelial cells demonstrated an increase in reverse transendothelial migration, reaching greater than 50% of total transendothelial migration events [10]. It is important to note that this study did not address the migration of neutrophils in the tissue parenchyma, and this will be an interesting area for future study. These findings of neutrophil reverse migration in mice and in vitro are paralleled by monocyte studies that demonstrated reverse transendothelial migration with similar kinetics and regulation by JAM-C [56, 57]. This suggests a possible conservation in the mechanisms that mediate reverse transendothelial migration of neutrophils and macrophages.

Several differences have been observed between zebrafish and mammalian reverse migration that await further investigation. While all of the reverse migration events described thus far in mice involve transendothelial migration, some neutrophils are able to disperse throughout the body of the zebrafish larva without entering the vasculature. Most apparent is that nearly all wound responsive neutrophils perform reverse migration in zebrafish, whereas approximately 10% do so under the observed conditions in mice [9, 10]. We believe that this discrepancy may be the result of using larva versus adult animals, species-specific differences, or the type of inflammatory stimulus. However, the high percentage of reverse migrating neutrophils may provide a substantial benefit in studies of reverse migration. Mammalian studies have supported the utility of using zebrafish to study reverse migration; however, neither system has yielded a definitive answer on the mechanisms that mediate reverse migration.

5. The Mechanisms Driving Reverse Migration

Recent work in zebrafish has implicated hypoxia-inducible factor-1α (Hif-1α) in neutrophil reverse migration. Pharmacologic stabilization of Hif-1α or the expression of a dominant active Hif-1α impaired resolution of inflammation by neutrophil reverse migration [48]. While this is a promising first step, it does not appear that Hif-1α is the dominant factor regulating reverse migration.

The observed behavior of reverse migrating neutrophils, as described above, can allow us to speculate on the potential signaling mechanisms that are relevant to this process. As reverse migration occurs both during active inflammation and as part of the local resolution of neutrophil-mediated inflammation, there may be two temporally distinct phases of this process. During the early response to a wound, velocity and directionality are equivalent during forward and reverse neutrophil migration, suggesting that reverse migration is an active process [8]. Therefore, a passive mechanism, such as the loss of wound-derived chemoattractants and the random dispersal of neutrophils, is not likely to be involved.

We speculate that the signals that trigger neutrophils to perform reverse migration could include a competing chemoattractant "pulling" them away from the wound, a chemorepellent "pushing" them away from the would, or both (Figures 2(a) and 2(b)). Because neutrophils often migrate back to the vasculature after an inflammatory response, chemoattractants emanating from the blood or endothelium are attractive targets for promoting migration away from a wound (Figure 2(a)). Interestingly, high concentrations of chemoattractants, including IL-8 (CXCL8), can repel neutrophils in vitro and in vivo [58]. Other leukocytes can also be repelled by high chemokine concentrations. T cells are repelled by high concentrations of SDF-1 (CXCL12) in vivo and in vitro [59], and monocytes can be repelled by high concentrations of eotaxin-3 (CCL26) [60]. Thus, it is also plausible that the wounded tissue could be a source of both chemoattractants and chemorepellents in competition with each other. Previous studies of leukocyte chemorepulsion suggest that a wound chemoattractant at sufficiently high concentration could also act as a chemorepellent. As a neutrophil approached the wounded tissue, the concentration of chemorepellent would increase, potentially overwhelming the effect of the chemoattractant and driving the neutrophil away from the wound (Figure 2(b)).

Neutrophils respond to chemokines in a hierarchical manner, preferring some over others, when faced with competing gradients [61, 62]. The oscillatory migration of neutrophils between wounds and the vasculature suggests that a mechanism of competing chemoattractant gradients between these locations is likely (Figure 2(c)) [8, 9]. In this scenario, the signals promoting migration away from a wound (Figures 2(a)-2(b)) would compete with the signals attracting neutrophils to wounds. One complicating factor for this model is that "fresh" neutrophils continue to arrive at wounds after some have already reverse migrated, suggesting that neutrophil intrinsic regulation may also be involved in their oscillatory behavior and eventual departure. Therefore, we believe that the most likely explanation for the oscillatory behavior during the early wound response is a combination of competing chemokine gradients and neutrophil-autonomous changes in chemokine receptor sensitivity. It is known that neutrophils will internalize and downregulate G protein coupled receptors, including many chemokine receptors, after stimulation [63]. In this model, as neutrophils approach a high concentration of chemoattractant at the wound or vasculature, receptor desensitization would occur and promote migration towards the competing gradient (Figure 2(d)). Failure to internalize the CXCR4 receptor prevents downregulation of CXCR4-mediated signalling and is responsible for the retention of neutrophils in the bone marrow or caudal hematopoietic tissue of WHIM syndrome patients and a zebrafish model of WHIM syndrome, respectively [24, 64–67]. This suggests that the dynamic regulation

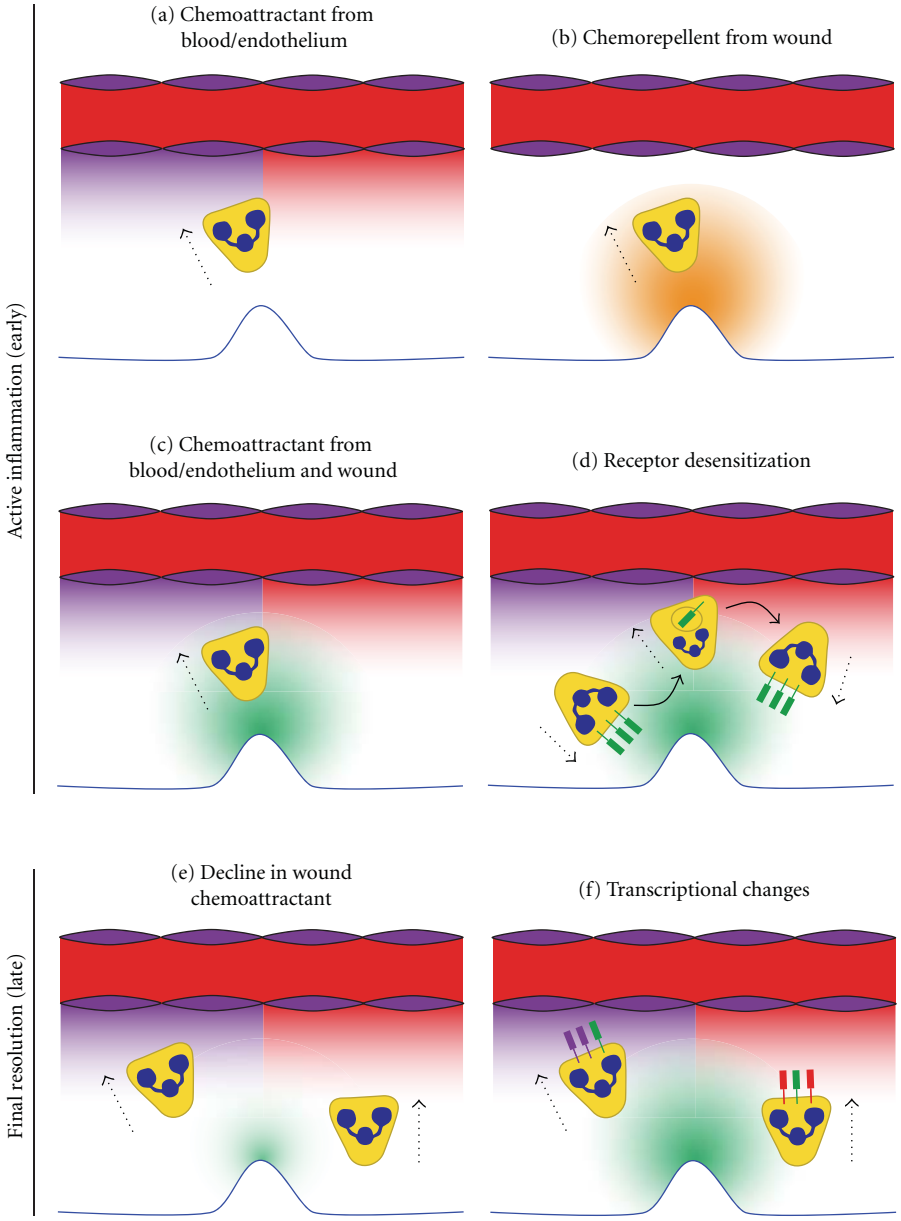

Figure 2: Proposed mechanisms for reversed migration. This diagram illustrates how chemoattractant gradients from the blood (red), endothelium (purple), and wound (green) or chemorepellent gradients (orange) may influence reverse migration. The color of a chemoattractant receptor matches the gradient to which it responds. (a)–(d) Reverse migration in the early wound response. (a) Demonstration of neutrophil reverse migration towards blood or endothelium-derived chemoattractants. (b) Reverse migration of a neutrophil away from a wound-derived chemorepellent. There could be competition between wound-derived chemoattractants (not shown) and chemorepellents promoting reverse migration, or neutrophils may perform fugetaxis from areas of high chemoattractant concentration. (c) Oscillatory behavior of neutrophils suggests competing gradients of chemoattractants may exist between the wound and vasculature. (d) Receptor desensitization, via internalization or other mechanisms, may allow neutrophils to oscillate between the wound and the vasculature while others are still actively responding to the wound. (e)–(f) Mechanisms that promote resolution of neutrophil-mediated inflammation at wounds. (e) During the healing phase, wounded tissue may gradually produce less neutrophil chemoattractant, shifting the balance to favor reverse migration. (f) Neutrophils that responded to a wound may initiate transcriptional changes favoring reverse migration from the wound. Potential changes include altered expression or sensitivity of chemoattractant receptors.

of chemokine receptor surface expression or its sensitivity to signaling is critical for allowing neutrophils to follow the appropriate gradient of chemoattractant, which could be necessary for performing reverse migration.

The eventual permanent departure of neutrophils from the wound site indicates the second phase of the reverse migration response. During this later phase, the wounded tissue may gradually produce less chemoattractant or more

chemorepellent as it heals, shifting the balance towards reverse migration (Figure 2(e)). The time it takes for neutrophils to leave a wound, which can be several hours, makes it possible that transcriptional changes may also be involved. In a mechanism that may be cooperative with declining chemoattractant gradients, neutrophils could be programmed to activate transcriptional changes that favor reverse migration after responding to an inflammatory stimulus. The result could modify chemokine receptor expression or sensitivity, shifting the balance towards reverse migration (Figure 2(f)). Clearly, there are many possible mechanisms internal and external to the neutrophil that may drive reverse migration, making this an area ripe for rapid advancement.

6. Potential Roles for Reverse Migrated Neutrophils

While current studies have not demonstrated a definite role for reverse migrated neutrophils, the recent findings of several groups have challenged the idea that neutrophils are short-lived cells with narrowly defined functions. Because reported neutrophil half-lives were less than 12 hours and there was no knowledge of neutrophil reverse migration, neutrophils were not thought to have immunomodulatory roles other than through the cytokines and effectors that they produced at sites of inflammation. However, recent reports have challenged the short lifespan of neutrophils. Although controversial, a recent study used ^2H_2O labeling to determine that the in vivo half-life of human neutrophils was 3.8 days (total lifespan: approximately 5.4 days) [68–71]. Others have also reported neutrophil lifetimes that were longer than 24 hours. Neutrophils that underwent reverse transendothelial migration, trafficked to lymph nodes, or were cocultured with TNF-α/IL-17 stimulated synovial fibroblasts had their expected lifetimes extended [51, 52, 72]. Zebrafish neutrophils that underwent reverse migration could also be found for at least 2 days after they had left a wound [9]. While evidence supports the existence of reverse migration and prolonged neutrophil life in vivo, data supporting either a proinflammatory or anti-inflammatory role for reverse migrated neutrophils remain plausible.

An intriguing correlation between studies of reverse migrated neutrophils and immunomodulatory neutrophils is that these populations appear to have an activated phenotype that is characterized by elevated CD11b and elevated CD54 expression (Table 1) [10, 51–55]. $CD11b^{high}$ neutrophils were found to transport fluorescent antigen to lymph nodes and survive for an extended period. Although direct interaction with lymphocytes was not documented, neutrophil depletion resulted in increased IL-5 production, which suggested that neutrophils could be altering cytokine production by $CD4^+$ T cells [52]. A more recent study found that $CD11b^{high}CD54^{high}CD62L^{low}CD16^{high}$ neutrophils were induced after injecting healthy human subjects with LPS or in severely injured trauma patients, and that this neutrophil population was capable of inhibiting antigen-dependent and- independent T cell proliferation. Catalase treatment or Mac-1 integrin (CD11b/CD18) blocking decreased the neutrophil inhibitory function, and imaging revealed that H_2O_2 was produced at neutrophil T cell contacts, suggesting a model in which activated neutrophils could form a synapse-like structure and deliver proliferation-inhibiting H_2O_2 to T cells (Figure 3) [54]. Another interesting population of $CD11b^{high}CD54^{high}$ neutrophils was recently found in the marginal zone of the spleen. These neutrophils expressed MHC class II, which is normally found on professional antigen presenting cells and had the ability to promote antibody diversification and production by splenic B cells. These neutrophils populated the splenic lymphoid follicles after acquiring gut-associated-microbial products, suppressed T cell proliferation, and promoted antibody production to T cell-independent antigens (Figure 3) [55]. Taken together, these findings support the idea that neutrophils are able to acquire material from extravascular tissue, return to and survive in lymphoid tissue, and modulate the adaptive immune response. The ability to retrieve antigen outside of the bloodstream and the surface phenotype consistent with reverse migrated neutrophils suggests the possibility that neutrophils could perform reverse migration during their trip back to lymphoid tissues.

Table 1: Comparison of surface phenotypes between studies of neutrophil reverse migration and immunomodulation by neutrophils. CD11b (integrin α_M), which is a component of Mac-1 integrin, and CD54 (ICAM-1) were the surface molecules with the most overlap between these studies. Blank spaces indicate that the expression of this molecule was not addressed by a particular study. The (↑) indicates that expression of the indicated molecule was elevated over the appropriate control sample of neutrophils (non reverse migrated, not responsible for neutrophil effects on lymphocytes, etc.).

	CD11b	CD54
Reverse migration		
Buckley et al. 2006 [51]	↑	↑
Woodfin et al. 2011 [10]		↑
Immunomodulation		
Maletto et al. 2006 [52]	↑	
Ostanin et al. 2012 [53]	↑	↑
Pillay et al. 2012 [54]	↑	↑
Puga et al. 2012. [55]	↑	↑

While the effects of neutrophils on the adaptive immune system described above could be viewed as anti-inflammatory or immunomodulatory, a proinflammatory role has also been proposed for reverse migrated neutrophils [9, 10, 51]. Several lines of evidence lend support to this hypothesis, which stems from the observations that severe, localized trauma can lead to multiple organ failure and neutrophil reverse migration. Neutrophils are thought to be important in the pathogenesis of multiple organ failure, which is associated with states of injury and heightened inflammation [73, 74]. While the reverse migration of neutrophils away from a site of inflammation may result in local resolution of inflammation, the observation by Yoo and Huttenlocher that reverse migrated neutrophils disperse in tissues throughout the body of zebrafish suggests the possibility that neutrophils could be promoting

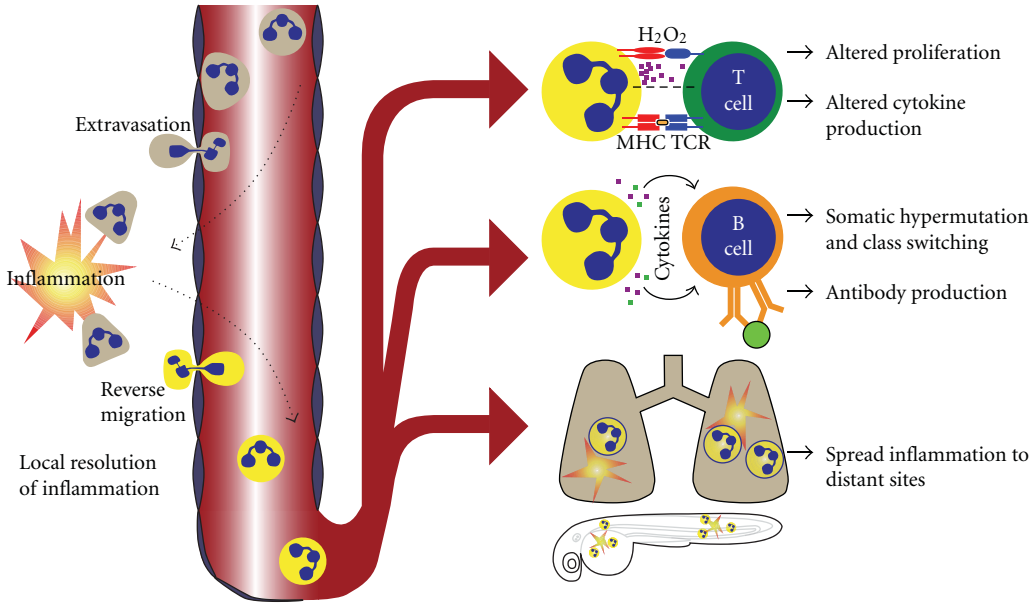

Figure 3: Potential functions of reverse migrated neutrophils. On the left side of the illustration, neutrophils (tan) are responding to an extravascular inflammatory stimulus. After responding to the stimulus, neutrophils perform reverse migration (yellow) and enter the vasculature. This process has been suggested as a mechanism to resolve inflammation at the local level. We are proposing the following as potential functions of reverse migrated neutrophils. Neutrophils may modulate T-cell proliferation and cytokine production in an antigen-independent or-dependent manner. Integrin-mediated neutrophil-T cell contact, hydrogen peroxide, and T cell receptor (TCR) signaling have demonstrated importance in neutrophil-mediated regulation of T cell function. Neutrophils may promote antibody diversification, class switching, and production by splenic B cells through the secretion of cytokines. Reverse migrated neutrophils may travel to distant tissues and induce additional inflammation. Reverse-migrated neutrophils were implicated in inducing pulmonary inflammation in mice.

inflammation or tissue damage in these sites (Figure 3) [9]. Additionally, proinflammatory conditions such as ischemia-reperfusion injury, rheumatoid arthritis, and chronic colitis generate elevated numbers of neutrophils with the reverse-migrated surface phenotype [10, 51, 53]. Mice with chronic colitis were found to have neutrophils that presented antigen to T cells in an MHC II-restricted manner, resulting in increased T cell proliferation and proinflammatory cytokine production [53]. Furthermore, after ischemia-reperfusion injury in mice, pulmonary edema was observed, and neutrophils with a reverse migrated surface phenotype could be found in the lung, suggesting that this neutrophil population may promote inflammation at sites distant from the actual injury (Figure 3) [10]. Reverse migrated neutrophils also produce elevated amounts of reactive oxygen species, which are thought to play a key role in the pathophysiology of multiple organ failure [10, 51, 73, 74]. Although a pro- or anti-inflammatory role for reverse migrated neutrophils remains uncertain, many lines of evidence support the idea that neutrophils can retrieve antigen from extravascular tissues, move to distant organs, and influence the adaptive immune response, leading us to believe that neutrophil reverse migration could be playing a role in these neutrophil functions.

7. Conclusions and Future Directions

The last five years have yielded exciting developments in the study of neutrophil biology, including the process of reverse migration, which are rapidly changing the view that neutrophils are short-lived cells with narrowly defined effector functions. It seems that in at least some circumstances neutrophils can regulate T cell activity and present antigen in the context of MHC II, functions which were previously ascribed to macrophages and dendritic cells, the professional antigen presenting cells [52–55]. While evidence in support of the existence of reverse migration in mammals continues to grow, the mechanisms driving reverse migration and the functions of reverse migrated neutrophils remain to be further defined.

Continued progress towards these goals will require additional characterization of neutrophil forward and reverse migration coupled with technical advances. In order to fully understand the signaling that drives reverse migration, we must better characterize the signaling that is recruiting neutrophils to wounds. Currently, we know that a gradient of H_2O_2 drives the early recruitment of neutrophils to wounds. However, neutrophils still arrive at wounds with a 30–60 minute delay in the absence of wound-produced H_2O_2, indicating that other chemoattractants are likely involved at later time points [46]. Additionally, the chemoattractants or chemorepellents that drive neutrophil reverse migration remain entirely unknown. The intracellular signaling that differentiates forward from reverse migration is also unexplored. Fully characterizing this signaling hierarchy would be facilitated by chemical or genetic screening strategies with the ability to read out changes in neutrophil-mediated inflammation.

Advances in our imaging capabilities in zebrafish will also be critical to further progress in reverse migration studies. While we can observe the movements of neutrophils responding to wounds in zebrafish, we currently know little about their other functions in vivo. Tools that allow live imaging of neutrophil activation and effector functions—reactive oxygen species production, phagocytosis, and degranulation—will be particularly valuable. Biosensors that allow the activity of critical signaling pathways to be monitored will help to integrate the roles of extracellular cues and neutrophil effector functions on their wound responses. A FRET-based Rac activity biosensor, which has been applied to the study of primordial germ cell protrusion and migration, is an example of the type of probe that will be integral in understanding the signaling pathways controlling reverse migration [75, 76].

Determining the function of reverse migrated neutrophils should also be a priority. Recent reports that neutrophils can modulate B cell and T cell functions demonstrate the importance of characterizing these interactions in vivo. Approaching this question with 2–4-day-old zebrafish larvae is not possible, as they have not yet developed an adaptive immune system [16]. As a result, the functions of zebrafish B and T cells in response to inflammatory stimuli are poorly characterized. Techniques that allow simultaneous in vivo imaging of neutrophils, B cells, T cells, and effector molecules in more developed zebrafish would allow a more definitive determination of how these interactions shape immune responses.

In order to fully understand the role of neutrophil reverse migration, it will be necessary to determine how it impacts immune homeostasis and disease. Models of immunodeficiency and inflammatory disease have been developed in zebrafish larvae [22, 23, 77, 78]. However, a model of neutrophil autonomous inflammatory disease has not yet been developed in zebrafish. These and future disease models can be used to determine if neutrophil reverse migration is altered in pathologic states. Additionally, determination of the signaling that drives reverse migration will allow this process to be inhibited, which will be informative in understanding how it may influence pathology.

While rapid progress has been made in the characterization of reverse migration in zebrafish and mice, much remains to be learned about the underlying mechanisms and functional consequences of this process. However, the availability of powerful tools for genetic manipulation and in vivo imaging makes it clear that the use of transparent zebrafish larvae will allow researchers to continue probing the secrets of neutrophil behavior in vivo.

Authors' Contribution

T. W. Starnes and A. Huttenlocher wrote the paper.

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Review Article

Through the Looking Glass: Visualizing Leukemia Growth, Migration, and Engraftment Using Fluorescent Transgenic Zebrafish

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Zebrafish have emerged as a powerful model of development and cancer. Human, mouse, and zebrafish malignancies exhibit striking histopathologic and molecular similarities, underscoring the remarkable conservation of genetic pathways required to induce cancer. Zebrafish are uniquely suited for large-scale studies in which hundreds of animals can be used to investigate cancer processes. Moreover, zebrafish are small in size, optically clear during development, and amenable to genetic manipulation. Facile transgenic approaches and new technologies in gene inactivation have provided much needed genomic resources to interrogate the function of specific oncogenic and tumor suppressor pathways in cancer. This manuscript focuses on the unique attribute of labeling leukemia cells with fluorescent proteins and directly visualizing cancer processes *in vivo* including tumor growth, dissemination, and intravasation into the vasculature. We will also discuss the use of fluorescent transgenic approaches and cell transplantation to assess leukemia-propagating cell frequency and response to chemotherapy.

1. Zebrafish Models of Leukemia

Zebrafish models of hematological malignancies exhibit striking similarities with human and mouse disease [1–7], yet afford unique avenues of study due to imaging modalities that permit direct visualization of fluorescently labeled blood cells within live animals. As with mouse and human disease, zebrafish leukemias are distinguished from lymphomas by the infiltration of leukemic cells into the marrow. Lymphomas are predominantly located as masses throughout the body, including lymph nodes in mouse and human, and have no or little infiltration into the marrow [8]. Leukemias are also classified as acute or chronic. Acute leukemias are arrested at early stages of maturation, are highly proliferative, and advance quickly in patients [8]. By contrast, chronic leukemias are arrested at later stages of maturation and resemble functional, yet abnormal, blood cell counterparts. Although characterized by increased circulating white blood counts, chronic leukemias are often much slower growing and take months or years to progress. Leukemias can be further subdivided based on the blood lineage in which cells have become transformed [8]. To date, zebrafish models of Acute Lymphoblastic Leukemias (ALL), Acute Myeloid Leukemia (AML), and Myeloproliferative Neoplasms (MPN) have been described.

Zebrafish first emerged as a powerful genetic model of leukemia with the description of transgenic approaches in which cMYC was overexpressed in developing thymocytes [7]. Utilizing the *rag2* promoter to drive both MYC and GFP expression, transgenic zebrafish T-cell acute lymphoblastic leukemias (T-ALLs) could be easily visualized in live animals. In this model, fluorescently labeled T cell precursors resident in the thymus were the T-ALL-initiating cell type and disseminated widely over the course of tumor progression [7].

Moreover, GFP+ thymocytes exhibited stereotypical homing to the nasal placode, periocular space, and kidney marrow when assessed by serial fluorescent imaging over days [7]. Subsequent studies developed conditional approaches to create fluorescent transgenic zebrafish models of T-ALL that utilized CRE-Lox or tamoxifen-inducible MYC-ER strategies [5, 9]. Interestingly, withdrawal of tamoxifen and subsequent inactivation of MYC expression led to regression of fluorescently labeled T-ALL; however, leukemia regression was not observed in *pten* mutant fish or those that overexpressed activated *Akt* [9]. These data indicate that *Akt* pathway activation is sufficient for tumor maintenance in this model. Additional studies have utilized fluorescence imaging to assess synergy between MYC and Bcl2 [5, 10] and NOTCH1-ICD [1]. Moreover, human *NOTCH1-intracellular domain-EGFP* transgene expression induces fluorescently labeled T-ALL with a long latency of >6 months in mosaic and stable transgenic zebrafish [6]. Finally, forward genetics screens that utilize ENU (N-Ethyl-N-nitrosourea-) induced mutagenesis are easily performed in zebrafish due to their large clutch size and accessible observation of phenotypes. Utilizing this approach, the Trede group mutagenized *Tg(lck:GFP)* transgenic fish and visualized animals for fluorescently labeled T-ALL onset in F1 and F2 animals, identifying both dominant and recessive mutations that affect T-ALL onset [11]. Mapping of mutations that are found in these mutant lines will likely uncover novel mechanisms that drive T-ALL onset and growth in both zebrafish and man.

Many exciting new models of hematopoietic malignancy have been created including B-cell acute lymphoblastic leukemia (B-ALL), acute myeloid leukemia (AML), and myeloproliferative neoplasm (MPN). For example, Sabaawy et al. developed a model of B-ALL by overexpressing *EGFP-TEL-AML1* from a ubiquitous transgene promoter. In this model, 16 of 545 transgenic animals developed B-ALL by 8–12 months of age [2]. Zhuravleva et al. generated transgenic zebrafish in which the *MYST3/NCOA2* fusion gene was expressed under control of the *spi1* promoter [12]. 2 of 180 *MYST3/NCOA2-EGFP* mosaic transgenic animals developed AML at 14 and 26 months. Two models of MPN have also been developed. Le et al. utilized CRE/Lox techniques to conditionally activate *kRASG12D* in developing embryos [3]. A subset of these animals went on to develop myeloproliferative neoplasm with a latency of 66.2 ± 23.1 days (n = 10 of 19 fish). Forrester et al. also developed a conditional CRE/Lox transgenic approach to model MPN [13]. Specifically, *NUP98-HOXA9* was conditionally activated in *pu.1* expressing cells, leading to 23% of adult *NUP98-HOXA9*-transgenic fish developing MPN by 19–23 months of age. Finally, several investigators have utilized heat-shock transgenic approaches to uncover early developmental effects of fusion oncogenes in blood development, including *AML1-ETO, RUNX1-CBF2T1,* and *TEL-JAK2* [4, 14, 15]. These heat-shock approaches drive transgene expression during early development and often result in aberrant arrest of cells in early stages of blood development. However, the development of frank leukemia in heat-shock inducible transgenic lines has yet to be reported. Taken together, zebrafish have fast emerged as a novel animal model of leukemia and are poised to contribute to our understanding of the molecular pathogenesis of human disease.

2. Fluorescent Transgenic Approaches to Label Leukemia Cells

Many studies have employed the use of stable transgenic zebrafish to drive oncogenic transgene expression in a tissue-specific manner including pancreatic adenocarcinoma [16], hepatocellular carcinoma [17], melanoma [18–20], embryonal rhabdomyosarcoma [21], and leukemia. By and large, investigators have used oncogene fusions with GFP to create tumors that are fluorescently labeled. For example, we and others have generated *EGFP-Myc, NOTCH1-GFP, EGFP-TEL-AML1*, and *MYST3/NCOA2-EGFP* fusions to drive leukemogenesis while also fluorescently labeling leukemic cells [2, 6, 7, 12]. Although these approaches have been largely successful in generating fluorescently labeled leukemias, it is worth noting that fluorescent protein expression is linked with oncogene localization within the cell and protein stability. For example, MYC is a nuclear transcription factor with a half-life of ~30 minutes in non-transformed cells. Thus, the EGFP-MYC fusion protein is rapidly turned over in normal thymocytes prior to GFP maturation into a functional fluorescent molecule, precluding the use of fluorescence to identify stable transgenic *Tg(rag2:EGFP-Myc)* animals at 5 days of life. However, the *EGFP-Myc* transgene is stabilized following transformation leading to weak, nuclear fluorescent protein expression in T-ALL. Fluorescent protein fusions can also exhibit reduced transforming activity depending on cellular context. For example, we have developed a zebrafish model of *kRASG12D*-induced embryonal rhabdomyosarcoma but have been unable to model this disease using the same transgene promoter to drive expression of a GFP fusion with kRASG12D. By contrast, others have used similar RAS fusion constructs to generate fluorescently labeled hepatocellular carcinoma, pancreatic adenocarcinoma, and melanoma [16, 17, 19, 20]. To obviate issues surrounding the function of fluorescent protein-oncogene fusions, it is possible to utilize dual transgenic approaches to drive both the oncogene and fluorescent protein within the same cell types. For example, *Tg(rag2:Myc)* lines could be bred to *Tg(rag2:GFP)* fish. The resulting progeny would develop T-ALL that expresses high fluorescent protein expression.

Although stable transgenic zebrafish have been used to develop robust models of cancer, mosaic transgenic approaches provide many unique benefits for modeling cancer in zebrafish. First, stable transgenic zebrafish are often prone to developing early onset cancers, making maintenance of stable lines difficult. Second, the creation of stable transgenic zebrafish is time-consuming and requires crossing putative transgenic animals to identify founder fish. Although the transgenesis with Tol2 transposase has facilitated the creation of stable transgenic lines, complex breeding strategies are required to introduce additional transgenes and/or mutant alleles into a given background. Such approaches often require multiple generations to develop strains of interest. By contrast, mosaic transgenesis relies on the ability of

multiple, linearized transgenes to incorporate into the genome as concatamers when microinjected into one-cell stage zebrafish, ultimately culminating in the coexpression of transgenes in developing disease. We have successfully used this approach to show that *kRASG12D* collaborates with *p53* loss to induce early onset embryonal rhabdomyosarcoma [22] and work from Feng et al., elegantly showed that mosaic transgenesis can be used to modify *Myc*-induced T-ALL through coinjection of activated *Akt* [10]. We have used similar approaches to develop T-ALLs that coexpress MYC and various fluorescent reporters including AmCyan, GFP, zsYellow, dsREDexpress, and mCherry [23, 24]. In these experiments, embryos are coinjected with *Myc* and fluorescent protein under transcriptional control of the *rag2* promoter. A small cohort of animals develop fluorescently labeled thymi that eventually progresses into T-ALL. Using this approach, we have been able to create T-ALLs in various genetic backgrounds, permitting the creation of syngeneic strain fish that develop multicolored T-ALL (Figure 1) [23]. Finally, we have recently utilized mosaic transgenesis to coexpress Notch1a-ICD, MYC, and GFP by coinjection of three transgenes simultaneously into one-cell stage animals [1]. In summary, while some fluorescent transgenic approaches can be limited by fusion stability, early onset of cancer, and genetic background, other fluorescent transgenic approaches have been able to overcome these limitations. Such approaches provide rapid assays to identify collaborating oncogenic/tumor suppressor pathways in leukemia.

3. Cell Transplantation Approaches to Visualize Tumor Cell Engraftment

Investigators have utilized cell transplantation of fluorescently labeled cancer cells into sublethally irradiated adult zebrafish to assess tumorigenicity [7]. For example, Traver et al. optimized cell transplantation of both blood and leukemic cells into gamma-irradiated animals [7, 25]. Specifically, recipient fish were irradiated with 20–25 Gy two days prior to cell transplantation and then injected with fluorescently labeled donor cells into the peritoneal cavity or sinus venosis. For T-ALL, animals can be injected with 1×10^6 cells and assessed for fluorescently labeled leukemia engraftment at 10 days posttransplantation [7, 25]. Imaging of engraftment can be further facilitated by transplantation into optically clear strains of zebrafish that lack iridiphores and melanocytes—aptly named *casper* [26]. *Casper* fish were created by breeding together *roy* and *nacre* mutants and must be maintained as double homozygous mutant animals. These fish are transparent as adults, facilitating detailed imaging of cell migration, metastasis, and kinetics of tumor growth. For example, recent work has shown that blood cells can be tracked and counted within the circulation of live adult fish using an integrated optical system that combines a laser scanning confocal microscope and an *in vivo* flow cytometer [27].

Although transplantation of donor cells into irradiated recipients is a powerful tool to assess short-term engraftment potential, long-term engraftment of cells >20 days posttransplantation is often not possible due to the recovery of the host immune system and subsequent attack of engrafted cells [23, 28]. To avoid immune rejection, Mizgirev and Revskoy recently developed syngeneic zebrafish strains and created robust models of transplantable, chemically induced hepatocellular carcinomas, hepatoblastomas, cholangiocarcinoma, and pancreatic carcinoma [29–31]. Specifically, syngeneic zebrafish were created by fertilizing eggs with UV-inactivated sperm, then subjecting eggs to heat-shock [29]. Female gynogenic diploid animals were raised to adulthood and the process repeated. The resulting progeny were genetically similar and could be maintained by incrossing or mating male fish back to the founding mother. Several lines were created using this method including clonal golden strain 1 and 2 (CG1 and CG2). Adoptive transfer of chemical-induced cancers and *Tg(rag2:EGFP-Myc-)* induced T-ALLs from CG2-strain fish could engraft disease into syngeneic recipients [31]. Moreover, fluorescently labeled rhabdomyosarcoma and T-ALL cells arising in CG1 strain fish could also engraft into nonirradiated, recipient fish [23, 24]. Taken together, these results illustrate the power of cell transplantation and use of syngeneic zebrafish to study leukemia cell engraftment.

4. Cell Transplantation Approaches to Examine Tumor Cell Homing and Intravasation into Vessels

Blood cells and their dynamic cell movements can be easily visualized in live fluorescent transgenic zebrafish. For example, researchers have tracked the migration of various blood lineages including erythroid and macrophage progenitors [25, 32–34]. Importantly, hematopoietic stem cell (HSC) movement can also be followed in *Tg(CD41:eGFP)*, *Tg(cmyb:GFP)*, *Tg(runx1:GFP),* and *Tg(lmo2:GFP)* transgenic zebrafish larvae [35–40]. Moreover, fluorescently labeled blood cells can also be tracked in adult fish [27, 41]. Capitalizing on cell transplantation approaches, investigators have also utilized fluorescence imaging to visualize normal hematopoietic cell homing in live animals. For example, Bertrand et al. visualized HSC homing to the caudal hematopoietic tissue by transplanting *Tg(CD41:eGFP; gata1:dsRed)* cells into irradiated recipients [36]. We have also described the homing of *Tg(lck:GFP)*+ T cells back to the thymus following transplantation of cells into larval wildtype fish [42]. While malignant GFP+ T-ALL lymphoblasts also migrate to the thymus, they exhibit robust and specific homing to the olfactory bulb [6, 7]. These studies demonstrate the ease of visualizing cell migration and homing to specific anatomically defined sites within live animals using fluorescently labeled normal hematopoietic and leukemic cells.

Intravasation of cancer cells into the vasculature is a critical step in cancer progression, allowing the spread of tumor cells beyond the site of origin [43]. The extent to which lymphoblasts disseminate is the clinically defining characteristic of T-lymphoblastic lymphoma (T-LBL) and acute T-lymphoblastic leukemia (T-ALL) [8]. In T-LBL, transformed lymphoblasts are confined to mediastinal masses, while frank leukemia involves dissemination of cells to the marrow.

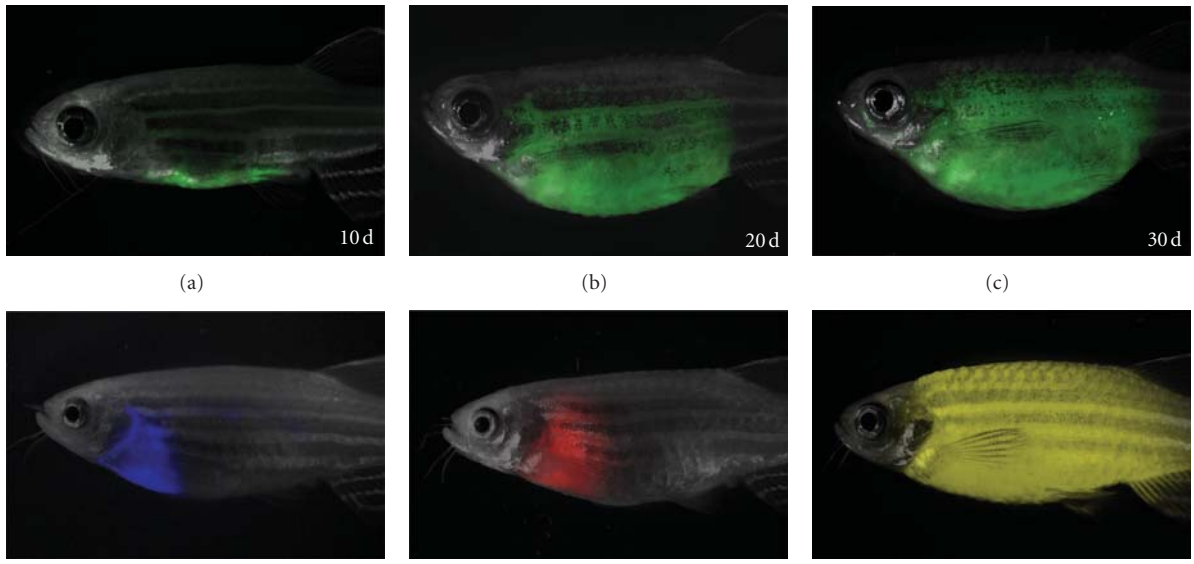

Figure 1: Fluorescently labeled Myc-induced T-ALLs from CG1-strain zebrafish engraft into nonirradiated CG1-strain recipients. (a)–(c) GFP-labeled T-ALLs were isolated from primary leukemic fish, and 1×10^3 FACS sorted GFP-labeled leukemia cells were transplanted into nonirradiated CG1-strain animals and scored for engraftment at 10, 20, and 30 days posttransplantation. (d)–(f) T-ALL transplant recipients that express Amcyan (d), dsRED (e), and zsYellow (f) under the *rag2* promoter. Panels are merged images of fluorescent and brightfield photographs. Images were originally published in [23].

Remarkably, this disease transition was recently visualized in zebrafish transplanted with fluorescently labeled lymphoblasts [10]. For example, RFP+ lymphoblasts from *Myc*-induced T-ALL were able to intravasate into *Tg(fli:GFP)*-labeled vasculature, while cells that overexpressed the anti-apoptotic protein Bcl2 were unable to enter the vasculature and, thus, were arrested in a T-LBL state (Figure 2) [10]. Remarkably, treatment of transgenic zebrafish that overexpressed MYC and Bcl2 with an antagonist to Sphingosine-1-Phosphate (S1P1), a T-cell adhesion and migration protein, promoted invasion into the vasculature [10]. These elegant studies by Feng et al. were the first to directly visualize the molecular mechanisms governing the transition of T-LBL to T-ALL and underscore the power of imaging dynamic cellular processes in fluorescently labeled animals.

5. Fluorescence Imaging to Visualize Leukemia Responses to Drug Treatment and Gamma-Irradiation

Fluorescence imaging of transplanted cancer cells can also be used to visualize response to chemotherapy and radiation. For example, the Revskoy group recently showed that GFP-labeled T-ALL cells could be serially transplanted into syngeneic strain larvae [31]. Treatment of transplant recipients with vincristine or cyclophosphamide reduced tumor burden (Figure 3) and extended lifespan significantly [31]. These experiments established that high-throughput cell transplantation assays can generate large cohorts of animals for drug screens and showed that zebrafish T-ALL responds to the same drugs that are used to treat human T-ALL patients [31]. In addition, fluorescently labeled cells can be assessed for response to radiation. For example, we have shown that engrafted GFP-labeled T-ALLs that coexpress *EGFP-bcl2* and the *Myc* transgene failed to undergo apoptosis following 20 Gy of gamma-irradiation [44]; however, T-ALLs that express only *Myc* were ablated by 4 days postirradiation, suggesting that *Myc*-induced T-ALL have an intact *p53* DNA damage pathway.

6. Cell Transplantation Approaches to Quantify Leukemia Propagating Cell Frequency and Aggression

Leukemia-propagating cells (LPCs) have the capacity to produce all the other cell types contained within the leukemia, are responsible for continued tumor growth, and ultimately drive relapse. Investigators have used fluorescence-activated cell sorting (FACS) to identify unique cell populations and limiting dilution cell transplantation to assess if molecularly defined leukemia cells retain LPC activity in human disease. For example, in AML a rare CD34+, CD38− cell enriches for leukemia-propagating potential [45, 46]. In T-ALL, it has been suggested that CD34+ CD7+ cell populations are enriched in LPCs [47]. Despite enormous efforts aimed at defining if and what cell surface markers define LPC activity, relatively little is known about the molecular mechanisms that drive leukemia propagating activity. For example, elegant work from Jean Soulier's group has shown xenograft transplantation of primary human T-ALL into

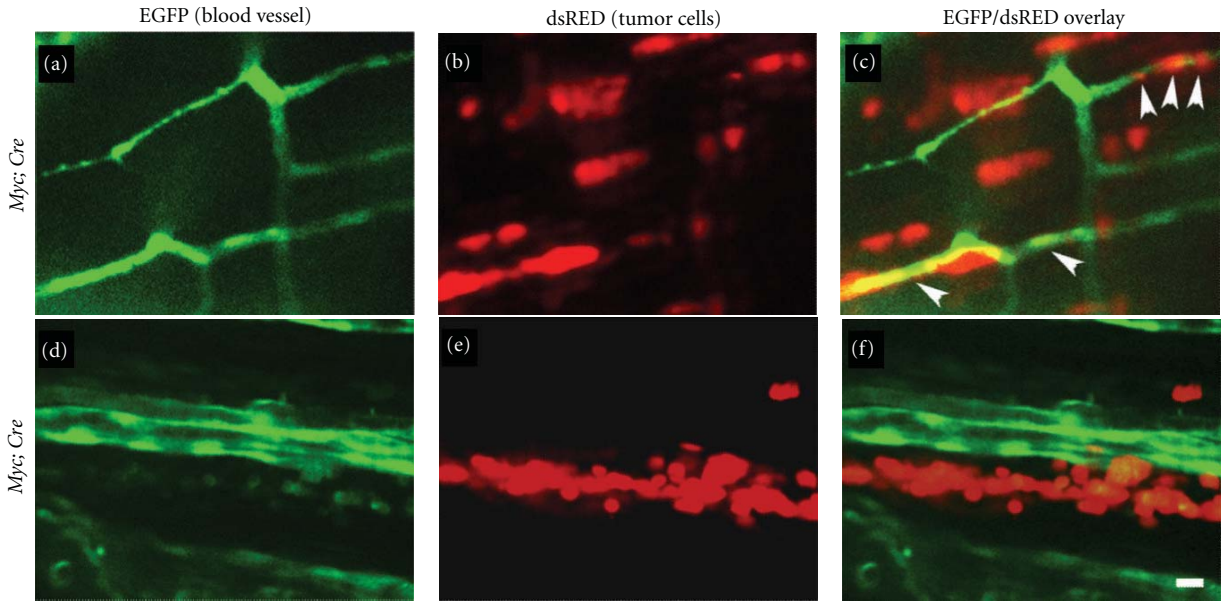

Figure 2: Zebrafish T-lymphoblasts overexpressing bcl2 spread locally but fail to intravasate into vasculature. (a)–(c) dsRED2-expressing lymphoma cells (b) from the *Myc; Cre* fish intravasate into EGFP-labeled vasculature (a) of the transplant host *Tg(fli1:EGFP); Casper* by 6 days posttransplantation (see arrowheads in (c)). (d)–(f) In contrast, dsRED2-expressing lymphoma cells (e) from the *Myc; Cre; bcl2* fish fail to intravasate vasculature (d) of the transplant hosts by 6 days posttransplantation (compare (f) with (c)). Note aggregates of the *Myc; Cre; bcl2* lymphoma cells in (e) and (f). Scale bar is 10 μm. Reprinted from [10].

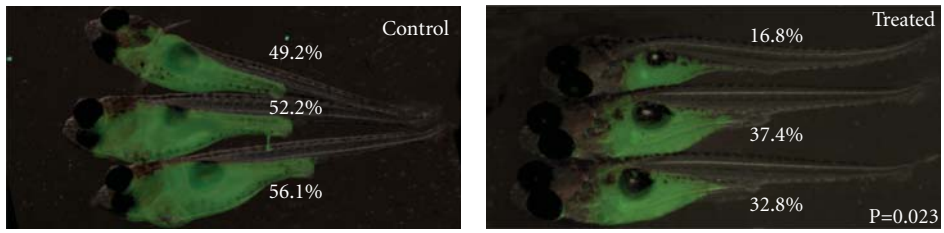

Figure 3: Syngeneic zebrafish transplant models of T-ALL are a powerful tool for drug discovery: T-ALL growth is suppressed by cyclophosphamide treatment. Approximately 200 cells/5 nL were engrafted into 5-day-old syngeneic CG2 larvae. Engrafted animals were treated with cyclophosphamide (400 mg/L dissolved in fish water) beginning 5 days posttransplantation. Images of control (a) and treated animals. (b) Tumor growth was assessed based on the percentage of body taken over by GFP+ T-ALL and compared using t-test calculations. This work was performed in [31] and later published in [61].

immune-compromised mice selected for a small subset of clones found within the diagnosis leukemia [48]. These clones contained specific genomic lesions that likely increase leukemia aggression and increase the frequency of LPCs within the bulk of the leukemia mass [48]. Yet, despite the identification of recurrent genomic changes associated within continued clonal evolution, the mechanisms driving these relapse-associated processes are largely unknown.

The process by which leukemic cells acquire mutations to increase aggression and frequency of LPCs has been difficult to study in human and mouse models of disease. However, recent work from the Trede group has utilized serially passaged fluorescently labeled zebrafish T-ALLs to demonstrate that leukemias become more aggressive and develop with shortened latency [49]. To assess genetic changes acquired between the primary and evolved clones, array comparative hybridization studies were completed to identify recurrent genomic DNA alterations associated with increased aggression. An average of 34 new copy number aberrations (CNAs) were identified in T-ALLs following serial passaging, a majority of which were also found in human T-ALL [49]. Clonal evolution can also result in increased numbers of LPCs contained within the leukemia mass [48]. To directly assess LPC frequency within the bulk of the tumor mass, we have pioneered high-throughput limiting dilution cell transplantation approaches and showed that 1% of Myc-induced T-ALL cells has the capacity to remake leukemia in syngeneic recipient animals [23, 24]. Following serial passaging, a subset of clones can increase LPC activity with up to 16% of cells now capable of inducing leukemia in transplant recipient animals [23]. Similar array CGH studies as described by Rudner et al. [49] are currently underway

to identify recurrent CNAs associated with modulating LPC frequency in zebrafish T-ALL. Taken together, we believe that unbiased genetic approaches, when coupled with limiting dilution cell transplantation assays in zebrafish, will likely uncover the mechanisms driving relapse-associated changes in aggression and LPC frequency in human disease.

7. Conclusion and Challenges for the Future

Zebrafish has fast emerged as a powerful model of leukemia. When coupled with fluorescent transgenic approaches and powerful imaging techniques, these models are uniquely positioned to uncover mechanisms driving tumor dissemination, progression, and relapse. Moreover, the use of multifluorescent transgenic animals will allow for labeling of tumor cell compartments similar to those defined in RAS-induced rhabdomyosarcoma models [21, 50] and for the visualizing of leukemia growth in relation to supportive cell types including vasculature, fibroblasts, and macrophages. Moreover, though not the focus of this paper, cell transplantation approaches that utilize fluorescently labeled, human leukemia cells into either zebrafish embryos or adults will likely provide novel experimental models to assess tumor growth and response to therapy [51–60], capitalizing on the numbers of disease animals that can be created by microinjection and direct visualization of tumor growth *in vivo*.

Conflict of Interests

The authors declare no competing financial interests.

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Review Article

Pathogen Recognition and Activation of the Innate Immune Response in Zebrafish

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The zebrafish has proven itself as an excellent model to study vertebrate innate immunity. It presents us with possibilities for *in vivo* imaging of host-pathogen interactions which are unparalleled in mammalian model systems. In addition, its suitability for genetic approaches is providing new insights on the mechanisms underlying the innate immune response. Here, we review the pattern recognition receptors that identify invading microbes, as well as the innate immune effector mechanisms that they activate in zebrafish embryos. We compare the current knowledge about these processes in mammalian models and zebrafish and discuss recent studies using zebrafish infection models that have advanced our general understanding of the innate immune system. Furthermore, we use transcriptome analysis of zebrafish infected with *E. tarda, S. typhimurium*, and *M. marinum* to visualize the gene expression profiles resulting from these infections. Our data illustrate that the two acute disease-causing pathogens, *E. tarda* and *S. typhimurium*, elicit a highly similar proinflammatory gene induction profile, while the chronic disease-causing pathogen, *M. marinum*, induces a weaker and delayed innate immune response.

1. Introduction

The use of adult zebrafish (*Danio rerio*) and their transparent offspring as hosts to model infectious diseases caused by human pathogens, or closely related animal pathogens, has recently provided novel insights into pathogenesis, which in many cases could not have been achieved using mammalian models [1–6]. The power of the zebrafish model lies in its suitability for genetic approaches, high-throughput screening, and live imaging studies. Fluorophore-marked transgenic lines are now available that allow unprecedented visualization of pathogen interactions with macrophages and neutrophils, the major phagocytic innate immune cell types of zebrafish larvae [7–11]. As early as one day after fertilization (dpf), zebrafish embryos display phagocytic activity towards microbial infections [12] and are able to mount an innate immune response with a transcriptional signature that resembles responses in mammalian or cell culture systems [13]. Adaptive immunity becomes active after approximately three weeks of development [14]. Therefore, innate immunity can be studied during the earlier zebrafish embryonic and larval stages in the absence of T- and B-cell responses. In this paper we focus on signaling pathways involved in pathogen recognition and activation of the innate immune response in zebrafish embryos and larvae. We compare the knowledge of the zebrafish innate immune system with that of human and mammalian models and discuss results from transcriptomic analyses that show clear specificity in responses to different bacterial pathogens, such as *Salmonella* and *Mycobacteria* species.

2. Pattern Recognition Receptors

The innate immune system is the host's first line of defense against infection; therefore, its main role is to recognize invading pathogens early and trigger an appropriate proinflammatory response [15]. The innate immune system utilizes a limited number of germline-encoded pattern recognition receptors (PRRs) to recognize evolutionary conserved structures on pathogens, named pathogen-associated molecular patterns (PAMPs) [15]. PRRs are also capable of indirectly sensing the presence of pathogens [16, 17].

This occurs when infection, inflammation, or other cellular stresses cause host factors to be present in aberrant locations, or to form abnormal molecular complexes, so called danger-associated molecular patterns (DAMPs) [17]. PRRs located on the cell surface are scouting the extracellular environment for the presence of microbes. PRRs located on endosomes identify microbes that have entered the phagolysosomal degradation pathway, and cytoplasmic PRRs recognize intracellular cytosolic pathogens or components of internalized microbes [18]. Upon PAMP recognition, PRRs signal the presence of infection and initiate proinflammatory and antimicrobial responses by activating several intracellular signaling pathways [19], ultimately leading to activation of gene expression and synthesis of a broad range of molecules. These include proinflammatory and chemotactic cytokines and antimicrobial peptides [20]. The different families of PRRs present in both humans and zebrafish and their downstream signaling pathways are summarized in Figure 1 and will be discussed below.

2.1. Toll-Like Receptors. The most extensively studied class of PRRs are the Toll-like receptors (TLRs), a family of 10 proteins in human. TLRs are named after the *Drosophila* Toll protein, which functions in dorsoventral patterning and antifungal responses [23]. TLRs are integral glycoproteins which possess an extracellular or luminal, ligand-binding domain with leucine-rich repeat (LRR) motifs and a cytoplasmic signaling Toll/Interleukin-1 (IL-1) receptor homology (TIR) domain [20, 24]. In mammals, the main cell types expressing TLRs are antigen-presenting cells (APCs), including macrophages and dendritic cells, and B lymphocytes [18]. However, most cell types are capable of expressing TLRs, for instance, in response to a localized infection [25]. In mammals, TLR4 recognizes Gram-negative bacteria via the lipid A portion of lipopolysaccharide (LPS), while TLR2 recognizes Gram-positive bacteria via lipoteichoic acid (LTA), lipoproteins, and peptidoglycan, and TLR5 recognizes the motility apparatus protein flagellin, which can be present on both Gram types [18]. Other TLRs are specialized in recognizing nuclear acids in endosomal and phagosomal compartments. TLR3 can detect viral replication by binding to double-stranded RNA (dsRNA), TLR7 and TLR8 specifically recognize single-stranded RNA (ssRNA) of RNA viruses, and unmethylated CpG DNA present in the genomes of viruses and bacteria is detected by TLR9 [18]. Ligand binding by a TLR will induce it to form homomeric or heteromeric oligomers, which triggers intracellular signal transduction via their TIR domains [18]. The mammalian TLR signaling pathway uses five different TIR-domain-containing adaptor molecules: MYD88, MAL/TIRAP, TRIF/TICAM1, TRAM/TICAM2, and SARM [19, 24]. Among these, MYD88 is the most universal adaptor, since it is used for downstream signaling by all TLRs, with the exception of TLR3 [26]. Downstream signaling via central intermediate molecules such as TRAF6 will eventually lead to the activation of transcription factors, mostly members of the ATF, NFκB, AP-1, IRF, and STAT families, regulating the expression of a battery of antimicrobial and proinflammatory genes [26].

Putative orthologs of mammalian TLRs have been identified in zebrafish, in addition to some fish-specific family members [27, 28]. A genome duplication during the evolution of teleost fish most likely explains why zebrafish have two counterparts for some of the mammalian TLRs (e.g., *tlr4ba/tlr4bb* for *TLR4* and *tlr5a/tlr5b* for *TLR5*), but it is still unknown whether this increase in the number of receptors is associated with diversification in PAMP recognition [4]. Only some of the zebrafish TLR ligands are currently known [29]. The specificity of TLR2, TLR3, and TLR5 is conserved between mammals and fish, recognizing lipopeptides, dsRNA, and flagellin, respectively [13, 30, 31]. Additionally, the fish-specific TLR22 has been shown to recognize dsRNA and PolyI:C [31]. However, zebrafish TLR4 cannot be stimulated by LPS, illustrating that not all ligand specificities are conserved between mammals and zebrafish [32, 33]. Signaling intermediates in the pathway downstream of mammalian TLRs have also been identified in zebrafish, including homologs of four of the adaptor proteins, Myd88, Mal/Tirap, Trif/Ticam1, and Sarm, and the central intermediate Traf6 [34]. Among these, Myd88 and Traf6 have been functionally studied by knockdown analysis in zebrafish embryos, showing their requirement for a proinflammatory innate immune response to microbial presence [13, 35–37]. Furthermore, triggering of the innate immune response in zebrafish embryos also leads to induction of members of the ATF, NFκB, AP-1, IRF, and STAT families of transcription factors [13, 38].

2.2. NOD-Like Receptors. Pathogens that escape the surveillance of cell surface and endosomal PRRs may end up in the cytosol, where nucleotide-binding-oligomerization-domain-(NOD-) like receptors (NLRs) detect their presence by intracellular PAMPs and DAMPs [39]. The NLRs constitute a family of 23 proteins in humans. Their defining features are the presence of a centrally located NOD domain responsible for oligomerization, a C-terminal LRR capable of ligand-binding, and an N-terminal protein-protein interaction domain, such as the caspase recruitment domain (CARD), pyrin (PYD), or baculovirus inhibitor repeat (BIR) domain [40]. Two of the NLRs, NOD1 and NOD2, can sense bacterial presence by directly or indirectly detecting molecules produced during synthesis or breakdown of peptidoglycan [40]. NOD1 recognizes g-D-glutamyl-meso-diaminopimelic acid (iE-DAP), a dipeptide produced mostly by Gram-negative bacteria, whilst NOD2 can recognize both Gram types, since it is activated upon binding to muramyl dipeptide (MDP), a more common component of peptidoglycan [41, 42]. Interestingly, both NOD1 and NOD2 have recently been implicated in detection of parasites lacking peptidoglycan, indicating that these receptors can recognize a broader range of pathogens than was originally assumed [43, 44]. Upon ligand-binding, NOD1 and NOD2 recruit the serine/threonine kinase RIPK2 (also known as RIP2) via CARD-CARD interactions, eventually leading to the activation of NFκB [45, 46]. In addition, NOD1/2 stimulation also induces MAP kinase signaling [47]. Synergistically, with TLR activation, NOD1/2 signaling cascades induce the expression

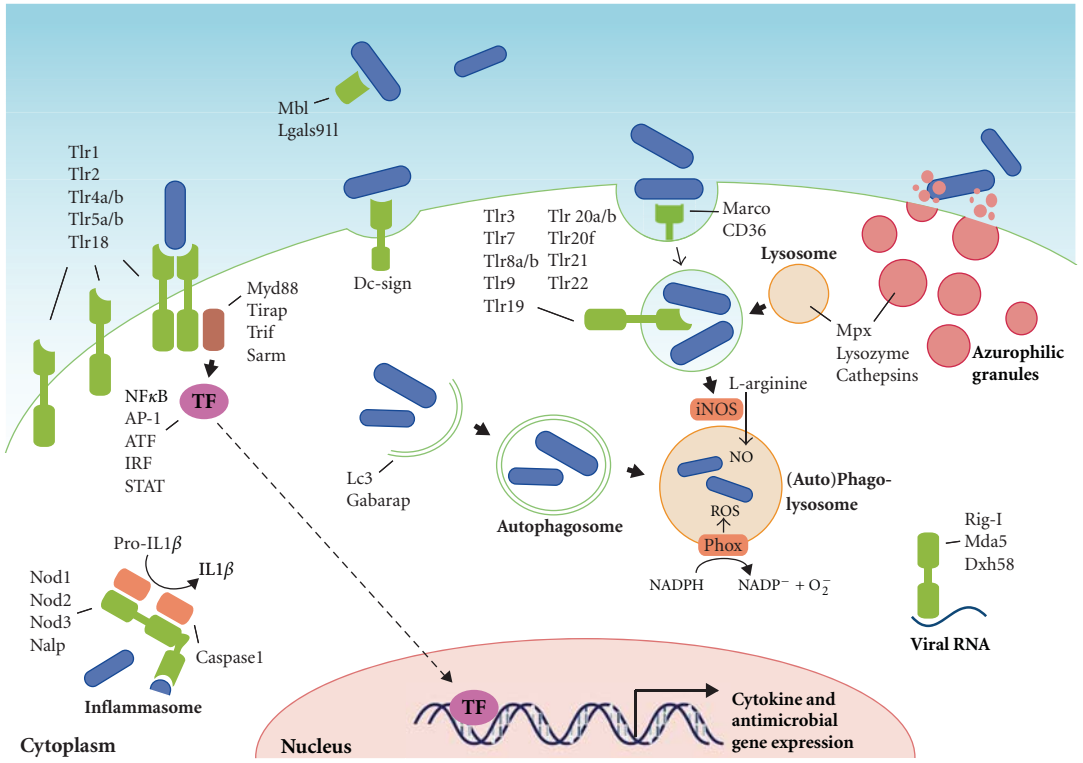

Figure 1: Pattern recognition receptors and effector mechanisms of the innate immune system. The localization of Tlrs on the cell surface or on endosomes is hypothetical and based on the known or proposed functions of their homologs in other fish or mammals. The ability of PRRs (depicted in green) to recognize PAMPs present on various types of microorganisms, like bacteria, viruses, and fungi, has been simplified here by depicting microorganisms as rod-like bacteria (in blue). PAMP recognition by PRRs leads to activation of transcription factors (TFs), which translocate to the nucleus and initiate transcription of cytokine genes, antimicrobial genes, and other immune-related genes. Defense mechanisms such as autophagy, ROS and NO production, and degranulation can be immediately activated upon microbial recognition, without de novo gene transcription.

of cytokines and chemokines, such as TNF, IL6, IL8, IL10, and IL12, as well as the production of antimicrobial peptides [46, 48, 49].

Other NLRs, such as IPAF, NALP1, and NALP3, mainly function to create a multiprotein complex known as the inflammasome, in which they associate with an adaptor called ASC (apoptosis-associated speck-like protein containing a CARD) and with procaspase 1 [50]. Oligomerization of the proteins in an inflammasome via CARD-CARD interactions ultimately leads to the cleavage of procaspase 1 into its active form, caspase 1, which is then available to catalyze the cleavage of accumulated pro-IL1β and pro-IL18 into their secreted forms, biological active IL1β and IL18 [40]. The NLR family member incorporated into these complexes determines which PAMPs and DAMPs are recognized by the inflammasome. A role for NALP3 has been established in the recognition of ATP [51], uric acid crystals [52], viral RNA [53], and bacterial DNA [54]. Both NALP1 and NALP3 share NOD2's ability to respond to MDP [55]. Furthermore, NALP1 can associate with NOD2 (Hsu 2008), showing a role for NOD2 in MDP-triggered IL1β activation, separate from its role as an inducer of proinflammatory gene expression.

Although the function of NLR family members in zebrafish is not widely studied, it is known that the canonical members of the mammalian NLR family, NOD1, NOD2, and NOD3 (or Nlrc3) are conserved. Additionally, a subfamily of NLRs resembling the mammalian NALPs and a unique teleost NLR subfamily are present [34, 56]. Confirmation of the antibacterial role of NOD1 and NOD2 in zebrafish was achieved by gene knockdown, resulting in higher bacterial burdens and decreased survival of embryos following *Salmonella enterica* infection [57]. Moreover, *nod1/2* depletion significantly decreased expression of dual oxidase (DUOX), required for production of reactive oxygen species (ROS) [57]. These findings illustrate that the family of Nod-like receptors and their downstream signaling pathways are important for antibacterial innate immunity, both in mammals and in zebrafish.

2.3. RIG-I-Like Receptors. Another family of cytosolic PRRs, the RIG-I-like receptors (RLRs), consists of three members: RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation-associated factor 5), and LGP2 (laboratory of genetics and physiology 2). All three members are DExD/H box RNA helicases that can detect the presence of RNA from a broad range of viruses [58]. While expressed at low levels in most tissues, their expression is greatly increased upon viral infections or interferon (IFN) exposure [59, 60]. The RNA helicase domain of RLRs has the capacity to hydrolyze

ATP and bind to RNA [61]. Furthermore, RIG-I and MDA5 contain a tandem of CARDs, which facilitate protein-protein interactions [60]. LGP2 lacks the two CARDs and is thought to function as a negative regulator of RIG-I and MDA5 signaling [62]. Following recognition of viral RNA, the CARDs of RIG-I and MDA5 become available for binding to a common mitochondrial signaling adaptor, IPS-1 or MAVS [63]. The subsequent signaling cascade culminates in the induction of transcription factors like interferon regulatory factor 3 (IRF3), IRF7, and NFκB [64]. Activation of these transcription factors leads to the production of type I IFN, which binds to the IFN receptor to initiate expression of interferon-stimulated genes (ISGs) [65]. Amongst these ISGs are antiviral proteins, immune-proteasome components, all three RLRs, members of the TLR family, transcription factors like IRF7, and various proinflammatory cytokines and chemokines [65]. As such, the RLR-induced pathway works cooperatively with TLR signaling to prepare the cell for elimination of viral infections [58].

Zebrafish homologs of RIG-I, MDA5, and DXH58 were identified in a genome search [66]. However, *in silico* analysis of the predicted proteins revealed that the domain distribution differs between humans and zebrafish [66]. For instance, whilst human RIG-I contains two CARDs, one DExD/H domain and a Helicase C domain, zebrafish RIG-I consists of a single CARD and a DExD/H domain [66]. Whilst functional studies of the RLR pathway are scarce, it is clear that zebrafish and other teleosts possess a strong antiviral IFN system, which shares a common evolutionary origin with mammals [67, 68]. The mitochondrial RLR adaptor, IPS-1/MAVS, was recently cloned from salmon and zebrafish, and overexpression in fish cells led to a constitutive induction of ISGs [68]. Furthermore, MITA, another adaptor functioning downstream of IPS-1/MAVS and upstream of Tank-binding kinase 1 (TBK1), was cloned from crucian carp (*Carassius auratus*) and shown to activate zebrafish IFN promoter gene constructs, dependent on IRF3 or IRF7 [69].

2.4. Scavenger Receptors. Scavenger receptors are a large family of transmembrane cell surface receptors, present on macrophages, dendritic cells, mast cells [70], and some endothelial and epithelial cell types [71]. Although originally defined for their role in uptake of low-density lipoproteins (LDL), they are now known to act as PRRs for a wide variety of PAMPs, like LPS, LTA, CpG DNA, yeast zymosan, and microbial surface proteins [72]. Commonly, PAMP binding to a scavenger receptor will induce the cell to directly phagocytose the pathogen [73]. Upregulation of scavenger receptor expression via TLR signaling can be a mechanism to increase phagocytic activity [74]. Moreover, scavenger receptors can also contribute to cytokine production as coreceptors for TLRs [75, 76]. Some of the C-type lectins, discussed below, also display scavenger receptor activity.

Based upon their multidomain structure, scavenger receptors are divided into eight subclasses (A-H) (Murphy 2005). Subclasses A and B are the most extensively studied, but members from other subclasses have also been shown to recognize bacterial PAMPs [72]. SR-A, the founding member of subclass A, functions as a phagocytic receptor for bacterial pathogens like *Staphylococcus aureus*, *Neisseria meningitides*, *Streptococcus pneumonia*, and *Escherichia coli* [77–79]. Macrophage receptor with collagenous structure (MARCO), another subclass A member with established PRR activity [80], functions as a phagocytic receptor for *S. pneumonia* [81] and *N. meningitidis* [82]. MARCO was shown to cooperate with TLR2 to trigger macrophage cytokine responses to the mycobacterial cell wall glycolipid trehalose dimycolate (TDM) and *Mycobacterium tuberculosis* [83]. CD36, the most prominent member of subclass B, is a sensor for LTA and diacylated lipopeptide (MALP-2) and also acts as a coreceptor for TLR2 [75]. CD36-mediated phagocytosis of *S. aureus* was shown to be required for initiation of TLR2/6 signaling [84]. SR-BI (or CLA-1), also in subclass B, can bind to LPS and was implicated in phagocytosis of both Gram-negative and Gram-positive bacteria [85]. As well as their antibacterial roles, CD36 and SR-BI are also known for increasing the pathogenesis of malaria and hepatitis C virus (HCV). CD36 can function as a receptor for erythrocytes that have been parasitized by *Plasmodium falciparum*, adhering these cells to the venular endothelium of various organs (Pluddemann 2007). Furthermore, SR-BI is used by *Plasmodium* sporozoites and HCV as an entry site into hepatocytes [72].

Many homologs of the mammalian scavenger receptor family can be identified in the zebrafish genome, but a systematic analysis is still awaited. A zebrafish homolog of human MARCO was identified as a specific marker for macrophages and dendritic cells from adult zebrafish [86], and this gene is also myeloid specific in zebrafish embryos [87]. Expression of the *cd36* gene was upregulated after exposing zebrafish to haemorrhagic septicemia rhabdovirus [88]. In contrast, *cd36* expression was downregulated by *Mycobacterium marinum* infection in adult zebrafish and larvae [22].

2.5. C-Type Lectins. The C-type lectin receptors (CLRs) are a large family of carbohydrate-binding proteins that are highly conserved amongst mammals [89]. The diversity of the CLR family is illustrated by the fact that up to 17 groups are present in vertebrates, with some consisting of soluble serum proteins, whilst others consist of transmembrane proteins. These are mainly expressed in myeloid cells (macrophages and dendritic cells) but also in natural killer cells [90, 91]. The best known CLR in serum is mannose-binding lectin (MBL), a member of the collectin class, which binds to a variety of sugar moieties present on viruses, bacteria, fungi, and protozoa and activates the complement system [92]. In terms of their function as PRRs, the transmembrane CLRs that are expressed on myeloid cells are the most interesting. Transmembrane CLRs can be divided into two groups: the mannose receptor family and the asialoglycoprotein receptor family [93]. CLRs recognize pathogens mainly via ligand binding to mannose, fucose, and glucan carbohydrate structures, which means that together they are capable of recognizing most classes of human pathogens [93]. Like scavenger receptors, CLRs can act as phagocytic receptors

for nonopsonized bacteria, leading to their destruction in acidified phagolysosomes [73]. The best-studied member of the asialoglycoprotein receptor family is Dectin-1, which mediates phagocytosis of yeast and the yeast-derived protein zymosan [94]. Phagocytosis induced by CLRs like Dectin-1 is not only important for the lysosomal breakdown of pathogens, but also for antigen presentation [95, 96]. Besides their role in phagocytosis, CLRs can directly induce gene expression upon carbohydrate recognition. PAMP recognition by Dectin-1, Dectin-2, and macrophage-inducible C-type lectin (Mincle) ultimately leads to activation of NFκB [97–99]. Where Dectin-1 associates with the kinase Syk to activate NFκB [100], Dectin-2 and Mincle are dependent on Fc receptor Υ-chain as an adaptor molecule [98, 99]. Other CLRs, for example, DC-specific ICAM3-grabbing nonintegrin (DC-SIGN), induce specific gene expression profiles upon pathogen recognition by modulating TLR signalling [93]. When DC-SIGN recognizes mannose or fucose moieties on pathogens such as *Mycobacteria*, HIV-1, measles virus, and *Candida albicans*, it activates a Raf-1-dependent signaling pathway that modulates TLR-induced NFκB activation, increasing the production of IL8 and anti-inflammatory IL10 production [101].

Only a few homologs of CLRs have been described in zebrafish. A homolog of the complement activating mannose-binding lectin (MBL) was associated with resistance against *Listonella anguillarum* [102]. Expression of another soluble lectin, *lgals91l,* is enriched in zebrafish embryonic myeloid cells and is dependent on the Spi1/Pu.1 transcription factor that plays a crucial role in myeloid cell development in vertebrates [87]. A membrane type collectin, CL-P1 (collectin placenta 1), was shown to be involved in vasculogenesis during zebrafish embryogenesis [103]. In humans, CL-P1 is mainly expressed on vascular endothelial cells and has been shown to act as a scavenger receptor mediating the phagocytosis of bacteria and yeast [104]. A putative homolog for DC-SIGN has recently been proposed and is upregulated in immune-related tissues following infection by *Aeromonas anguillarum* [105]. Finally, putative homologs for the mammalian C-type lectin NK cell receptors have been identified in zebrafish and are differentially expressed on cells from the myeloid and lymphoid lineages [106].

3. Effector Mechanisms of the Innate Immune Response in Zebrafish

While the adaptive immune system requires several days before reacting to invading microbes, the innate immune system consists mostly of defenses that are constitutively present and activated immediately upon infection (Figure 1). The general inflammatory response is a crucial innate defense mechanism. A state of inflammation is necessary for proper function of host defenses, since it focuses on circulating immune cells and antimicrobial components of the plasma at the site of infection. Below, we focus on the effector mechanisms involved in the cell-mediated part of the innate immune response. In addition, soluble serum proteins, including complement factors and other acute-phase proteins, make an important contribution to the innate defenses, and strong induction of their encoding genes has been observed in adult and embryonic zebrafish infection models [13, 36, 38, 107–109].

3.1. Secreted Peptides and Lipid Mediators of the Innate Immune Response. Cytokines, including interleukins, chemokines, and interferons, are small secreted proteins that steer the host's immune system into a cytotoxic, humoral, cell-mediated, or allergic response [110]. Since this paper focuses on innate immunity, we will mainly discuss the cytokines produced by or acting on phagocytic cells. A distinction can be made between cytokines that promote a state of inflammation and cytokines that are anti-inflammatory. The main proinflammatory cytokines produced by phagocytes are TNFα, IL1α, IL1β, IL6, and IL8 [110]. TNF-α is processed as a membrane-bound protein and, when required, the active soluble factor is cleaved off by the TNF-α converting enzyme (TACE) [111]. Similarly, IL1α and IL1β are synthesized as inactive precursors that are only secreted as active cytokines after inflammasome-mediated cleavage by caspase 1 [112]. The most potent anti-inflammatory cytokine in humans is IL10, which deactivates the proinflammatory cytokine production by macrophages and T cells [113]. The IL10/IL12 balance, maintained by cells of the innate immune system, determines whether adaptive immunity polarizes towards a Th1 (promoted by IL12) or Th2 response. A Th1 response, which activates the bactericidal activities of macrophages, is the most important for controlling intracellular pathogens. The single type II IFN, IFNγ, is also required for activating macrophage bactericidal functions, while type I IFNs (IFNα and IFNβ) and type III IFN (IFNλ) function in mounting antiviral responses. Finally, eicosanoid lipid mediators also promote (e.g., prostaglandins and leukotrienes) or inhibit (e.g., lipoxins) inflammation, thus synergizing with or antagonizing cytokine functions.

Many of the cytokine subfamilies are conserved between zebrafish and mammals [34]. However, there has been extensive expansion and diversification of members of the chemokine gene family in zebrafish, and their specific functions are yet to be determined [114]. Several of the main cytokines, like IL1β, IL6, and IL10, have been cloned and characterized [115–117]. Furthermore, the zebrafish homolog of interleukin 10 receptor 1 (IL10R1) has recently been identified and seems to contain all the protein domains that are required for its function in anti-inflammatory signaling [118]. The proinflammatory chemokine IL8 (CXCL8) and it receptors, CXCR1 and CXCR2, are also conserved between mammals and zebrafish [119]. In addition, a second IL8/CXCL8 lineage has been identified in both zebrafish and common carp (*Cyprinus carpio*), and the chemotactic properties of carp IL8/CXCL8 molecules of both lineages were demonstrated by *in vitro* chemotaxis assays using carp leukocytes [120]. Both pro- and anti-inflammatory cytokines are upregulated upon infection of zebrafish embryos with pathogens such as *S. typhimurium* [13], *P. aeruginosa* [121], and *E. tarda* [122, 123].

The role of TNF during *Mycobacterium marinum* infection of zebrafish embryos was studied by knockdown analysis of the TNF receptor (*tnfrsf1a*), which revealed that intracellular bacterial burdens, granuloma formation, and necrotic death of macrophages are increased in the absence of TNF signaling [124]. The importance of TNF signaling during *M. marinum* infection was further illustrated when the same model was used to show that a strict balance between pro-inflammatory TNF and anti-inflammatory lipoxins is vital for control of mycobacterial infections, with either too much or too little TNF expression leading to a more severe outcome of the disease [1]. Another study using the zebrafish model indicates that TNF-α is a potent activator of endothelial cells, leading to the production of chemokines, whilst it has little effect on the activation status of phagocytes [125]. This suggests that fish TNF-α mainly functions in the recruitment of leukocytes to the site of infection, rather than activating them.

The three IFN groups present in humans are not conserved unambiguously in zebrafish and other fish species. The type II group of IFNs in zebrafish consists of IFNγ1 and IFNγ2 [126]. Expression levels of the corresponding genes did not change upon infection of zebrafish embryos with *E. coli* or *Y. ruckeri*, but was increased by *M. marinum* infection [126, 127]. Viral infection induced their expression in adult zebrafish but not in embryos [126]. IFNγ1 and IFNγ2 were shown to bind to different receptor complexes, and Janus kinase 2a (Jak2a), but not Jak2b, was shown to be required for intracellular transmission of the IFNγ signal. Two groups of antiviral IFNs, named IFNϕ1 and IFNϕ2, exist in zebrafish, and structural analysis showed that these are evolutionarily closer to type I than to type III human IFNs [34, 67, 128]. IFNϕ1 and IFNϕ2 signal via distinct receptor complexes [67, 129]. All zebrafish IFNϕ genes induce the expression of genes that are predicted to be involved in antiviral activities [67].

3.2. Phagocytosis, Autophagy, and Lysosomal Destruction. Internalization of microorganisms is triggered when they are recognized by phagocytic receptors, mainly by scavenger receptors discussed above. This type of direct phagocytosis is termed nonopsonic phagocytosis, while opsonic phagocytosis relies on host-derived proteins that coat the surface of the microbe thereby enhancing phagocytosis efficiency. Opsonins include complement fragments, most notably C3b, which are recognized by complement receptors [130]. Mannose binding lectin, which can initiate C3b formation, and antibodies that bind to Fc receptors (IgG) or that activate complement (IgM) are also considered opsonins. Regardless of which receptor initiates the process, phagocytosis requires the activation of kinases and Rab GTPases that control alterations in the phospholipid membrane and remodeling of the actin cytoskeleton [131]. In macrophages, fusion of the resulting vesicle with early and late endosomes will decrease the pH of the immature phagosome and alter the proteins present on its membrane. Ultimately, maturing phagosomes turn into phagolysosomes when lysosomes fuse with them, mixing their contents [132]. Lysosomes are highly acidic endocytic vesicles (pH $<$ 5.5), containing active proteases and lipases, and hydrolytic enzymes such as cathepsin D [133]. In addition, phagolysosomes also contain bactericidal peptides (defensins) and have the ability to generate toxic oxidative compounds that help microbial degradation [134]. Most of our knowledge about phagosome maturation comes from studies of phagocytosis in macrophages, and much less is known about phagosome maturation in neutrophils. While macrophage phagosomes fuse with endosomes and lysosomes, neutrophil phagosomes obtain their bactericidal properties by fusing with secretory vesicles and granules [135, 136]. In contrast to phagosome maturation in macrophages, neutrophil phagosomes do not acidify in order to become microbicidal [135, 136].

Many intracellular pathogens, like *M. tuberculosis*, *S. typhimurium*, and *Legionella pneumophila*, have evolved the ability to prevent phagosome maturation in macrophages and survive inside these vesicles [137]. To a certain extent, such pathogens can also withstand the hostile environment of the (phago)lysosome. Other pathogens like *Listeria monocytogenes*, *Francisella tularensis*, and many viruses can escape the phagosome and enter the cytosol [138]. *Mycobacterium marinum*, a pathogen studied extensively in zebrafish to model human tuberculosis, can survive inside phagosomes but also escape into the cytosol and spread to neighbouring cells by actin-based motility [139, 140]. Phagosomal escape has also been observed for the human pathogen *M. tuberculosis* and is dependent on a virulence factor, the ESX-/RD1 secretion system, shared by all pathogenic mycobacteria [141]. Together, these data indicate that host cells face numerous pathogens that have developed multiple strategies to avoid the pathway of phagolysosomal degradation. To counter such threats, cells may use autophagy to clear microbes and microbe-containing vesicles from the cytosol. Autophagy is well known as a metabolic process that recycles nutrients by degrading intracellular organelles and proteins. Only recently, it has been recognized that autophagy also plays an important role in the innate immune response against intracellular pathogens [142]. Autophagy is initiated when an autophagosomal isolation membrane is formed around its target, enclosing it entirely in a double-membrane vesicle. This process relies on class III phosphatidylinositol 3-kinase (PI3-kinase) and autophagy-related genes (Atgs), such as Atg6 (or Beclin-1) [143]. The hallmark of autophagosomes is the presence of Atg8 (or LC3) in their membranes, which is essential for membrane elongation [144]. Similar to a maturing phagosome, the autophagosome also fuses with lysosomes to achieve its degradative properties [145]. In addition, autolysosomes acquire unique antimicrobial properties due to the function of autophagic adaptor protein p62, which delivers cytosolic components to autolysosomes where they are processed into potent antimicrobial peptides [146]. As reviewed elsewhere [147], pathogen-targeted autophagy can be induced by several TLRs and NLRs, TNF-α, NFκB, and many other immune-related signalling molecules.

The transparency of zebrafish embryos and availability of fluorescent macrophage and neutrophil reporter lines allow for study of the process of phagocytosis in great detail

[7, 148–150]. It was recently shown that zebrafish embryonic macrophages efficiently engulf *E. coli* bacteria from blood- and fluid-filled cavities, while neutrophils are hardly capable of phagocytosing bacteria present in fluids [150]. However, neutrophils did prove to be highly phagocytic when moving over bacteria present on tissue surfaces. This shows that the type of immune cell that clears an infection not only depends on the PAMPs present on the invading microbe, but also on the characteristics of the infection site. An *in vivo* phagocytosis assay was used to show that functions of Wasp1, Wasp2, Abi2, and cofilin regulator 14-3-3ζ (Ywab) in bacterial phagocytosis are conserved in zebrafish [151]. The recent generation of a transgenic zebrafish line with GFP-tagged LC3 has enabled *in vivo* visualization of the interactions between microbes and this core component of the autophagy machinery [152]. The importance of autophagy in the innate immune response of zebrafish remains to be studied, but we have shown that LC3-labeled structures accumulate around *M. marinum* infection sites in zebrafish embryos (Figure 2). Furthermore, autophagy-related genes were induced in adult zebrafish infected with *Citrobacter freundii* and zebrafish embryos infected with *S. typhimurium* [37, 153].

3.3. Oxidative Defenses in Leukocytes. In several systems, it has been shown that neutrophils are the first immune cells to arrive at the site of infection or wounding. They facilitate their migration by exocytosing granules that contain metalloproteinases and other enzymes that degrade the extracellular matrix [154]. Upon recognition of pathogens, neutrophils release their antimicrobial granules, called azurophils, into phagosomes or the extracellular environment [155, 156]. Azurophils are packed with acidic hydrolases and antimicrobial proteins, such as lysozyme, cathepsins, and myeloperoxidase (MPO) [157]. The primary function of MPO is to react with hydrogen peroxide(H_2O_2), which subsequently oxidates chloride, tyrosine, and nitrite to form hypochloric acid (HOCl), tyrosine radicals, and reactive nitrogen intermediates [158]. These highly reactive chemicals attack the surface membranes of microbes. Additionally, microbes can be bound by neutrophil extracellular traps (NETs), which are fibrous networks of granule proteins and chromatin released by neutrophils [159].

While MPO is mostly produced in neutrophils, all professional phagocytes produce high levels of reactive oxygen species (ROS), including superoxide, H_2O_2, and hydroxyl radicals, produced by the enzymes NADPH oxidase (NOX) and dual oxidase (DUOX) [160]. The NOX of phagocytes (Phox) is only activated upon exposure to microorganisms or other pro-inflammatory stimuli [161]. When active, Phox is located in the phagosomal membrane and catalyzes the respiratory burst, which consists of the large-scale production of ROS that helps degrade phagocytosed microbes by nonspecifically oxidizing protein, DNA, lipid, and carbohydrate [162]. H_2O_2 produced during the respiratory burst can also function as a substrate for MPO activity. The oxidative enzyme DUOX may even combine the two functions, by generating H_2O_2 as a substrate for its own peroxidase domain [160].

Nitric oxide (NO) is produced from the amino acid L-arginine by nitric oxide synthase (NOS) enzymes and functions as a signaling molecule in numerous biological processes as well as having antimicrobial activity [163]. There are two constitutively expressed NOS enzymes, neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3), and one inducible NOS (iNOS or NOS2) that is important in innate immunity. Regulation of NOS2 plays an important role in the inflammatory response, and many cells of the immune system are capable of producing NO [164, 165]. NO has cytostatic and cytotoxic antimicrobial effects when high amounts are excreted by immune cells into mammalian tissues, most likely via reactive nitrogen species (RNS) which are generated when NO interacts with O_2 [166]. These RNS subsequently lead to lipid peroxidation, DNA damage, oxidation of thiols, and nitration of tyrosine residues [167]. It has recently been shown that Nos2a, the zebrafish homolog of NOS2, is also required for the expansion of hematopoietic stem cells and progenitor cells during infection, leading to increased numbers of the required immune cells [168]. This discovery further adds to the importance of NOS2 in the inflammatory response.

The oxidative defense mechanisms need to be tightly controlled, since high levels of reactive chemicals like ROS and RNS cause tissue damage at sites of infection. Therefore, the resolution phase of inflammation is critical in order to restore the tissue to its normal state and prevent chronic inflammation. The molecules produced during oxidative defenses are often self-limiting and help initiate resolution of inflammation by inducing neutrophil apoptosis [160, 169]. Furthermore, iNOS-induced NO production can be countered by activation of arginase (ARG), which depletes the substrate for iNOS by converting L-arginine to the harmless compounds urea and L-ornithine, thus creating conditions more favorable for wound healing [163, 170].

The zebrafish homolog of MPO, officially named MPX, is specifically expressed in neutrophils during embryonic development. Transgenic reporter lines driven by the *mpx* promoter have made the zebrafish a highly suitable model organism to study neutrophilic inflammation [8, 171]. In fact, using one of these lines, it was demonstrated for the first time that H_2O_2 produced in the context of wounding not only functions as an antiseptic compound, but also forms a gradient that is required for rapid attraction of leukocytes [172]. However, this H_2O_2 gradient is only generated at wounds and does not occur at infected tissues [173]. The formation of this H_2O_2 gradient was shown to be dependent on the oxidase activity of Duox. The Src family kinase Lyn has been identified as the redox sensor that mediates neutrophil migration towards the wound [174]. The innate immune function of Duox and the importance of ROS in zebrafish were further established by studies showing that knockdown of Duox impaired the ability of zebrafish larvae to control enteric *Salmonella* infections [175]. It has also been shown that zebrafish Phox is important in controlling the *in vivo* growth of the pathogenic fungus *Candida albicans* [176]. A 5,5-dimethyl-l-pyrroline N-oxide- (DMPO-) based immunospin trap technique has been adopted for in situ detection of ROS production in zebrafish embryos [177].

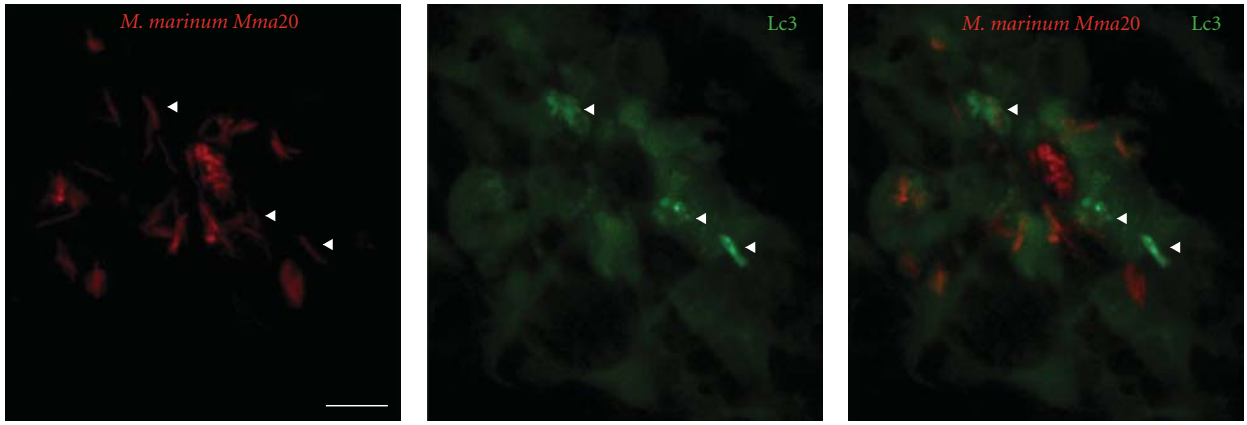

Figure 2: *In situ* detection of autophagy by Lc3 accumulation. CMV::LC3-GFP transgenic [15] zebrafish embryos (28 hpf) were injected into the caudal vein with 200 colony-forming units (CFU) of *M. marinum Mma20* expressing a pMST3::mCherry vector. Confocal images were taken of a tail region of the developing larva at 3 days after infection (3 dpi), a point at which the *M. marinum* infection (a) has been established. Low levels of Lc3-GFP signal (b) can be observed throughout the cells, whilst brighter regions (indicated by arrowheads) are only observed upon Lc3 accumulation and formation of autophagic membranes associated with bacteria (c). Scale bar: 10 μm.

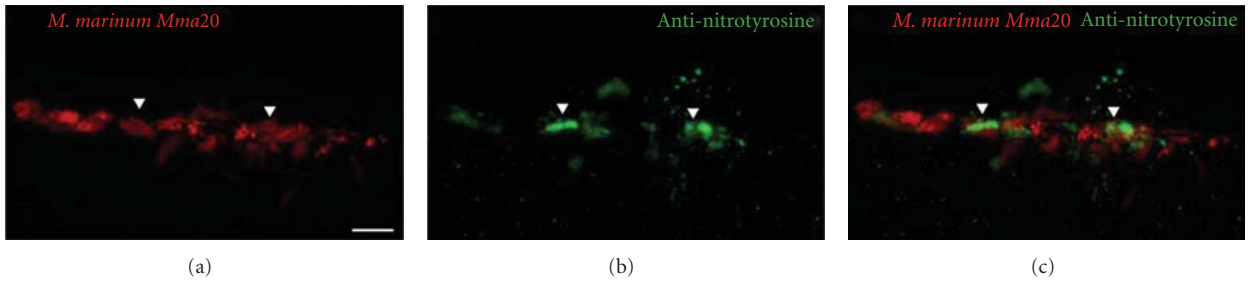

Figure 3: *In situ* detection of reactive nitrogen species. Wild-type zebrafish embryos (Albino; 28 hpf) were injected into the caudal vein with 200 colony-forming units (CFU) of *M. marinum Mma20* expressing a pMST3::mCherry vector. Confocal images were taken of a tail region of the developing larva at 3 days after infection (3 dpi), a point at which the *M. marinum* infection (a) has been established. Embryos were fixed in 4% paraformaldehyde at 3 dpi, and immunohistochemistry was performed, using an antinitrotyrosine antibody that detects tissue nitration (b) [21]. Colocalization (c) between bacteria and extensive tissue nitration can be observed at this time point. Scale bar: 10 μm.

DMPO is a chemical substrate that binds to reactive oxygen, which can later be detected with an anti-DMPO antibody. This protocol detects the build-up of the conjugated product, thereby showing a cumulative ROS production. Furthermore, a respiratory burst assay has been developed for zebrafish embryos, which was used to demonstrate that macrophages and neutrophils are the ROS-producing cells in zebrafish [178]. A similar method is available to image the production of NO in zebrafish embryos, using a diaminofluorescein probe that only becomes fluorescent in the presence of NO [179]. As mentioned before, nitration of tyrosine residues is a hallmark of NO production. Forlenza et al. (2008) used an antinitrotyrosine antibody on common carp tissue to visualize the tissue nitration that occurs at sites of *Trypanoplasma borreli* infection [21]. We used the same antibody for immunohistochemistry on zebrafish embryos to visualize the production of RNS in response to *M. marinum* infection (Figure 3). This technique also visualizes the nitrosative stress that the host tissue suffers upon release of RNS. The resolution of inflammation that should prevent tissue damage following such stresses has also been studied in zebrafish. This has led to new insights on the mechanisms underlying resolution, including apoptosis and retrograde chemotaxis of neutrophils, with the oxygen-sensing transcription factor hypoxia-inducible factor-1α (Hif-1α) playing a role in the control of these mechanisms [171, 180].

4. Gene Expression Programs Reflecting Innate Immune Responses

4.1. Genome-Wide Expression Profiling. The availability of the zebrafish genome sequence facilitates the use of microarray and deep sequencing techniques for genome-wide expression profiling. Zebrafish embryos and larvae are useful for *in vivo* analysis of gene expression profiles upon infection, since large numbers can be pooled to level out individual variation. However, pooling should be done with caution, and it is advisable to verify conclusions by analysis at the single-embryo level [123]. A protocol has been developed for single embryo RNA isolation that gives sufficient RNA

Table 1: Transcriptome profiling studies on infection models in adult and embryonic zebrafish.

Bacterial species	Strain	Infection model	Reference
Mycobacterium marinum	M; E11	Adult (IP)	Meijer et al.*[182]
Mycobacterium marinum	*Mma*20; E11	28hpf (CV); Adult (IP)	Van der Sar et al. [22]
Mycobacterium marinum	M; E11	Adult (IP)	Hegedus et al.* [107]
Salmonella enterica serovar Typhimurium (*Salmonella typhimurium*)	SL1027; LPS derivative SF1592 (*Ra*),	28hpf (CV)	Stockhammer et al.** [13]
Streptococcus suis	HA9801	Adult (IP)	Wu et al. [183]
Salmonella enterica serovar Typhimurium (*Salmonella typhimurium*)	SL1027; LPS derivative SF1592 (*Ra*),	28hpf (CV)	Ordas et al.** [38]
Edwardsiella tarda	FL6-60	28hpf (CV)	Van Soest et al. [123]
Citrobacter freundii	Not specified	Adult (IM)	Lu et al. [153]

* and **: these studies used the same samples but applied microarray analysis and deep sequencing, respectively.
(IP): intraperitoneal; (CV): caudal vein; (IM): immersion.

for microarray or RNA sequencing [181]. Expression profiling can be done either at whole organism level or on FACS-sorted immune cells from transgenic lines. The latter approach was used to determine the transcriptional signature of early myeloid cells [87]. Microarray analysis of zebrafish adults and embryos infected with various pathogens has provided insights into the transcriptome during infection and has provided leads for further functional studies (Table 1). The transcriptional response of both zebrafish embryos and adults showed clear conservation with host responses detected in other vertebrate models and human cells. Genes that were induced upon infection included receptors involved in pathogen recognition, signaling intermediates, their downstream transcription factors (like NFκB and AP-1), and inflammatory mediators. Furthermore, these studies led to the identification of novel immune responsive genes and infection markers, for example, the DNA-damage-regulated autophagy modulator 1 gene (*dram1*), which was identified in a knockdown study of Traf6, a central intermediate in TLR and TNF receptor signaling [37].

4.2. Comparison of Gene Expression Profiles Induced by Different Bacterial Pathogens. To illustrate the similarities and differences in the innate immune response against different bacterial pathogens, Figure 4 shows a comparison of the gene expression profiles of zebrafish infected with *Edwardsiella tarda*, *S. typhimurium*, and *M. marinum*. *E. tarda* is a Gram-negative, naturally occurring fish pathogen that belongs to the Enterobacteriaceae family. Inside its host, *E. tarda* is able to resist complement activity and can survive inside macrophages [184]. It causes a progressive disease when injected into the caudal vein of 28 hours after fertilization (hpf) embryos, leading to mortality within 2 days after infection (dpi) [123]. *S. typhimurium* (short for *S. enterica* serovar Typhimurium), also belonging to the Gram-negative Enterobacteriaceae family, causes salmonellosis in a broad range of hosts. *S. typhimurium* is a facultative intracellular species that can survive within phagocytic and nonphagocytic cells. Following internalization, it survives and replicates in a modified phagosome, known as the *Salmonella*-containing vacuole. Like *E. tarda*, injection of *S. typhimurium* into the caudal vein at 28 hpf leads to a progressive disease which leads to mortality of the embryo during the first 30 hours after infection (hpi) [13, 185]. In contrast, *M. marinum* injection at the same stage leads to a chronic infection that persists during larval development. *M. marinum* is a natural pathogen of teleost fish and a close relative of *M. tuberculosis*, the causative agent of tuberculosis in humans. *Mycobacteria* have a thick, waxy, acid-fast staining cell wall containing characteristic lipids that are important for virulence. Both *M. marinum* and *M. tuberculosis* have the ability to replicate inside macrophages, eventually causing them to undergo apoptosis. Dependent on secreted virulence factors that are conserved between *M. marinum* and *M. tuberculosis*, other macrophages are attracted to the initial infection site. These become infected by phagocytosing the apoptotic remains, which ultimately leads to the formation of a granuloma [186]. Using the zebrafish embryo model, Ramakrishan et al. have provided new insights demonstrating the importance of the innate immune system to control *M. marinum* infection during early stages of pathogenesis [1, 2, 124, 187, 188].

Complementary to previously reported transcriptome data (Table 1), here we present new data comparing the gene expression profiles induced by *E. tarda*, *S. typhimurium,* and *M. marinum* under similar conditions (Figure 4). We injected 200 colony-forming units (CFUs) of each pathogen into the caudal vein of 28 hpf zebrafish embryos and analyzed the response at 8 hpi. Since *M. marinum* develops a chronic infection, we also sampled at 4 dpi, a time point at which granulomas are present. Finally, we compared the transcriptome profile of the embryonic samples with data from a previous study, in which adult zebrafish were infected with the same strain of *M. marinum* [22].

The two progressive Gram-negative pathogens, *E. tarda* and *S. typhimurium*, induced a strong early immune response at 8 hpi, while the chronic *M. marinum* infection hardly induced any response at this time point. At 4 dpi, the transcriptome profile of *M. marinum*-infected embryos did

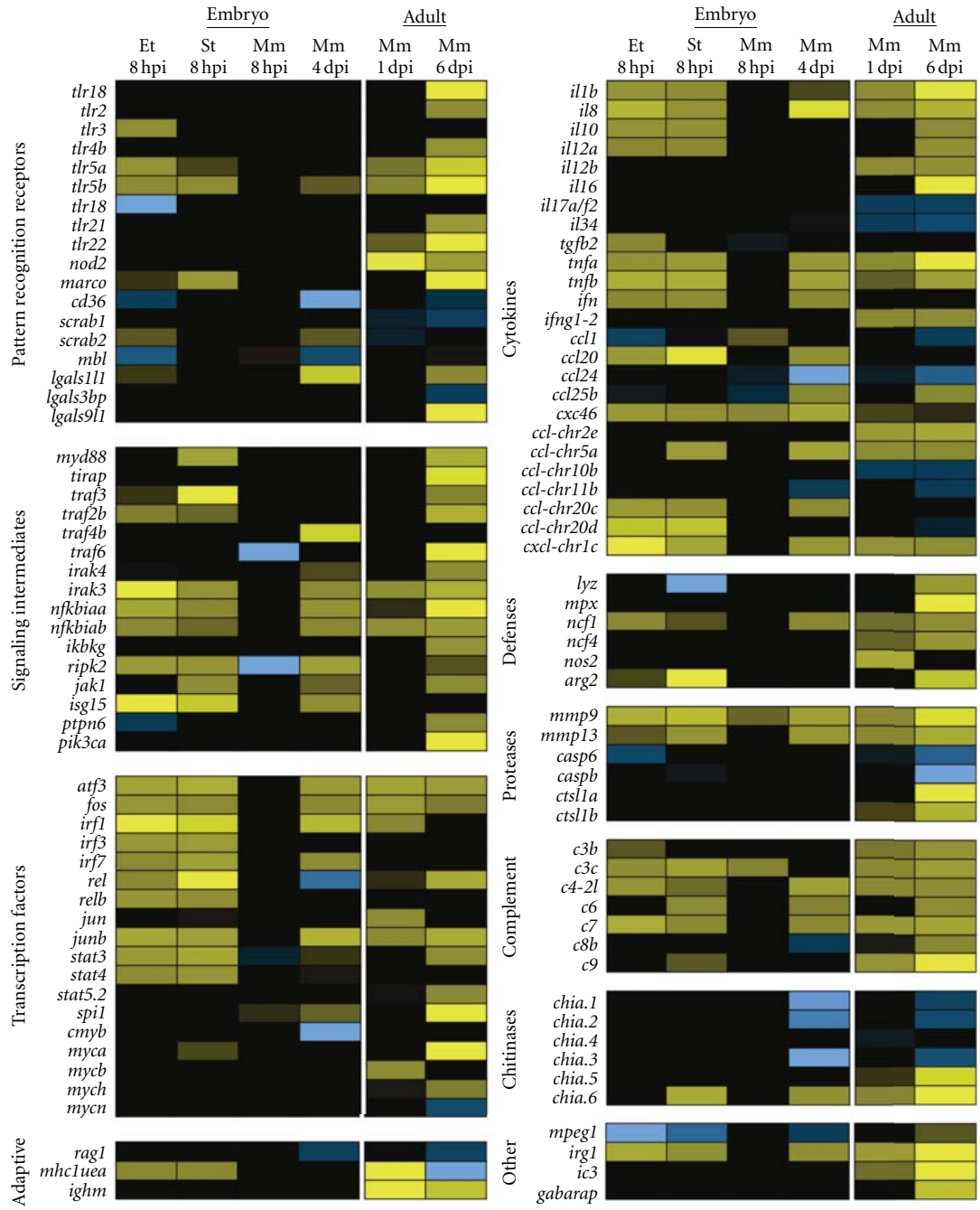

Figure 4: Comparison of the zebrafish innate immune response to different bacterial pathogens. Gene expression profiles of zebrafish embryos and adults infected with *E. tarda FL6-60* (Et), *S. typhimuriumSL*1027(St), and *M. marinum Mma20* (Mm) are depicted in a heat map. Embryos were infected with 200 CFU of each pathogen into the caudal vein at 28 hpf and snap frozen individually at 8 hpi for *E. tarda* and *S. typhimurium*, and at 8 hpi and 4 dpi for *M. marinum*. Triplicate samples for each infection condition were compared with samples from control embryos (injected with PBS) using a common reference microarray design. The raw data were deposited in the Gene Expression Omnibus database under accession number GSE35474. The data derived from embryonic infections were compared with data from a study in which adult zebrafish were infected intraperitoneally with *M. marinum Mma20,* after which RNA samples were taken at 1 dpi and 6 dpi [22]. The dose of the *Mma20* strain used in the adult infection study was lethal within days after the final sampling point at 6 dpi. Only genes relevant to this paper were included in the heatmap. All selected genes are represented by a minimum of two probes that showed significant up or downregulation (significance cut-offs for the ratios of infected versus control groups were set at 2-fold with $P < 10^{-5}$). Upregulation is indicated by increasingly bright shades of yellow, and downregulation is indicated by increasingly bright shades of blue. It should be noted that the genes listed in this figure are named according to sequence homology with mammalian counterparts and in most cases have not yet been confirmed functionally.

show an immune response, although it was still weaker than the response to *E. tarda* or *S. typhimurium* infection at 8 hpi. In adults, the immune response to *M. marinum* infection has been shown to develop in a similar manner, with hardly any induction of proinflammatory genes at 1 dpi and a stronger response at 6 dpi, when the fish began to show symptoms of disease [22]. Infections with *E. tarda* and *S. typhimurium* resulted in a remarkably similar transcriptome. Nevertheless, subtle differences were observed, like the upregulation of Tlr3 that was specific to *E. tarda* infection in this data set, and the variation in the panel of cytokines expressed upon these infections.

Interestingly, various PRRs, for example, Tlr5a and 5b, showed increased expression upon infection, most likely indicating an elevated state of awareness needed to identify the invading pathogens. In contrast, the fish-specific Tlr18, the scavenger receptors CD36, scarb1, and scarb2, and the C-type lectin Mbl were downregulated in some conditions. In many cases, signaling intermediates downstream of PRRs were upregulated, relaying and possibly amplifying the activating signals they receive from their respective receptors. A wide range of transcription factors with well-established functions in immunity (e.g., Atf3, Jun and Fos, Rel, and the IRF and Stat family members) were significantly upregulated under all conditions tested, except for the 8 hpi time point of *M. marinum* infection, whereas we observed upregulation of transcription factors of the oncogenic Myc family mainly in adult fish. The hematopoietic transcription factor Spi1 (Pu.1) was upregulated in *M. marinum* infection of embryos and adults. Genes for the key pro-inflammatory cytokines, like TNFα (two genes in zebrafish: *tnfa* and *tnfb*), IL1β, and IL8, and for the anti-inflammatory cytokine IL10 were induced by infection with any of the three pathogens. Other cytokines appeared to be more specific for certain pathogens or might not be expressed at the specific time point of infection that we sampled.

We also observed increased expression of genes involved in effector mechanisms. However, upregulation of the genes encoding lysozyme, myeloperoxidase, and iNos was detectable only in adult zebrafish infected with *M. marinum*. Infection with any of the three pathogens led to increased gene expression of *ncf1*, a subunit of the neutrophil NADPH oxidase complex. Proteases are an important part of the innate immune response, functioning in reorganizing the extracellular matrix to allow leukocyte migration, in degradation of microbes, and in processing of cytokines. In adult zebrafish infected with *M. marinum*, we observed upregulation of cathepsin-like 1a and 1b (*ctsl1a* and *ctsl1b*), members of lysosomal cathepsin family that aids in the destruction of microbes. Expression levels of *casp6* and *caspb,* members of the cysteine-aspartic acid protease (caspase) family involved in apoptosis, were downregulated at different stages of infection in adults and embryos. The matrix metalloproteinase (mmp) genes 9 and *mmp13* proved to be excellent markers for infection, since their gene expression was induced by *E. tarda*, *S. typhimurium* and *M. marinum*.

Our data further suggest that complement activation plays an important role during the early innate immune response, since a large number of complement factor genes show increased expression upon infection. Upregulated expression of the autophagy marker genes *lc3* and *gabarap* in adults infected with *M. marinum* hints towards a role for autophagy in the control of this infection. Intriguingly, a macrophage-expressed gene with unknown function in immunity, *mpeg1* [87], is downregulated during the embryonic immune response against all three pathogens. The mouse homolog of this gene encodes a perforin-like protein that is expressed in mature macrophages and prion-infected brain cells [189]. We have also observed specific upregulation of genes with as of yet unknown function in immunity, like immunoresponsive gene 1 (*irg1*). This gene is highly conserved in vertebrates and has high homology to bacterial methylcitrate dehydrogenase [190]. We also included some genes involved in adaptive immunity in our comparison, the lymphocyte marker *rag1*, the immunoglobulin heavy chain gene *ighm,* and the antigen-presenting major histocompatibility complex class I UEA gene (*mhc1uea*). Even though no cells of the adaptive immune system are present yet, embryos infected with *E. tarda* or *S. typhimurium* increase the expression of the MHC I gene. Finally, upon infection with *S. typhimurium* and *M. marimum*, we observe up and downregulation of chitinases, a family of genes which has been attributed a role during the host-microbial interactions involved in the development of acute and chronic inflammatory conditions [191].

5. Discussion

Zebrafish infectious disease models have started to make an important contribution to the understanding of host-pathogen interaction mechanisms. A good example is the discovery of the mechanism whereby a mycobacterial virulence factor (ESAT6) induces *mmp9* expression in host epithelial cells neighboring infected macrophages, which enhances macrophage recruitment and formation of granuloma-like aggregates that provide a replication niche for mycobacteria [2]. The combination of genetics and *in vivo* imaging in zebrafish embryos is unparalleled in other vertebrate models. Furthermore, zebrafish embryos provide an ideal model for high-throughput *in vivo* screening of antimicrobial drug candidates or novel vaccine candidates [192, 193]. Knowledge of the zebrafish immune system is also important in high-throughput screening for cancer in zebrafish embryos [194]. However, many aspects of zebrafish immunity still require further characterization and validation.

Currently available transgenic lines clearly distinguish macrophages (marked by *csf1r*/*fms* and *mpeg1*) from neutrophils (marked by *mpx* and *lyz*) in embryos and larvae, but there is insufficient knowledge of surface markers to identify different macrophage and neutrophil subpopulations. Similar to mammals, there is evidence of the existence of subpopulations of classically activated macrophages (M1: high producers of proinflammatory mediators, ROS, and NO) and alternatively activated macrophages (M2: high producers of anti-inflammatory mediators) in fish [195]. The polarization of macrophages towards these subtypes plays

a critical role in the pathology of both infectious diseases and cancer [196]. Furthermore, different subpopulations of mammalian neutrophils (N1 and N2) have been recently described that display pro- and antitumorigenic properties [197] and that probably will also turn out to have distinctive functions during infectious disease pathology. Tumor implants in zebrafish embryos were shown to attract a heterogeneous population of leukocytes, including cells that express arginase, a marker of alternatively activated macrophages [177]. In addition, the neutrophil markers *mpx*, *mych*, and *lyz* do not show complete overlap [177, 198], and markers such as *cxcr3*.2 and *ptpn6*, which are macrophage specific in one-day-old embryos, also label a subset of neutrophils at later stages [87]. Future development of transgenic lines that can distinguish these multiple myeloid subsets would further strengthen the use of zebrafish models for innate immunity and infectious disease studies.

As detailed in this paper, counterparts of the major vertebrate PRRs and downstream signaling components have been identified in zebrafish, but relatively few have thus far been functionally studied in infectious disease models. Recently, new PRRs have been described in mammals, like the INF-inducible dsRNA-activated protein kinase R (PKR) [199], the cytosolic DNA sensor DNA-dependent activator of IFN-regulatory factors (DAI) [200], and a cytosolic DNA receptor named AIM2 (absent in melanoma 2) [201]. Thus far, only the zebrafish homolog for PKR has been identified. Furthermore, autophagic adaptors known as sequestosome 1/p62-like receptors (SLRs), conserved between zebrafish and human, have recently been suggested as a new category of PRRs, since they have the ability to recognize and capture targets for immune-related autophagy [202].

Various datasets derived from transcriptome analyses have shown the specificity of immune responses to different pathogens. In future studies, the analysis of these responses can be refined by FACS sorting of immune cell populations from infected embryos, using labeled pathogens in combination with transgenic lines for different immune cell types. For example, it now comes within reach to aim at dissecting the differences in gene expression between *M. marinum*-infected macrophages inside a granuloma and recently attracted uninfected macrophages. In addition, simultaneous profiling of pathogen and host genes will be a challenging approach to help unravel the complex mechanisms underlying host-pathogen interactions. Transcriptome analysis only reveals altered RNA levels upon infection, and therefore, the application of proteomic and epigenetic analyses are needed to study the regulation of immune responses on different levels. Transcriptome studies have revealed infection responsiveness of many genes that have not yet been well studied (for example, *dram1*, *mpeg1*, *irg1*, and *irg1l*, mentioned above) and an emerging immune function for several chitinase-like proteins during infection [13, 37, 123]. Many zebrafish infection models have been described here and in other recent papers [4, 203, 204] that can be used to investigate the functions of these genes in different pathogenic interactions, either using morpholino knockdown in embryos or using stable knockout lines which nowadays can be identified very efficiently by high-throughput resequencing of mutant libraries or by targeted knock-down approaches using technologies such as zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) [205].

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Review Article

Histocompatibility and Hematopoietic Transplantation in the Zebrafish

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The zebrafish has proven to be an excellent model for human disease, particularly hematopoietic diseases, since these fish make similar types of blood cells as humans and other mammals. The genetic program that regulates the development and differentiation of hematopoietic cells is highly conserved. Hematopoietic stem cells (HSCs) are the source of all the blood cells needed by an organism during its lifetime. Identifying an HSC requires a functional assay, namely, a transplantation assay consisting of multilineage engraftment of a recipient and subsequent serial transplant recipients. In the past decade, several types of hematopoietic transplant assays have been developed in the zebrafish. An understanding of the major histocompatibility complex (MHC) genes in the zebrafish has lagged behind transplantation experiments, limiting the ability to perform unbiased competitive transplantation assays. This paper summarizes the different hematopoietic transplantation experiments performed in the zebrafish, both with and without immunologic matching, and discusses future directions for this powerful experimental model of human blood diseases.

1. Introduction

In the past few decades, the zebrafish has emerged as an outstanding vertebrate animal model for studying developmental hematopoiesis (reviewed in [1, 2]). In this same time frame, the understanding of the biology of adult hematopoietic stem cells has also blossomed, predominantly due to hematopoietic transplantation experiments performed in mice (reviewed by Orkin and Zon in [3]). To capitalize on the advantages of the zebrafish model (small size, high fecundity, rapid maturation, external fertilization, and the ability to perform large-scale genetic and chemical screens), a zebrafish hematopoietic transplantation assay was needed.

Developing a transplantation assay in the zebrafish required a different approach than that used in mice. While differential expression of CD45 isoforms is generally used to distinguish between donor and recipient cells in murine transplant assays, these reagents are not available for zebrafish. Instead, scientists have utilized transgenic technology to make zebrafish expressing green fluorescent protein (GFP) or other fluorochromes under the influence of an ubiquitous or a tissue-specific promoter. These fluorescently labeled donor cells are transplanted into fluorochrome-negative recipients, and engraftment is monitored at various time points after transplant.

2. A History of Hematopoietic Transplantation in Zebrafish

2.1. Adult Marrow Cells into Embryos. The first hematopoietic transplant experiments in zebrafish were performed by Traver et al., whose work was published in 2003 [4]. This landmark paper was the first to report the evaluation of zebrafish kidney marrow cells including separation of the major blood cell lineages by flow cytometry, a method which

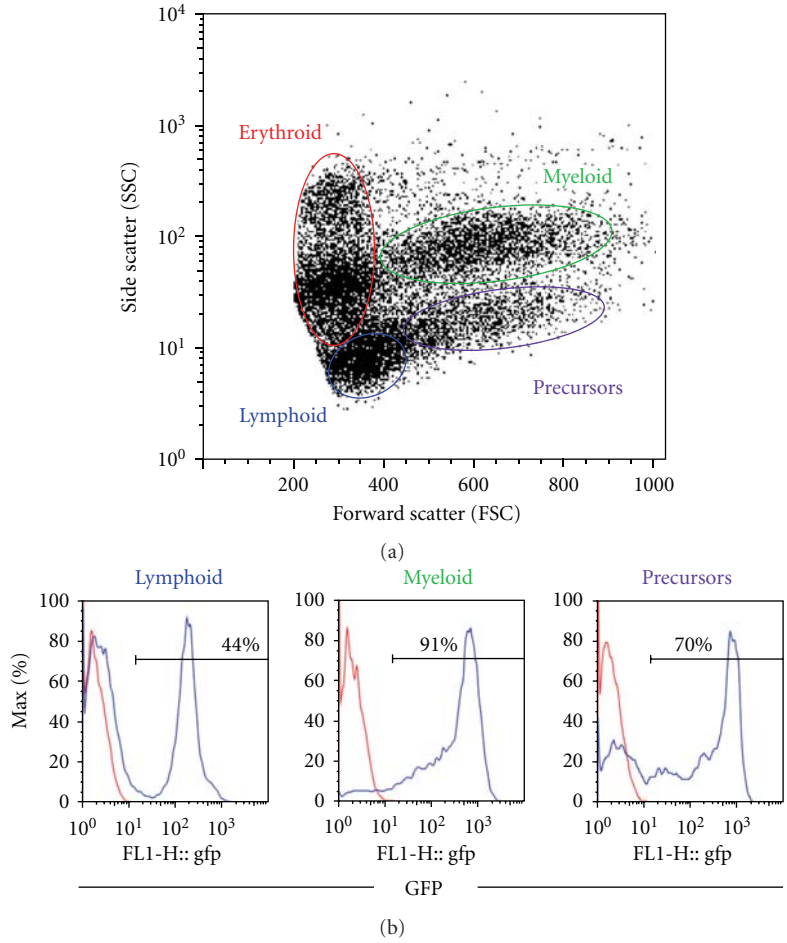
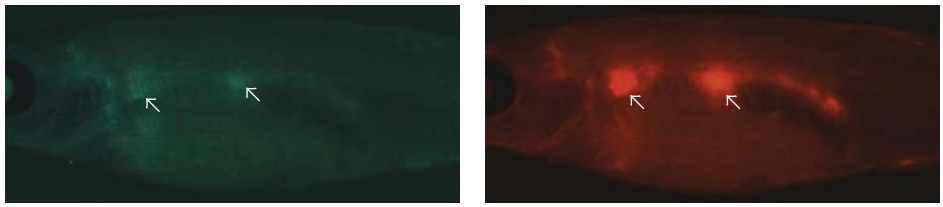


Figure 1: Flow cytometry analysis of zebrafish whole kidney marrow from a marrow transplant recipient. Zebrafish transplant recipients were irradiated and injected with 5×10^5 marrow cells from a transgenic *β-actin:GFP* donor. Whole kidney marrow from a representative recipient was dissected 3 months later and analyzed by flow cytometry. (a) The forward scatter (FSC) versus side scatter (SSC) profile of zebrafish whole kidney marrow shows four cell populations: erythroid, lymphoid, myeloid, and precursor cells. (b) Histograms for GFP expression of cells within the lymphoid, myeloid and precursor gates show multilineage engraftment with GFP$^+$ donor cells (blue lines). The red lines show GFP expression in a wild-type-negative control fish.

is currently the standard procedure for identifying multilineage engraftment after hematopoietic transplantation in zebrafish (Figure 1(a)). In addition, hematopoietic transplantation was used to rescue two different mutant embryos. The Vlad tepes (*gata1*$^{-/-}$) mutation is homozygous lethal by 14 days after fertilization, and these embryos have a complete absence of erythroid cells [5]. Approximately 100–1000 whole kidney marrow (WKM) cells from a *gata1-GFP* transgenic donor were injected into the circulation of *gata1*$^{-/-}$ zebrafish embryos 48 hours after fertilization (hpf). While untransplanted control embryos did not survive past 14 dpf, 20–60% of the transplant recipients survived long term, up to 8 months after transplant. All surviving recipients had circulating GFP$^+$ red blood cells, indistinguishable from the *gata1-GFP* donors [4].

Taking these embryonic transplant experiments one step further, donor marrow was isolated from double transgenic *β-actin-GFP/gata1-dsRED* fish, in order to monitor donor-derived cells from multiple lineages. The *β-actin-GFP* transgene is expressed by almost all zebrafish cell types, including all leukocytes. Erythrocytes do not express *βactin*, so they are marked by the *gata1-dsRED* transgene instead. For these experiments, the transplant recipients were *bloodless (bls)* mutants, a dominant, partially penetrant mutation resulting in absent primitive hematopoiesis, but preserved adult hematopoiesis [6]. Injection of double-positive WKM cells into 48 hpf *bls* mutants allowed independent tracking of GFP$^+$ leukocytes and dsRED$^+$ erythrocytes in the recipient embryos [4]. Sustained multilineage donor-derived cells were visible in the circulation of transplant recipients at 8 weeks after transplantation, indicating successful engraftment of long-term hematopoietic repopulating cells.

2.2. Adult Marrow Cells into Adult Recipients. Following up on their transplantation experiments into embryos, Traver et al. subsequently performed transplantation of WKM cells into adult recipients [7]. After using ionizing radiation as pretransplant conditioning to ablate the recipient's hematopoietic cells, including the immune system, approximately 1×10^6 *β-actin-GFP/gata1-dsRED* donor marrow



(a) (b)

Figure 2: Direct visualization of engrafted GFP$^+$ and mCherry$^+$ marrow donor cells in *casper* recipients. 40×10^3 WKM cells from a transgenic *ubiquitin:GFP* donor were mixed with 80×10^3 WKM cells from a transgenic *ubiquitin:mCherry* donor and injected into the circulation of a *casper* recipient fish. The photos are taken 4 weeks after transplantation and show engraftment of (a) GFP$^+$ and (b) mCherry$^+$ cells in the kidney (white arrows).

cells were delivered into the recipient's circulation by direct intracardiac injection. When irradiated with 40 Gy, a lethal dose, all the untransplanted animals died by 14 days after irradiation. However, >70% of the animals receiving WKM cells after irradiation were rescued, and survived at least 30 days after irradiation. As in the experiments with embryonic transplant recipients, GFP$^+$ leukocytes and dsRED$^+$ erythrocytes were visible in the circulation of the engrafted adult recipients using fluorescence light microscopy [7]. FACS analysis of recipient WKM showed robust multilineage engraftment with >86% GFP$^+$ cells up to 8 weeks after transplant (Figure 1(b)).

2.3. Embryonic HSCs into Embryos. Similar to murine embryonic HSCs, the first HSCs in the developing zebrafish are located in the aorta-gonad-mesonephros (AGM) [8]. Initial experiments to identify these HSCs in zebrafish relied upon anatomic similarities with murine embryonic HSCs. Cells expressing *cmyb, runx1,* and CD41 are observed in the ventral wall of the dorsal aorta in zebrafish embryos 24–36 hpf [9–12], similar to the expression noted in the ventral wall of the aorta in murine embryos [13]. These *cmyb*+ and *runx1*+ cells were presumed to be embryonic definitive HSCs, although functional evaluation of these cells was lacking. Using CD41 as another marker of embryonic HSCs, Bertrand et al. sorted CD41$^+$/gata1$^-$ donor cells by flow cytometry from *CD41-eGFP/gata1-dsRED* double transgenic embryos at 72 hpf [14]. These cells were then injected into the sinus venosus of age-matched wild-type embryos. Within one day after transplant, donor-derived cells were observed in the caudal hematopoietic tissue (CHT) and thymi of recipients. Although the transplanted donor cells had been dsRED negative, subsequent erythroid differentiation of engrafted cells revealed dsRED$^+$ cells in the circulation of recipients [14]. These experiments helped to prove that CD41$^+$ cells in the AGM are capable of colonizing definitive hematopoietic organs, namely, the thymus and CHT, in developing zebrafish, and therefore, this population includes the first developing HSCs in the embryo.

2.4. A Competitive Transplantation Assay for Chemical Screening. Capitalizing on the relative ease of *in vivo* chemical screening using the zebrafish model, Li et al. have utilized a competitive hematopoietic transplantation assay to search for chemicals that enhance hematopoietic engraftment (manuscript submitted). Marrow cells from βactin-GFP fish were incubated *ex vivo* in chemicals from a panel of more than 2000 known bioactive compounds. After pretreatment, the βactin-GFP WKM was mixed at a standard ratio with WKM from commercially available red Glofish, and transplanted into *casper* recipient fish [15]. Normally kidney marrow fluorescence is not visible in an adult animal due to the presence of pigmentation in the skin. However, *casper* fish are homozygous for two pigment mutations, *roy* and *nacre*, and therefore have transparent skin, allowing visualization of engrafted fluorescent marrow cells *in vivo*. Unlike prior studies examining engraftment at a single time point by FACS analysis of multilineage WKM populations, this screen also followed the level of GFP$^+$ and RFP$^+$ cells in the kidney of anesthetized recipients at several time points after transplant (Figure 2). The ratio of green : red marrow cells by fluorescence microscopy *in vivo* was highly correlated with the green : red ratio measured by flow cytometry of the dissected WKM cell preparation. All chemicals identified in the screen that stimulated enhanced engraftment were also tested in murine transplants to validate the effects in an immune-matched mammalian transplant assay. In total, ten compounds were identified in the screen that resulted in enhanced green : red ratio, and these are currently undergoing further evaluation.

3. Importance of Immune Matching in Hematopoietic Transplantation

None of the transplantation experiments described to this point took into account any aspect of immunologic matching, as isogenic and congenic fish lines were not available. This fact highlights another significant difference between murine and zebrafish marrow transplants, namely that murine donors and recipients are congenic and hence immunologically identical. In contrast, although many commonly used zebrafish lines (e.g., AB, Tubingen, and wik) have been repeatedly incrossed through decades of laboratory use, attempts to generate truly isogenic or congenic zebrafish lines have largely failed due to inbreeding depression such that these fish lines could no longer be maintained [16]. In addition, sex skewing of clutches, whereby a generation of siblings was all the same sex, has also hindered the ability

to maintain highly inbred fish lines. Despite this disadvantage, significant progress has still been made developing hematopoietic transplantation methods in the zebrafish over the past decade, as described above.

As more sophisticated transplantation experiments are designed to ask more complex questions about stem cell biology, the need for immune matching becomes more critical. When transplanting any allogeneic tissue into an adult recipient with a competent immune system, one would expect a lack of immune matching to result in rejection of the transplanted tissue (reviewed in [17]). In the zebrafish, immune matching is not required in embryonic recipients younger than 5 days after fertilization, as thymic development is not apparent until then [18]. By 4–6 weeks after fertilization, the cellular and humoral immune system is fully functional and would be capable of rejecting any transplanted tissue that was not histocompatible [19, 20]. Pretransplant conditioning with radiation is commonly used to suppress the immune system of adult murine and zebrafish recipients, and in the case of hematopoietic transplants to give the added advantage of clearing the marrow niche. For zebrafish recipients receiving a sublethal dose of radiation, the transplanted tissue is still rejected once the recipient's immune cells recover, approximately 4 weeks after irradiation [21].

Another consequence of immune mismatch between transplant donors and recipients occurs uniquely in the setting of hematopoietic transplantation. When engrafted immune cells recognize the recipient as "nonself," an immune response is mounted against the recipient's tissues resulting in graft-versus-host disease (GVHD), a phenomenon that is also observed clinically in human allogeneic bone marrow transplant [22]. Therefore, the importance of immune matching in hematopoietic transplantation impacts not only initial engraftment, but also the health and survival of the recipient if the engrafted hematopoietic cells attack the host.

4. Methods to Quantitate Hematopoietic Engraftment

Comparing the function of two HSC populations involves a competitive hematopoietic transplantation assay where both populations engraft in the same transplant recipient (reviewed by Purton and Scadden in [23]). This experimental design is required when mutant marrow cells from one donor are hypothesized to have defective hematopoietic engraftment. The mutant cells are transplanted into the recipient together with a radio-protective dose of wild-type marrow cells. If the mutant HSCs are defective, the wild-type HSCs will out-compete them, and the donor chimerism of the recipient will highly favor the wild-type donor cells. Without these wild-type HSCs to rescue the recipient, lack of engraftment of the mutant cells would likely result in the recipient's death, and there would be no blood or marrow cells to evaluate at the end of the experiment. Using a competitive experimental design ensures that all the recipients survive until the end of the experiment and their data are included in the final analyses. In the event that the mutant marrow has normal HSC function, the donor chimerism would reveal an equal mix of engrafted hematopoietic cells from both donors. Immune matching of both donors and the recipient is an essential component of any competitive hematopoietic transplantation assay. Otherwise, one cannot rule out biased immune rejection of one donor's cells compared to the other, and the engraftment "winner" may merely reflect immunologic differences and not a difference in stem cell biology.

A variation of the competitive hematopoietic transplantation assay is the limit dilution assay. This method is the gold standard for quantitating HSC content and also requires all donors and recipients to be immunologically matched. This assay involves transplantation of serially diluted marrow cells such that fewer and fewer marrow cells are given to subsequent transplant recipients, while a constant number of wild-type marrow cells are given simultaneously to radioprotect the recipients. Engraftment and donor chimerism are evaluated for each recipient, and then Poisson statistics are used to calculate the number of long-term repopulating cells contained in the original marrow population [24]. The ability to perform these competitive and quantitative experiments using zebrafish HSCs will be essential to characterize stem cell mutants and asking questions about HSC biology. Therefore, a better understanding of the histocompatibility genes in the zebrafish is needed so that these assays can be performed with proper immune matching.

5. Histocompatibility Antigens in Zebrafish Compared with Other Vertebrates

One of the first multimegabase regions of the human genome to be sequenced, the human major histocompatibility complex (MHC) locus, is located on chromosome 6p21.31 and contains over 200 identified genes within a 3.6×10^6 basepair span [25]. The classical class I and class II genes within the MHC region are the central cell surface proteins responsible for determining tissue histocompatibility of an allograft. This gene-dense region also contains a number of other genes important for the immune response, including antigen-processing genes such as proteasome subunit β type (PSMB), complement genes, and the peptide transporters TAP1 and TAP2 [26, 27].

Class I MHC molecules are polymorphic transmembrane proteins with three immunoglobulin-like domains that are expressed on virtually all cell types. They bind noncovalently to β2-microglobulin and present endogenously derived peptides to $CD8^+$ T lymphocytes (reviewed in [28]). Although class I and II proteins share a similar three-dimensional structure, class II MHC molecules are heterodimeric complexes consisting of an alpha chain and a beta chain, with each chain containing two immunoglobulin-like domains. They present lysosomally derived peptide antigens to $CD4^+$ T lymphocytes, and their expression is limited to B-lymphocytes, macrophages, and other antigen-presenting cells.

While most jawed vertebrate species possess linked class I and II genes located within a single chromosomal locus similar to the human MHC, the bony fishes are unique in

Table 1: Mean percentage of GFP$^+$ cells in engrafted recipient zebrafish receiving MHC-matched or -unmatched donor marrow.

	Only Chr 19 matched [35]		Chr 1, 8, 19 all matched	
Myeloid matched	47.86 ± 30.9	$P = 0.0002$	52.36 ± 25.43	$P = 0.0036$
Myeloid unmatched	6.45 ± 1.77		11.58 ± 7.03	
Lymphoid matched	10.51 ± 19.88	$P = 0.05$	9.51 ± 12.32	$P = 0.047$
Lymphoid unmatched	1.28 ± 0.38		3.47 ± 4.601	

Data are mean ± S.D.

that they have class I and II genes located on distinct chromosomes [29]. In the zebrafish, at least three relevant loci have been identified. Chromosome 19 contains class I genes as well as some antigen-processing genes, making the locus syntenic to the human MHC locus [30, 31]. However, there are no class II genes on chromosome 19. Instead the zebrafish class II alpha and beta genes are located on chromosome 8 [26, 32]. Chromosome 1 contains additional class I genes, termed "ze" genes, which appear most similar to mammalian nonclassical Class I genes [33]. Finally, the "L" genes, class I genes unique to teleost fish, are located on chromosomes 3 and 8, although they are less polymorphic than other class I genes, and their precise function is not clear [34]. While DNA sequence analyses of the zebrafish MHC genes show similarities with MHC genes of many species, virtually no data are available to evaluate the function or even the cell-surface expression of the class I and II genes in zebrafish. Prior to the transplantation experiments described below, no functional evaluation of any zebrafish MHC genes had been performed.

6. Immune-Matched Hematopoietic Transplants in Zebrafish

Following up on the adult marrow transplant experiments published in 2004 [7], subsequent adult transplantation experiments sought to evaluate long-term hematopoietic engraftment greater than 12 weeks after transplant. Having observed poor survival in random donor long-term hematopoietic transplantation experiments (J. L. O. de Jong and L. I. Zon, unpublished data), immune typing of the zebrafish MHC genes was a logical step to ensure that graft rejection and/or GVHD were not contributing to the recipient mortality. In these first hematopoietic transplant experiments with immune matching, the class I MHC genes at the chromosome 19 locus were typed for all the sibling progeny of a single mating pair [35]. Genotyping was achieved by preparing DNA from fin clips of individual fish, then using a panel of PCR primers to amplify MHC gene sequences. The amplified fragments were then sequenced to identify the specific MHC genes present in each individual animal. As expected, there were four MHC haplotypes represented within this family, and approximately 25% of the progeny fell into each of the four genotypes. WKM cells from β-actin-GFP$^+$ donor fish of each MHC genotype were transplanted into GFP-negative siblings of the same MHC genotype and also into unrelated wild-type recipients, presumed to be mismatched. Survival and donor chimerism were significantly improved in the matched recipients compared with the unmatched recipients (Table 1), indicating the importance of immune matching at the chromosome 19 MHC locus for hematopoietic engraftment [35]. These experiments were the first functional evaluation of any zebrafish MHC genes in a transplantation assay.

These first experiments did not specifically type for class II genes located on chromosome 8, or other class I genes on other chromosomes. It may be that coincidental matching at the class II locus occurred for a significant number of the related "matched" recipients in these experiments, thereby contributing to improved donor chimerism.

We expected that immune matching at the class II locus would also be important for hematopoietic engraftment. Therefore, we performed additional transplantation experiments matching the donors and recipients at three separate loci: the two class I loci on chromosomes 1 and 19 and the class II locus on chromosome 8. 2.5×10^5 WKM cells from β-actin-GFP$^+$ donor fish were transplanted into both completely matched recipients and unmatched, unrelated recipients. Long-term engraftment at 3 months after transplant showed similar donor chimerism results as the transplant experiments with matching at only the chromosome 19 locus (Table 1). These data suggest that matching of the class I genes at the chromosome 19 locus is the most important for tissue histocompatibility in a transplantation assay, and that the additional MHC loci on chromosomes 1 and 8 play a minimal role. Further experiments are underway to individually test the class I genes on chromosome 1 and the class II genes on chromosome 8 to determine the contribution, if any, of these loci to histocompatibility in tissue transplantation.

7. Optimizing Survival of Hematopoietic Transplant Recipients

Survival of zebrafish hematopoietic transplant recipients is often difficult to predict from one experiment to the next. We have implemented a number of changes to the initially published transplantation protocol to address the problem of poor survival after transplant. While lack of histocompatibility may play a role for some animals, a number of other factors also appear to be important. In our experience, younger fish have better survival than older fish, and optimal

recipients are approximately 3-4 months of age (J. L. O. de Jong and L. I. Zon, unpublished data). This may be due to colonization of older fish with bacterial or fungal pathogens that overwhelm and kill the immune-compromised host after transplantation. Maintaining excellent water quality is also critically important to recipient survival. We hypothesized that treatment with prophylactic antibiotics for a few days immediately after transplant might improve survival. However, placing transplant recipients "off system" in fish water containing antibiotics paradoxically caused decreased survival, as fish being treated in this way suffered from quickly deteriorating water quality and high ammonia levels (C. Lawrence, personal communication). While it is impractical to keep a therapeutic level of antibiotics in the large volume of water circulating through an entire aquatic system, the ability to maintain water quality at a consistently high standard resulted in improved survival of our transplant recipients, even without antibiotics.

Determining the appropriate radiation dose for pretransplant conditioning of recipient fish has also proven more challenging than initially anticipated. Water can greatly attenuate the radiation dose over a short distance. For example, at a depth of 1 cm of water, we have observed that the radiation dose at the bottom of the dish is decreased by about 10–15% compared with the radiation dose at the surface of the water (J. L. O. de Jong, unpublished data). Therefore, it is critically important that fish be placed in a minimal volume and depth of water to ensure that all recipients receive an equivalent radiation dose. The minimum lethal dose of radiation for zebrafish was first reported to be 40 Gy [7]. However, subsequent work showed that this dose was not optimal for pretransplant conditioning, as the mortality of fish was 100%, even after receiving a radio-protective dose of WKM cells. A sublethal dose of 25 Gy provided for maximal survival with engraftment, so this was the dose selected for most experiments [35]. This result suggests that while the hematopoietic compartment is the most radiation-sensitive tissue in the zebrafish, as in mammals, there is a narrow therapeutic index for lethal radiation damage to other tissues. To minimize the radiation injury to nonhematopoietic tissues, many protocols for murine and human bone marrow transplants utilize fractionated radiation dosing. We have now initiated a standard conditioning protocol of 30 Gy split into two equal fractions of 15 Gy, where the two fractions are given 24 hours apart. The survival of these recipients is comparable to animals receiving 25 Gy as a single dose (J. L. O. de Jong, unpublished data). Finally, we have observed that different fish lines have varying sensitivities to radiation. For example, when comparing fish from the AB strain that have been bred to homozygosity at the MHC loci, some were significantly more sensitive to a given radiation dose than others (Figure 3). This result suggests that a radiation dose-response titration should be performed for each strain of recipients to be transplanted in order to determine the optimal radiation dose. Alternatively, conditioning with chemotherapeutic medications such as cyclophosphamide [36] could be used, although these have not been tested for pretransplant conditioning of zebrafish donors.

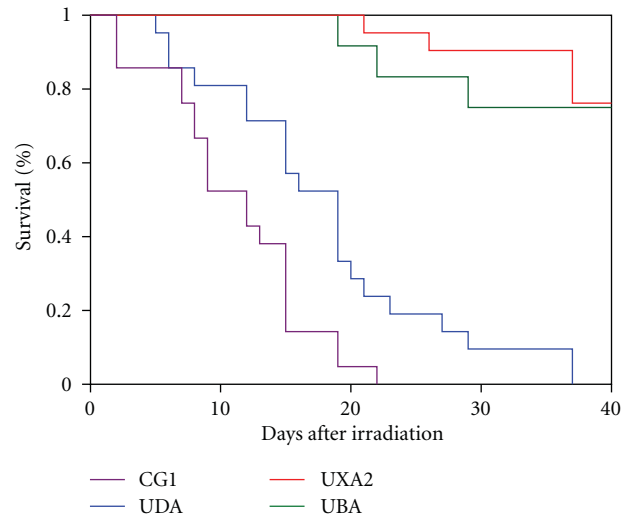


Figure 3: Survival of different zebrafish lines in response to radiation. Kaplan-Meier survival curves are shown for four different zebrafish strains after irradiation with a total dose of 25 Gy, delivered in two equal fractions of 12.5 Gy separated by 24 hours. Twenty one fish were irradiated in each group. CG1 is a clonal homozygous diploid fish line generated by parthenogenesis [21, 38]. UDA, UXA2, and UBA are inbred zebrafish lines derived from a single mating pair of AB parents [35]. Each line was named for the homozygous class I MHC gene at its chromosome 19 locus. The results demonstrate 100% mortality for the CG1 fish by day 22, and by day 37 for the UDA fish. In contrast, the UBA and UXA2 fish lines both had approximately 80% survival at 40 days after irradiation.

8. Future Directions for Hematopoietic Transplantation in the Zebrafish

Although HSC transplantation is a commonly used treatment modality for human diseases, including many malignancies, blood disorders, and immune deficiencies, this procedure continues to have high morbidity and mortality. Difficulties include selecting an optimally matched allogeneic donor, prolonged immune suppression with susceptibility to deadly infections, delayed and/or incomplete immune reconstitution, and maximizing the graft-versus-tumor effect while minimizing graft-versus-host disease. A zebrafish model for hematopoietic transplantation permitting *in vivo* investigation of these challenges would provide a basis to understand the biological mechanisms involved and identify possible solutions to address them.

8.1. Parthenogenesis to Develop Homozygous Diploid Fish Lines. The lack of isogenic and congenic fish lines is a serious handicap for future transplantation experiments with zebrafish. To overcome this barrier, gynogenetic fish lines have been utilized in recent years to successfully transplant liver tumors, acute lymphoblastic leukemia cells, and rhabdomyosarcoma tumor cells into unirradiated immunologically identical adult recipients [21, 37]. Developing these homozygous diploid clonal fish lines is labor intensive, time consuming and inefficient [38, 39]. However, once a robust

line is generated, it can be used to make transgenic donors with fluorochrome-labeled marrow cells. These donors could then be used to perform competitive HSC transplants using immunologically identical donors and recipients. Developing a homozygous diploid fish line from *casper* fish would be even more useful, as the advantages of analyzing engraftment at many time points could also be realized in the setting of an immune-matched competitive transplant. Efforts are currently underway to generate these fish.

8.2. Minor Histocompatibility Antigens. Further work will also be valuable to identify all the specific class I and II genes important for histocompatibility in the zebrafish, both for a basic understanding of zebrafish immunology, as well as the implications for optimizing future transplant experiments. When a zebrafish mutant has a postulated HSC defect, scientists need to have immune-matched recipients to test whether marrow cells from the mutant zebrafish have flawed engraftment in a competitive transplantation assay. Without immune matching, such an assay will be difficult to interpret. The ability to immunotype any random zebrafish, and thereby select appropriately matched donors and recipients would allow for a much quicker time frame to perform these experiments, compared with generations of inbreeding, which may be unsuccessful given the history of prior attempts to generate such inbred zebrafish lines. However, even having a donor with "perfect" matching at the MHC locus, human bone marrow transplant recipients are still at risk for GVHD, likely due to mismatched minor histocompatibility antigens on other chromosomes. Therefore, identifying both major and minor histocompatibility antigens throughout the genomes that are relevant for transplant rejection and GVHD in the zebrafish will be critical to prospectively determine optimally matched donors and recipients. This information will clearly be useful for zebrafish experiments, as described above. In addition, identifying significant minor histocompatibility antigens in the zebrafish would suggest minor histocompatibility antigens that may also be relevant for human bone marrow transplantation and GVHD. Such work may impact the selection of human bone marrow transplant donors to minimize this potentially devastating outcome after human BMT.

8.3. Developing a Zebrafish Model for GVHD. Finally, in the process of fully characterizing the zebrafish histocompatibility genes, we expect to identify recipients with GVHD. To date, we have observed transplant recipients that develop severe edema and ascites resulting in flaring of their scales. This condition in the zebrafish is generically termed "dropsy" and likely can result from a myriad of causes. We postulate that in the setting of hematopoietic transplantation, some of these recipient fish may have GVHD, although further work is needed to fully characterize the "dropsy" phenotype after transplant and confirm the pathophysiology of this diagnosis. By characterizing the GVHD phenotype in zebrafish and developing a zebrafish model of GVHD, one could exploit the advantages of genetic and small molecule-based screening to further characterize the pathways that regulate GVHD. Such experiments may discern mechanisms to minimize GVHD while maximizing the graft-versus-leukemia effect in bone marrow transplant patients.

9. Conclusion

As a model for human disease, the zebrafish holds numerous advantages. Gaining knowledge of the functional Class I and II genes in the zebrafish will enhance our understanding of basic zebrafish biology, as well as the ability to use this versatile animal model to ask questions about tissue transplantation, including hematopoietic stem cells, other normal tissues and cancers cells. This work will likely inform mammalian biology, improving our understanding of human HSCs, and has the potential to impact the treatment of patients undergoing bone marrow transplantation.

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Review Article

Zebrafish Thrombocytes: Functions and Origins

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Platelets play an important role in mammalian hemostasis. Thrombocytes of early vertebrates are functionally equivalent to mammalian platelets. A substantial amount of research has been done to study platelet function in humans as well as in animal models. However, to date only limited functional genomic studies of platelets have been performed but are low throughput and are not cost-effective. Keeping this in mind we introduced zebrafish, a vertebrate genetic model to study platelet function. We characterized zebrafish thrombocytes and established functional assays study not only their hemostatic function but to also their production. We identified a few genes which play a role in their function and production. Since we introduced the zebrafish model for the study of hemostasis and thrombosis, other groups have adapted this model to study genes that are associated with thrombocyte function and a few novel genes have also been identified. Furthermore, transgenic zebrafish with GFP-tagged thrombocytes have been developed which helped to study the production of thrombocytes and their precursors as well as their functional roles not only in hemostasis but also hematopoiesis. This paper integrates the information available on zebrafish thrombocyte function and its formation.

1. Introduction

Hemostasis is a defense mechanism to prevent loss of blood in the event of an injury in an organism that has a vasculature [1]. It consists of the platelet response to injury which results in platelet aggregation and plugging the wound, termed primary hemostasis, followed by the interplay of a complex cascade of coagulation factors on the platelet surface ultimately resulting in a fibrin clot, termed secondary hemostasis. After their primary hemostatic function platelets, also repair the damaged endothelium [2]. In primary hemostasis platelets adhere to collagen in the subendothelial matrix in response to injury and are subsequently activated by a complex signaling cascade resulting in secretion of their granular contents. These contents also result in the amplification of platelet aggregation at the site of injury and formation of a platelet plug which is stabilized further with help of fibrin [1]. This hemostatic plug prevents loss of blood from the site of injury. Thus, platelets that play a role in hemostasis and defects in platelet function have been shown to be involved in bleeding disorders as well as many pathophysiological conditions like thrombosis, inflammation, and even cancer [3]. Platelets have a number of receptors on their membrane surface that help regulate signaling pathways in platelets. A substantial amount of research has been done in studying platelet development and function mostly using human platelets [2–4] murine models [4], and identification of a number of factors and their roles in platelet function [2–4]. Recently, to identify novel factors involved in platelet function, N-ethyl-N-nitrosourea (ENU) mutagenesis and genomic screens of genes affecting platelet development and function have been attempted in mice [5]. However, they are expensive, less efficient, and have lower throughput. In humans, several novel quantitative trait loci associated with platelet-signaling pathways have been identified: however, these studies require additional functional evaluation using either animal models or human subjects [6]. Thus, study of platelet function requires a model system that is efficient, less costly, and amenable to higher-throughput screen, with hemostatic pathways similar to those found in humans [7]. The hemostatic system of invertebrates differs from that of vertebrates and therefore cannot be used as a model organism to study hemostasis [8]. In this regard, we wondered whether *Danio rerio* (Zebrafish) previously used as a genetic

model to study developmental biology could be used as a genetic model to study hemostasis especially platelet biology [1]. Its high fecundity, external fertilization, transparency at early stages of development, and availability of large-scale mutagenesis methods are some of the features that make it a useful model system, thus attracting our attention [9, 10]. However, the challenge was to prove whether zebrafish thrombocytes and their functional pathways are similar to those found in platelets. For this, characterization of thrombocytes and their functional pathways was required as well as technology suitable for large-scale screens. Therefore, we developed the required technologies ourselves and found them sufficient enough to warrant their utility for the study of hemostatic function. Recently, several groups utilized our zebrafish model to study hemostasis and discovered several factors regulating hemostasis [11]. This paper provides an overview on the zebrafish thrombocyte characterization and development as well as other advances made not only in our laboratory but also from other laboratories which have applied the knowledge and technology that we developed in studying thrombocyte biology.

2. Development of Zebrafish Model to Study Thrombocyte Function

Unlike mammalian platelets which are anucleated, zebrafish thrombocytes have a nucleus. Our work has shown morphological and functional similarities between the zebrafish thrombocytes and human platelets [12]. Zebrafish thrombocytes have a sparse cytoplasm with large nuclei. The ultrastructure analysis of thrombocytes demonstrated that the cytoplasm contains many vesicles that open to the cell surface, similar to the open canalicular system in mammalian platelets (Figure 1). To demonstrate thrombocyte function, we developed blood collection and thrombocyte aggregation assays using less than one microliter of blood and established that zebrafish thrombocytes are stimulated by agonists including collagen, ADP, ristocetin, and arachidonic acid consistent with the human platelet aggregation methods. The results from such analyses revealed that the receptors for collagen, ADP, vWF, and thromboxane are conserved [12]. By using immunological methods, we have shown that αIIb integrin receptor and GpIb are present on thrombocyte membrane. Cox1 and Cox2 enzymes involved in arachidonic acid metabolism have also been identified in zebrafish [13]. Recently, we have shown that the thrombin receptor PAR-1 and its paralogue PAR-2 are also present on thrombocytes [14]. Using antibody staining and RT-PCR, we have also shown the presence of vWF in thrombocytes [15]. In a recent review, Lang et al. provide a detailed result of BLAST searches between human adhesion proteins and zebrafish proteins confirming our evidence for their similarities [16]. Thus, receptors for both thrombocyte adhesion and aggregation have been shown to be conserved in zebrafish. Subsequently, we developed a laser-induced thrombosis assay to study thrombocyte function and established that thrombosis assays are physiologically relevant in this model [17]. This study resulted in three assays, time to occlusion of artery from the time of laser injury (TTO), time to attachment of first cell from the time of laser injury (TTA) and also time taken to dissolution of the aggregate (TTD). Several reviews regarding the development of the zebrafish model for the study of thrombocyte function using laser-induced thrombosis assays from our laboratory are available [18–21].

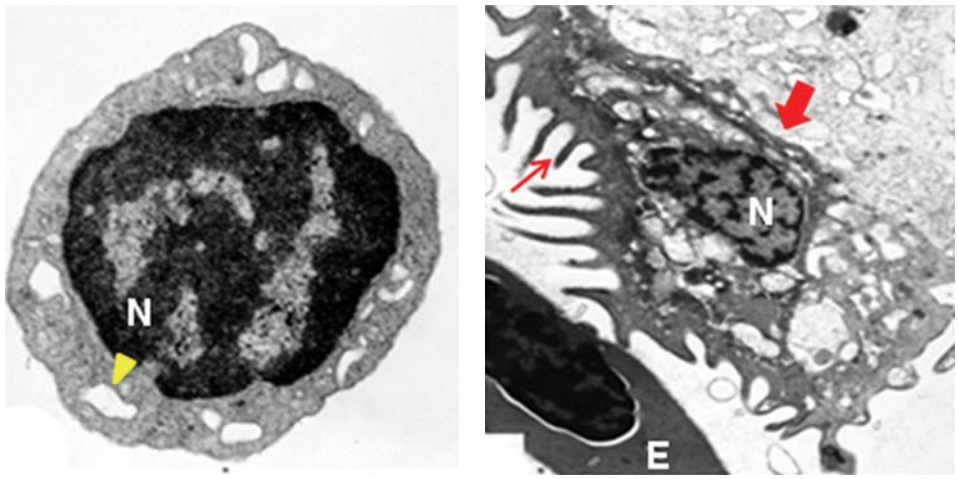
3. Cell Biology of Thrombocyte Function

To visualize thrombus formation, we wanted to perform intravital staining of the blood cells in zebrafish larvae by intravenous injection of lipophilic dye DiI-C18 (DiI) [22]. Surprisingly, we found only a few cells in the circulating blood were labeled in contrast to the entire blood cells. Subsequently, we identified that only a small proportion of thrombocytes in zebrafish blood was labeled by DiI alone, whereas all thrombocytes were labeled by mepacrine and, thus, giving two populations of thrombocytes (DiI+ and DiI−) (Figure 2). We found that DiI+ thrombocytes have higher levels of rough endoplasmic reticulum and thus higher protein synthesis than the DiI− thrombocytes. Furthermore, labeling the thrombocytes with BrdU for 24 hours resulted in BrdU-labeled circulating thrombocytes which were DiI+, but there were no BrdU-labeled thrombocytes that were DiI−. These results suggested that DiI+ thrombocytes were the first ones to appear in the circulation and, therefore, we called them young thrombocytes which are generated by their precursor cells by thrombopoiesis; by contrast, DiI− thrombocytes were called mature thrombocytes since in the circulation young thrombocytes presumably progress through the maturation process. By performing annexin V binding assays and estimating P-selectin levels on these two types of thrombocytes, we found that young thrombocytes are functionally more active than the mature thrombocytes [23]. In addition, we also found that young thrombocytes first appear at the site of injury and form their own clusters followed by the subsequent appearance of a mature thrombocyte cluster [23].

We have recently identified in a transgenic line initially developed by Weinstein's laboratory (National Institutes of Health, Bethesda, Maryland) for the purpose of imaging blood vessels (where GFP expression is driven by the endothelial cell-specific transcription factor, *fli1* gene promoter), circulating thrombocytes are labeled with GFP. In this line, we found two populations of thrombocytes, one DiI+, which has a less intense GFP expression, and one DiI− with a more intense GFP expression [24]. We also noted that the less intense GFP thrombocytes are first responders to injury and the more intense thrombocytes correspond to mature thrombocytes and have confirmed our previous findings using intravital microscopy [23].

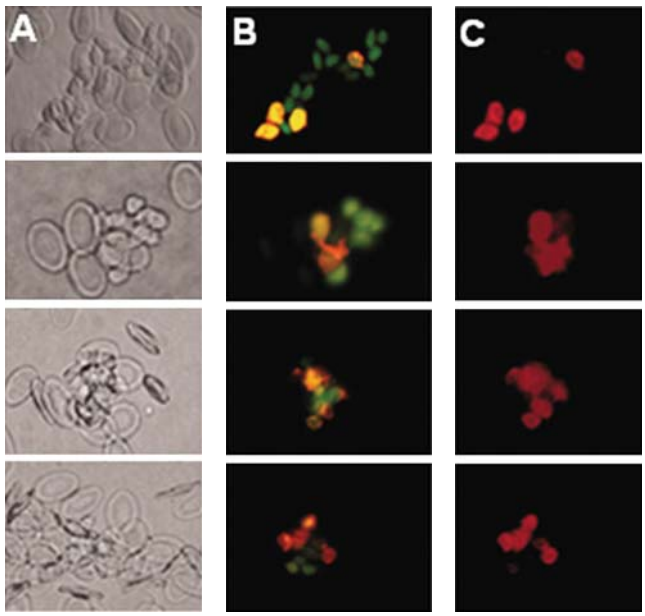
4. Thrombocyte Microparticles

Platelet microparticles are the microvesicles released by platelets upon activation and have been shown to be involved in thrombin generation [25]. These are 0.1–1.0 μm in diameter and posses most receptors found on platelets



(a) (b)

Figure 1: Zebrafish thrombocyte electron micrographs. (a) Zebrafish thrombocyte. Open canalicular like system is shown by arrowhead; N: nucleus; (b) An activated thrombocyte. Thrombocyte in an aggregation reaction; activated thrombocyte is shown by a thick arrow, thrombocyte in the aggregate shows filopodia shown by a thin arrow; E: erythrocyte [12].



(a) (b) (c)

Figure 2: Young and mature thrombocytes forming independent clusters in an aggregation reaction. Top to bottom, the panels show four different thrombocyte clusters. (a) bright field image; (b) DiI−labeled thrombocytes and mepacrine-labeled thrombocytes as green or orange; (c) DiI-labeled thrombocytes [23].

such as P-selectin, GPIb, and αIIbβ3 [26]. Microparticle formation from platelets is believed to occur when the asymmetry of the membrane phospholipid is lost and phophotidylserine is externalized [27, 28]. Platelet derived microparticles are thought to promote platelet interaction with subendothelial matrix in an αIIbβ3-dependent manner [29]. Elevated levels of microparticles are observed in many pathological conditions including meningococcal sepsis [30], disseminated intravascular coagulation [31], and myocardial infarction [32].

We recently identified thrombocyte microparticles in zebrafish and determined that they possess the membrane protein αIIb, which is also found in thrombocytes. Positive labeling of zebrafish microparticles with FITC annexin V suggests that microparticles could be a result of thrombocyte apoptosis [33]. To elucidate the role of microparticles in hemostasis, Kim et al. used CD41-GFP labeled zebrafish and studied microparticle aggregation/agglutination in the presence of different agonists. Thrombin, ADP, and collagen did not aggregate thrombocyte microparticles; however, ristocetin induced agglutination in microparticles derived from thrombocytes as well as non-thrombocytes, suggesting that the agglutination is dependent on vWF. During laser injury, we have shown that the thrombocyte microparticles are the first players to arrive at the site of injury (Figure 3), even before the young thrombocytes [33].

5. Genetics and Gene Knockdowns to Study Thrombocyte Function

ENU mutagenesis has been used extensively in forward genetic screens in an unbiased manner [1]. With the laser-induced thrombosis method a relatively high throughput screen is possible to select zebrafish mutants which have hemostatic defects. We proposed that such mutagenesis methods, combined with the laser-induced thrombosis method may lead to the discovery of novel thrombocyte-specific genes and so we pursued this approach. We performed a large-scale screen and found several mutants which have hemostatic defects; however, one mutant which we characterized has a defect in a novel orphan GPCR suggesting it plays a role in thrombocyte function (manuscript in preparation). Thus, we have established it is possible to

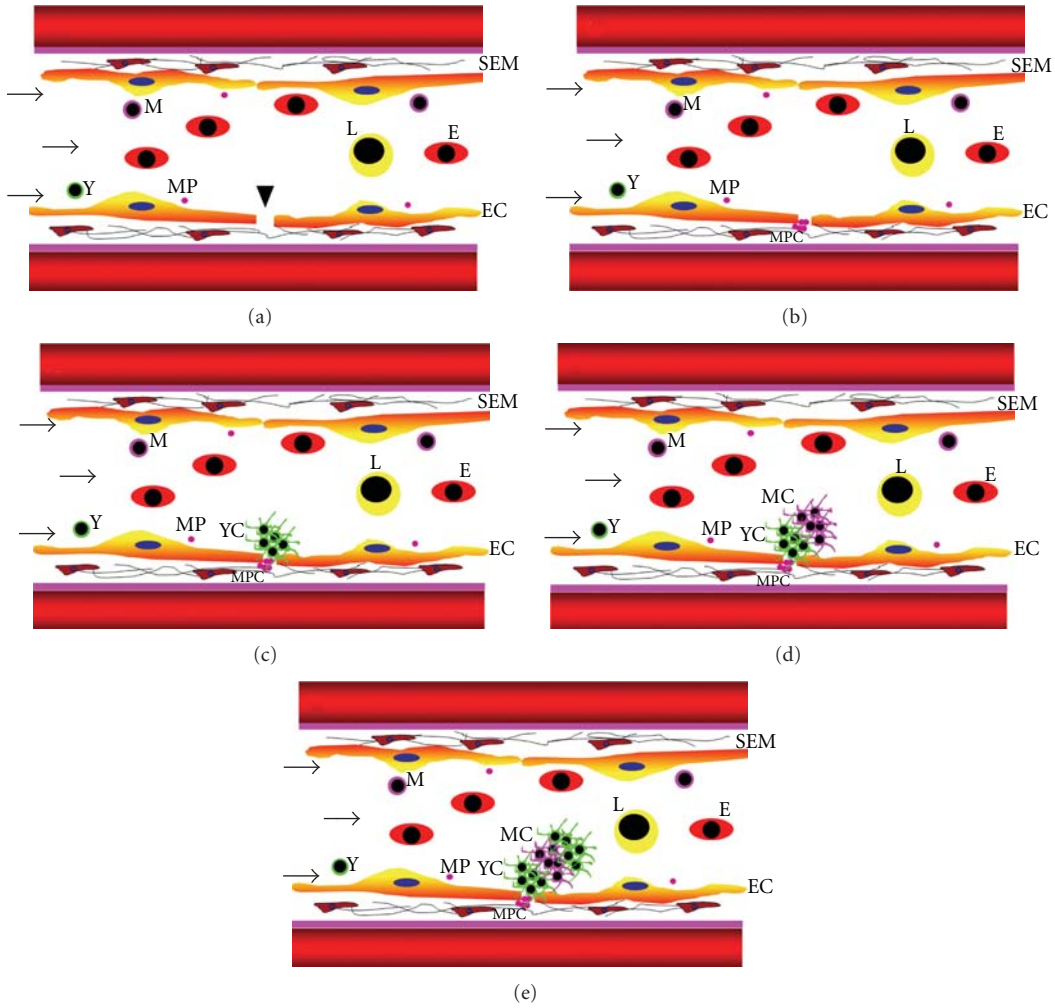


Figure 3: Schematic representation of sequential steps in growing arterial thrombus. Panels (a) through (e) show the sequence of events in thrombus growth. Arrowhead shows the site of laser injury in (a), (b) shows initiation of thrombus with the formation of microparticle (MP) clusters (MPC) followed by young thrombocyte (Y) clusters (YC) shown in (c) and then followed by a mixture of mature thrombocyte (M) clusters (MC) and YC as shown in (d) and (e) EC indicates endothelial cell; SE, subendothelial matrix; (e) erythrocytes; L, leukocytes. Arrows show the direction of blood flow.

conduct forward genetic screens for hemostatic function. Another mutant which has relevance to thrombocyte function is the *fade out* mutant which reiterates several aspects of Hermansky-Pudlak syndrome [34]. Furthermore, in the large-scale genome TILLING project spearheaded by Sanger Institute, several mutations in genes related to thrombocyte function were found. However, these will have to be sorted out and their functional evaluation performed in the near future.

We have also applied the knockdown technology developed by Ekker and his coworkers to study hemostatic function [35]. We used knockdown of clotting factors to establish the proof of principle and suggested that we could study the thrombocyte functions by knockdowns [1, 17, 36]. Knockdowns of thrombocyte-specific genes selected by microarray RNA analysis has resulted in identifying four genes (*acvr1, ift122, poldip2* and *ripk5*) all of whose deficiencies, in addition to other abnormalities, gave either a hemorrhagic phenotype or prolongation of TTO phenotype [37, 38]. Since then, several knockdowns affecting thrombocyte function have appeared (see Table 1). Schulte-Merker and his group silenced myosin light chain kinase gene *mlck1a* that is expressed in thrombocytes by knockdown and found this gene is important in thrombus formation [39]. By using knockdowns and our zebrafish thrombosis model, O'Connor et al. have identified four novel genes (*bambi, lrrc32, dcbld2* and *esam*) involved in platelet function [11]. These genes were selected from comparative transcript analysis of platelets and megakaryocytes together with nucleated blood cells, endothelial cells and erythroblasts. In this work, they used CD41-GFP zebrafish to estimate thrombocyte aggregation during arterial thrombosis by measuring thrombocyte surface area (TSA) which essentially provides similar information as the TTO assay. Another group has also used the zebrafish model to decipher the role of *prkca* (*PKCα*) and *prkcb* (*PKCβ*) genes in thrombocyte function; by knockdown

Table 1: Summary of the silencing of genes by knockdown methods affecting thrombocyte formation.

Gene	Functional evaluation	Phenotype	Reference
acvr1	Laser thrombosis	Hemorrhagic/Prolonged TTO	[36, 37]
ift122	Laser thrombosis	Hemorrhagic/Prolonged TTO	[36, 37]
poldip2	Laser thrombosis	Hemorrhagic/Prolonged TTO	[36, 37]
ripk5	Laser thrombosis	Hemorrhagic/Prolonged TTO	[36, 37]
mlck1a	Laser thrombosis	Prolonged TTO	[39]
bambi	Laser thrombosis	Prolonged TTA/reduced thrombus surface area	[11]
lrrc32	Laser thrombosis	Prolonged TTA/Reduced TSA	[11]
dcbld2	Laser thrombosis	Increased TSA	[11]
esam	Laser thrombosis	Increased thrombus size	[11]
prkca (PKCα)	Laser thrombosis	Reduced TSA	[40]
prkcb (PKCβ)	Laser thrombosis	Reduced TSA	[40]
itga2b (CD41)	Laser thrombosis/thrombocyte aggregation assays	Reduced TSA/Prolonged TTO/no aggregation of thrombocytes	[11, 41]
scl	Thrombocyte formation	Reduction in GFP+ cells in CD41-GFP transgenic zebrafish line	[48]
c-mpl	Thrombocyte formation	Reduction in GFP+ cells in CD41-GFP transgenic zebrafish line	[48]
runx1	Whole mount in situ hybridization/immunostaining	Accumulation of hematopoietic progenitors	[52]
miR-126/c-myb	Thrombocyte formation	Decrease in CD41 : EGFP+ thrombocytes in a double transgenic reporter line Tg (cd41 : EGFP) : Tg (gata1 : dsRed)	[55]
fog1	Thrombocyte formation	Failure to generate eGFP+ cells in CD41-GFP transgenic zebrafish line	[56]
mastl	Thrombocyte formation	Reduction in GFP+ cells in CD41-GFP transgenic zebrafish line	[58]
march2	Thrombocyte formation	Reduction in GFP+ cells in CD41-GFP transgenic zebrafish line	[59]
max	Thrombocyte formation	Reduction in GFP+ cells in CD41-GFP transgenic zebrafish line	[59]
smox	Thrombocyte formation	Reduction in GFP+ cells in CD41-GFP transgenic zebrafish line	[59]
pttg11p	Thrombocyte formation	Reduction in GFP+ cells in CD41-GFP transgenic zebrafish line	[59]
emilin1	Thrombocyte formation	Reduction in GFP+ cells in CD41-GFP transgenic zebrafish line	[59]
sufu	Thrombocyte formation	Reduction in GFP+ cells in CD41-GFP transgenic zebrafish line	[59]
arhgef3	Thrombocyte formation	Absence of GFP+ cells in CD41-GFP transgenic zebrafish line	[60]
ak3	Thrombocyte formation	Absence of GFP+ cells in CD41-GFP transgenic zebrafish line	[60]
rnf45	Thrombocyte formation	Absence of GFP+ cells in CD41-GFP transgenic zebrafish line	[60]
jmjd1c	Thrombocyte formation	Absence of GFP+ cells in CD41-GFP transgenic zebrafish line	[60]
tpma	Thrombocyte formation	Absence of GFP+ cells in CD41-GFP transgenic zebrafish line	[60]
nbeal2	Thrombocyte formation	Abrogation of thrombocyte formation	[61]
rgs18	Thrombocyte formation	Thrombocytopenia	[62]

expression of these genes, they showed that knockdown with either morpholino leads to attenuated thrombus formation [40]. This group has also used the TSA method but in their recent review they suggested that the manual TSA measurements may be time consuming and may not be accurate when using fluorescence measurements in CD41-GFP larvae although O'Connor et al. have calculated TSA for every minute of the time course and effectively used this method in their work [11, 41].

Although the knockdown methods combined with the laser-induced thrombosis method have the ability to demonstrate the function of the gene that plays a role in thrombosis, biochemical studies on thrombocytes cannot be performed because there is no way to study thrombocyte function by collecting blood samples from the larvae. In order to study the pathways involved in thrombocyte signaling, a knockdown in adult zebrafish was needed. Therefore, we used Vivo morpholino and created an adult Glanzmann's thrombasthenia phenotype by knockdown of the *itga2b* (*CD41*) gene [42]. With this advancement, it is now possible to study the biochemistry of thrombocytes after knockdown since we have already developed blood collection methods, thrombocyte assays and thrombocyte separation methods [14, 43, 44].

6. Cell Biology and Genetics of Thrombopoiesis

6.1. Development of Zebrafish Model for Thrombopoiesis. In zebrafish, hematopoiesis has been extensively studied [45, 46]. There are four distinct waves in the hematopoietic program of the developing zebrafish embryo. The first two waves start prior to 30 hpf in a region in zebrafish embryo

called intermediate cell mass (ICM) where macrophages and erythrocytes are generated, resulting in primitive hematopoiesis. The third and fourth waves are called definitive hematopoiesis and produce erythromyeloid progenitors and hematopoietic stem cells (HSCs), respectively. The third wave may start as early as 24 hpf but peaks at around 30 hpf in caudal hematopoietic tissue (CHT) also called the posterior blood island. The fourth wave starting at 32–36 hpf occurs within endothelial cells of the ventral wall of the dorsal aorta, comparable to mammalian aorta-gonad-mesonephros (AGM). The HSCs produced from the fourth wave colonize the CHT and adult hematopoietic organs, the kidney and thymus (Figure 4). Unfortunately, at the time we began our studies with zebrafish thrombocytes zebrafish thrombopoiesis, received little attention due to the lack of labeling of zebrafish thrombocytes and the inability to follow their development. Therefore, we took advantage of labeling of circulating thrombocytes *in vivo* by intravital microscopy in order to test when thrombocytes appear in the circulation during development. We found by DiI labeling, which specifically labels thrombocytes, that thrombocytes were present in the circulation around 36 hpf, almost coinciding with the fourth wave of hematopoiesis that occurs within the ventral wall of dorsal aorta suggesting precursors for thrombocytes must exist prior to 36 hpf [22]. Subsequently, Handin's laboratory developed a transgenic zebrafish (CD41-GFP zebrafish) where they used the *CD41* gene promoter to drive GFP expression. In this line they found green fluorescent cells flowing in the blood stream around 48 hpf; after this observation they asked us to test whether these cells aggregate using our thrombosis and thrombocyte aggregation assays. When we performed aggregation assays and laser injury thrombosis assays, a green fluorescent aggregate formed, establishing that Handin's green fluorescent cells were in fact thrombocytes [47]. Furthermore, it also provided the possibility of quantifying the intensity of the thrombocyte aggregates. Subsequently, knockdown of transcription factor gene *scl* and the receptor for a cytokine thrombopoietin *c-mpl* gene resulted in reduction of GFP-labeled thrombocytes, suggesting the presence of C-mpl receptor on zebrafish thrombocytes [48]. C-mpl receptor mRNA was shown to be present in the thrombocytes as early as 42 hpf [49]. Using this transgenic line, Lin et al. determined that the GFP+ thrombocytes were not present in the ICM and, therefore, are not part of primitive hematopoiesis [48]. However, they found nonmobile GFP+ thrombocytes between the dorsal aorta and caudal vein at 40 and 48 hpf that they suggested to correspond to the AGM although not having classical AGM features. The circulatory GFP+ thrombocytes appeared first at 48 hpf [48]. FACS analysis of the GFP+ cells from mesonephros detected two distinct populations: one with bright fluorescence (GFP^{High}), considered to be well-differentiated with typical thrombocyte morphology (scant cytoplasm and spindle shape), and the other with weak fluorescence (GFP^{Low}) and larger than GFP^{High} thrombocytes with undifferentiated morphology (round) and basophilic cytoplasm. Further studies by Kissa and coworkers revealed that the GFP^{Low} cells appeared first at 33 to 35 hpf as single cells between the dorsal aorta and the postcardinal vein

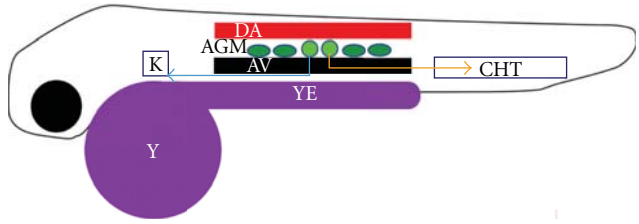


Figure 4: Schematic representation of thrombocyte development in zebrafish larva. DA, dorsal aorta; AV, axial vein; AGM, area corresponding to mammalian aorta- gonad- mesonephros; CHT, caudal hematopoietic tissue; K, kidney; Y, yolk; YE, yolk extension; filled small circles and ovals represent GFP^{Low} and GFP^{High} thrombocytes, respectively. The yellow and blue lines with arrows correspond to the routes of immigration of the thrombocytes. Thymus is not shown. Black circle and outline show the eye and the zebrafish body, respectively.

and they migrate subsequently to CHT and thymus via the axial vein rather than dorsal aorta [50]. Bertrand and his colleagues refined these studies and found that these cells appear as early as 27 hpf in the trunk randomly between axial vessels and confirmed their migration to CHT, thymus and pronephros along with the finding of their migration along the pronephric tubules [49]. These immigrants to kidney supposedly initiate adult hematopoiesis in the developing kidney. They also found migration of the GFP^{Low} cells between axial vessels and pronephric ducts and back to vessels. A recent study from Handin's laboratory revealed that the GFP^{Low} cells injected into irradiated adult zebrafish showed production of GFP+ cells in kidneys by long term multilineage reconstitution, suggesting that they have the features of HSCs while GFP^{High} cells did not reconstitute [51].

6.2. Identification of Factors Affecting Thrombocyte Development. Several transcription factors such as Fli-1, Fog1, GATA-1 (Zg1), NFE2, and Runx1 which have been found in megakaryocytes have also been identified in zebrafish [46]. *runx1* morpholino injected zebrafish embryos lack a normal circulation and accumulate immature hematopoietic progenitors [52]. The CD41-GFP cells were also found to express Runx1. Using CD41-GFP zebrafish, the truncated Runx1 developed normal CD41+ HSCs, indicating there is a Runx1-independent secondary pathway to generate HSCs [53]. Another factor, c-Myb a negative regular of megakaryocytopoiesis, has been identified in zebrafish [54]. Functional knockdown of *miR-126*, a key regulator of *c-myb* in zebrafish, resulted in an increase in erythrocytes and a decrease in thrombocytes, proving that the cell fate decision is regulated by the micro RNA [55]. Yet another factor Fog1, a cofactor that interacts with GATA-1 and GATA-2 has been shown to play a role in erythroid and megakaryocyte differentiation [56]. *fog1* morpholino injected in CD41-GFP zebrafish embryos failed to generate GFP+ mature thrombocytes suggesting that Fog1 is necessary for thrombocyte development [57]. Recently, thrombocyte maturation in the circulation has been studied in adult zebrafish, revealing that

the *gata1* promoter becomes weaker and *fli1* promoter gets stronger in mature thrombocytes and is conversely regulated in young thrombocytes [24].

In addition to these studies, a transient knockdown of *mastl* in zebrafish resulted in deficiency of circulating thrombocytes [58]. More recently, knockdowns of genes, *march2*, *max*, *smox*, *pttg1lp*, *emilin1*, and *sufu* resulted in a severe decrease in the number of thrombocytes indicating that these genes are important for thrombocyte development [59]. These genes were selected for knockdowns by genomewide analysis studies (GWAS) for genes adjacent to binding sites for GATA-1, GATA-2, Runx1, Fli-1, and SCL using primary human cells. Another study by Gieger et al. used meta-analyses of GWAS for mean platelet volume and platelet count and identified 68 genomic loci and from these loci four genes (*arhgef3*, *ak3*, *rnf145*, and *jmjd1c*) were silenced in zebrafish which led to the ablation of both primitive erythropoiesis and thrombocyte formation. Silencing of *tpma*, the orthologue of *tpm1* transcribed in megakaryocytes but not in other blood cells, abolished the formation of thrombocytes, but not erythrocytes [60]. In addition to these findings, silencing of *nbeal2* and *rgs18* in zebrafish resulted in reduction in thrombocyte formation [61, 62]. The silencing of genes by knockdown methods affecting thrombocyte formation is summarized in Table 1.

7. Future Studies

Despite the advances in genetic studies of thrombocyte function and development in zebrafish, many novel genes involved in thrombocyte origins and functions remain to be identified. For example, even though embryonic GFP^{Low} thrombocytes have been identified as HSCs and their role in repopulating the kidney for initiating the subsequent generation of thrombocytes from HSCs has not yet been investigated. Thus, we have no information regarding genes involved in the production of thrombocyte precursor cells in adult zebrafish. Likewise, studies of genes involved in maturation from young to mature thrombocytes, as well as genes controlling the production of thrombocyte microparticles are in the beginning stages. Since our laser-induced thrombosis assays for studying hemostasis have already found applications, we anticipate more such studies of this kind will be performed to assess the role of novel human genes relevant to hemostasis and thrombocyte development and function [35]. Recently developed technologies such as Genome TILLING [63, 64], zinc finger nuclease, or other nuclease/s (TALEN) based knockout methods [65–68] are also anticipated to complement the already available methods for studying functions of genes involved in thrombocyte function and production. However, large-scale silencing of genes to study thrombocyte development and production are still prohibitively expensive. Thus, future development of cost-effective gene silencing methodologies is required to attempt a functional genomics approach to analyze thrombocytes using the zebrafish model. Once the genes are identified, utilizing Vivo morpholino technology, we predict that characterization of the phenotypes by thrombocyte aggregation/adhesion functional assays, and determination of their mechanism of action will all be within reach in the next decade.

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Review Article

In Vivo Chemical Screening for Modulators of Hematopoiesis and Hematological Diseases

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In vivo chemical screening is a broadly applicable approach not only for dissecting genetic pathways governing hematopoiesis and hematological diseases, but also for finding critical components in those pathways that may be pharmacologically modulated. Both high-throughput chemical screening and facile detection of blood-cell-related phenotypes are feasible in embryonic/larval zebrafish. Two recent studies utilizing phenotypic chemical screens in zebrafish have identified several compounds that promote hematopoietic stem cell formation and reverse the hematopoietic phenotypes of a leukemia oncogene, respectively. These studies illustrate efficient drug discovery processes in zebrafish and reveal novel biological roles of prostaglandin E2 in hematopoietic and leukemia stem cells. Furthermore, the compounds discovered in zebrafish screens have become promising therapeutic candidates against leukemia and included in a clinical trial for enhancing hematopoietic stem cells during hematopoietic cell transplantation.

1. Introduction

Zebrafish has been used effectively as a vertebrate model for studying blood cell development and function (for reviews see [1–5]). It is an advantageous model because the optical clarity of its embryos, and their ex utero development enables easy and real-time detection of hematopoietic cells during development. A wide variety of tools and reagents have been developed for *in vivo* labeling and imaging of blood cells and for investigating blood cell function (for reviews of these methods and protocols, see [6–10]). In addition, transient and stable genetic manipulation can link hematopoietic genes to their functions [11–16]. Added to this arsenal of research tools available in zebrafish is *in vivo* chemical screening [17–20]. By exposing zebrafish embryos to a chemical library, bioactive compounds that affect any complex developmental and physiological processes may be identified. Furthermore, *in vivo* chemical screening may be used for uncovering chemical agents that modify a disease phenotype in a whole animal. The compounds that induce a unique biological effect may serve as invaluable probes for identifying critical components of biological pathways, and compounds that can reverse a disease phenotype *in vivo* may have therapeutic potential or shed light on an effective therapeutic target. This innovative approach has created a unique utility for the zebrafish model in chemical biology and contributed to its emerging role in drug discovery (for additional reviews see [21–24]).

2. Linking Genes to Their Functions: *In Vivo* Chemical Screens versus Genetic Screens

Both genetic and *in vivo* chemical screens may be used to dissect genetic pathways that regulate specific biological processes. However, an *in vivo* chemical screen offers the advantage of temporal control that a traditional genetic screen does not. In a genetic screen, gene function is affected from conception. Thus, the role of a gene in early embryonic development may preclude characterization of its roles during later stages. On the other hand, in a chemical screen, compounds that affect the function of a gene can be administered at specific time points and for fixed durations chosen by the investigator so that its roles at different developmental stages may

be distinctly determined. In addition, in a genetic screen, the roles of a protein family may sometimes be masked by functional redundancy of its family members. However, chemical modulators may exhibit similar activities against multiple members of a protein family and can, therefore, reveal their *in vivo* cumulative roles. It should be noted that some compounds may affect multiple cellular proteins and thus their on-target effects should be carefully verified using additional chemical agents as well as genetic manipulations. Taken together, *in vivo* chemical screens may complement traditional genetic approaches and uncover hematopoietic genes that cannot be identified in genetic screens.

3. Drug Discovery: *In Vivo* Phenotype-Based Chemical Screening versus Target-Based Approach

Currently, the most common approach for identifying potential therapeutics is the target-driven approach (for reviews see [25, 26]). This approach relies on *a priori* understanding of disease mechanisms to the point of knowing a specific cellular component to be targeted. Thereafter, lead compounds may be obtained using *in vitro* or cell-based assays to determine binding to or modulation of target activity. Typically, these leads will be further optimized using these assays again before being assessed for their *in vivo* efficacy and toxicity. Targets employed by this approach are often enzymes such as kinases that are likely to have small-molecule binding pockets (for more discussions on target druggability, see reviews [26, 27]). Proteins that do not have an obvious pocket, such as transcription factors that often act by recruiting other cofactors, are sometimes dubbed undruggable targets.

Target-based chemical screens performed *in vitro* or in cultured cells are usually very efficient and are able to sample through tens of thousands of compounds. Even so, many drug candidates so identified fail because of poor *in vivo* potency, intolerable side effects, or inability to demonstrate clinical efficacy (for reviews see [28, 29]). In comparison, chemical screens performed in a whole organism may identify working drugs with a higher rate of success since *in vivo* potency and toxicity are evaluated simultaneously during the primary screen [30]. Moreover, by design these screens directly identify compounds that have demonstrated their effectiveness of reversing a disease phenotype *in vivo*. Instead of examining one target as in the target-driven approach, *in vivo* screening is able to interrogate any potential therapeutic targets existing in a biological system that may mediate a disease phenotype, including targets that act in a non-cell-autonomous manner. In many circumstances, the mechanisms of disease pathology are not fully understood, so a target-driven approach is lacking. *In vivo* chemical screening, on the contrary, can be performed before a valid molecular target is identified.

Although *in vivo* screening has a demonstratedly good likelihood of finding efficacious drug candidates, figuring out their mode of action can be a challenge. A significant amount of effort is usually needed to identify the molecular target of the candidate compound. Nevertheless, due to several important advances in analytical research tools including mass spectrometry, proteomics, genomics, metabolomics, expressional profiling, and chemical informatics as well as novel *in vivo* labeling methods, the efficiency and success rate of target identification have improved significantly in recent years [31–34]. In addition, *in vivo* chemical screens are sometimes performed using chemical libraries consisting of known bioactive compounds, so that the signaling pathways mediating a disease phenotype can be uncovered relatively quickly once chemical suppressors of the phenotype are identified.

4. Efficient *In Vivo* Chemical Screening in Zebrafish

Some of the model organisms that may be used for *in vivo* chemical screening are *Drosophila*, *C. elegans* and embryonic/larval zebrafish (*Danio rerio*) (for a review see [35]). All of these models have the scalability required for high-throughput screening. Among them, zebrafish is the only vertebrate model and thus possesses the closest physiological similarities to humans.

Features of zebrafish that enable efficient *in vivo* chemical screening are multiple. First is their fecundity. One pair of zebrafish can produce 100–200 embryos each week, so even a medium size aquarium with a couple hundred fish can produce thousands to tens of thousands of embryos per week for screening. Second, zebrafish embryos are small. Generally 3–5 embryos can be arrayed in a well of a 96-well plate containing 100–200 μL of fish water. Further, most cell-permeable small molecules (with octanol:water partition values, or $\log P$, above zero) can penetrate zebrafish embryos even when they are inside the chorions [36]. Thus, compounds can be added directly into the water surrounding the embryos. For screens performed in 96-well plates at a 10-μM concentration, only 1-2 micrograms of each compound will be needed for screening. In addition, zebrafish develop quickly, embryos/larvae at 1–5 days after fertilization (dpf) already possess various functional physiological systems. The short developmental timeframe significantly condenses the time needed for experimentation. Figure 1 shows a schema of *in vivo* chemical screening in zebrafish.

The assays employed for *in vivo* screening will depend on the phenotype of interest. For example, transgenic lines expressing fluorescent proteins under the control of cell-type-specific promoters may be used to track the production or location of specific cell types. Thus, zebrafish *pu.1*, *gata1*, *mpo*, *lyzC*, *csf1r*, *rag2*, *lck*, *CD41,* or *scl* reporter lines among others may be used to identify chemical modulators of myeloid cells, erythrocytes, neutrophils, macrophages, T cells, thrombocytes, or hemangioblasts, respectively [37–45]. Whole-mount immunostaining and RNA *in situ* hybridization may also be used to detect cell proliferation or expression of cell differentiation markers. Even a wide range of physiological outputs and responses may be used as screening readouts, such as chemical-induced enterocolitis, injury-induced inflammation, host-pathogen interactions, and laser-induced thrombosis [46–52]. Some of these assays may

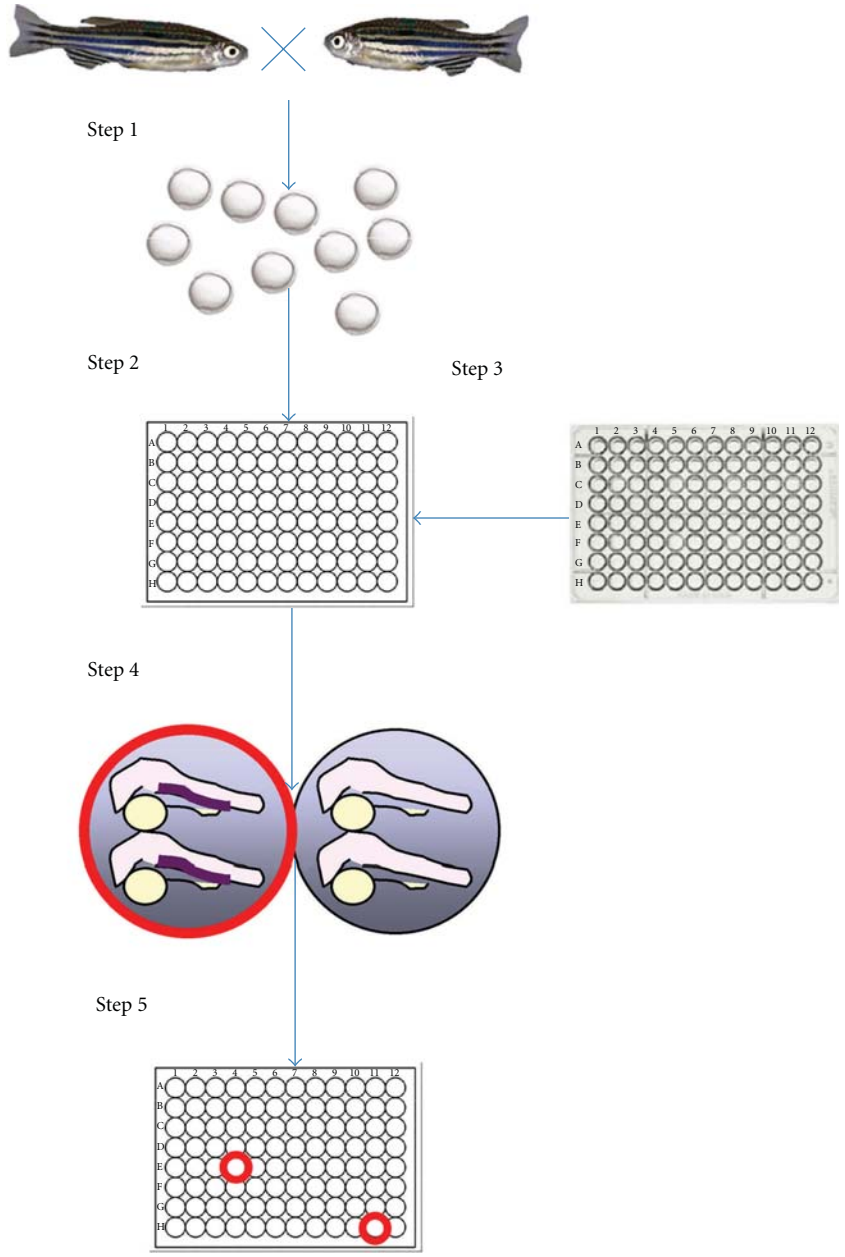

Figure 1: Chemical screening using zebrafish embryos. Step 1—Wild-type, reporter, or mutant zebrafish are crossed to obtain embryos. Step 2—Once reaching an investigator-specified developmental stage (usually between 0–5 days after fertilization), embryos are arrayed into multi-well plates either manually or by automation. Step 3—Compounds from a chemical library are added into the wells containing the embryos using a multichannel pipette or a pin-transfer device. Step 4—After reaching the developmental stage for phenotype manifestation, which is usually within hours to a couple of days after the compound treatment, embryos may be subjected to staining procedures, reporter, or functional assays to detect chemical-induced phenotypes or reversal of genetic phenotypes. The images shown here depict differential hematopoietic gene expression between the compound-treated (red circle) and vehicle-treated (black circle) embryos as detected by RNA *in situ* hybridization. Step 5—*In vivo* phenotypes can be detected by visual inspection or by automated imaging and recording. Thus, the whole screening procedure, once optimized, may be automated for high-throughput experimentation and finished within a few days. In addition, a wide range of phenotypes may be detected *in vivo*.

be processed by automated liquid handling machines or may be recorded using automated imaging systems and analyzed using customized software [51, 53–56]. Thus, conducting chemical screening in zebrafish provides great potential for identifying modulators of hematopoiesis and hematological diseases.

5. Considerations for Screening Designs, Hit Evaluation, and Translation to Humans

5.1. Screening Designs. As in any other types of chemical screens, the quality of the hits obtained in zebrafish screens can be directly influenced by the screening designs.

For example, if a screen is based on the reduction of the signals in a reporter assay, it may be prone to identifying false positives such as toxic compounds. In this case, a quick visual scan of embryo/larva viability before conducting the reporter assay may help exclude those nonspecific hits. In addition, since proper embryonic development depends on precise execution of multiple sequential processes, compounds added at different times will have the opportunity to affect different developmental steps. Thus, the timing and duration of chemical treatment are also likely to affect the screening outcomes. If a screen utilizes a transgenic line, additional validation steps should be incorporated to examine whether the hit compounds may affect the promoter used for driving the transgene or the stability of the transgene itself. For example, in one of the screens that we have performed, we have identified several hits that suppress the heat shock promoter used for driving the expression of an oncogene rather than the activity of the oncogene [20]. Whenever possible, positive controls should be used to validate that zebrafish models exhibit similar molecular machineries and pharmacological responses as humans do (if the screening purpose is drug discovery) for the biological processes under investigation. This confirmation beforehand will facilitate the likelihood of relevantly translating the findings from zebrafish screens to human conditions.

In addition, it is important to conduct a pilot screen using 100~300 compounds and one screening plate of untreated embryos/larvae to evaluate the robustness and potential variables of the screening methods, including the degrees of natural variations among different clutches of embryos/larvae. A pilot screen may also provide information as regard to the potential hit rates. On one hand, *in vivo* screening methods may cast a broad net for identifying compounds that elicit the phenotype-of-interest through various mechanisms. On the other hand, if the hit rates are higher than 1-2%, researchers may wish to incorporate secondary screening strategies or consider a different screening method in order to limit the hits to the ones that are likely to be of potential interest to the investigators. For example, we previously showed that immediately after the expression of the leukemia oncogene AML1-ETO, *gata1* expression is abolished, whereas *myeloperoxidase* (*mpo*) expression is increased at a later time point [57]. We conducted a chemical suppressor screen and identified various compounds that can restore *gata1* expression in the presence of AML1-ETO [20]. We have also verified the therapeutic potential of some of the hits identified in this screen, and these results will be discussed in more detail later. Conceivably, a chemical suppressor screen can also be performed based on the reversal of *mpo* upregulation in the same zebrafish model. The latter screening strategy may not only identify compounds that directly antagonize AML1-ETO's effects but also additional compounds that suppress the accumulation of mpo^+ cells through AML1-ETO-unrelated mechanisms. The choices of screening designs are subject to each investigator's discretion.

5.2. Hit Evaluation and Translation to Humans. The potency, effectiveness, and specificity of the confirmed hits obtained from zebrafish screens have already been demonstrated *in vivo*. Thus, these hits have a high probability of being effective in other *in vivo* systems. Both hematopoietic and other nonhematopoietic effects of these candidate compounds should be evaluated further in embryonic/larval zebrafish. The effects of the candidate compounds on cell differentiation, proliferation, or survival can be evaluated using whole-mount RNA *in situ* hybridization, whole-mount immunostaining or staining with lineage-specific cytological dyes such as Sudan Black for neutrophils and o-dianisidine for hemoglobin. These *in vivo* effects may be assessed facilely using embryonic/larval zebrafish. For example, we have found that AML1-ETO can reprogram hematopoietic cell fate decisions, converting the erythroid cell fate to the granulocytic cell fate. We have also found that nimesulide, a chemical suppressor of AML1-ETO, can reverse these effects in zebrafish. AML1-ETO has been shown to suppress erythroid differentiation in mammalian cells, and we have confirmed that nimesulide can also reverse AML1-ETO's effects in cultured cells [20]. The effects of candidate compounds on leukocyte or thrombocyte function can also be assessed in embryonic/larval zebrafish using an injury model for neutrophil chemotaxis, a bacterial infection model for phagocytosis, or a laser-induced coagulation assay [47, 58, 59]. Moreover, lineage-specific hematopoietic cells can be isolated from control and compound-treated embryos/larvae of various fluorescent reporter lines mentioned earlier by flow cytometry for transcriptional profiling analysis. Interestingly, the nonhematopoietic effects may sometimes provide instrumental information as to the mechanisms of action of the candidate compounds. For example, a candidate compound may cause a developmental phenotype similar to the phenotype caused by other genetic mutations or other chemicals with known bioactivities, suggesting that the candidate compound acts through a similar pathway as these other modulations do. The effects of the candidate compounds can also be evaluated in adult zebrafish using standard hematopoietic assays adapted from mouse models, including irradiation followed by hematopoietic cell transplantation and irradiation recovery assays, as well as leukemic cell xenograft and limiting dilution transplantation [37, 60–64]. The zebrafish provides the investigator the flexibility at which point to verify the effects of these compounds in mammalian systems. While the degree of conservation between zebrafish and mammals in hematopoiesis and in the functions of many hematopoietic cell lineages is high, conservation of humoral regulators and the adaptive immune system is presently less clear. However, rapid advancement in those areas is anticipated. For those biological processes already shown to be highly conserved, the translatability of the screening hits from zebrafish to humans will likely to be high.

6. Zebrafish Hematopoiesis and Hematological Disease Models in Zebrafish

6.1. Hematopoiesis. Zebrafish possesses a similar set of blood lineages as the mammals [11, 14, 63, 65–71]. The genes involved in blood cell development are also highly conserved

between zebrafish and mammals [72, 73]. Thus, it is a suitable model for investigating the genetic pathways regulating hematopoiesis and hematological diseases.

As in mammals, during embryonic development, zebrafish first exhibit a primitive wave of hematopoiesis and later produce several intermediate cell types that eventually contribute to definitive hematopoiesis (for more detailed reviews see [74, 75]). During primitive hematopoiesis, which begins around 11 hours after fertilization (hpf), zebrafish embryos produce myeloid and erythroid cells in two anatomically separate locations. Myeloid cells, which express the transcription factor *pu.1*, are formed in the anterior lateral plate mesoderm (ALM), while erythroid progenitors expressing the *gata1* transcription factor are formed in the posterior lateral plate mesoderm (PLM). It has been shown that hematopoietic cell fate in both blood islands is determined by the expression of these two genes. While knockdown of *pu.1* induces erythropoiesis in the ALM, knockdown of *gata1* promotes myelopoiesis in the PLM [76, 77]. These results indicate that primitive hematopoiesis in embryonic zebrafish produces bi-potent hematopoietic progenitor cells. Thus, these two synchronously specified blood populations may be useful for identifying important genes that regulate myeloid and erythroid cell fate determination. In a later section of this paper, we will discuss a study that utilizes these cells to uncover some of the AML1-ETO's oncogenic effects that lead to acute myeloid leukemia [20, 57].

In zebrafish, multipotent hematopoietic stem cells (HSCs) originate in the hemogenic endothelium of the aorta, which is equivalent to the aorta-gonad-mesonephros (AGM) in mammals [78]. Using *in vivo* lineage-tracing experiments, it has been shown that these newly emerged HSCs will subsequently colonize a transient hematopoietic tissue called the caudal hematopoietic tissue (CHT), which may be comparable to another mammalian embryonic hematopoietic site in the fetal liver [79–81]. Finally, HSCs from those regions will migrate to and seed both kidney (equivalent to bone marrow in mammals) and thymus, the final hematopoietic organs that remain through adult life [79–81]. As in mammals, zebrafish HSCs express *runx1* and *cmyb*, and *runx1* deficiency abrogates definitive hematopoiesis in fish [78, 82–84]. Several major signaling pathways that regulate HSC formation and homeostasis in mouse models also affect HSC formation in zebrafish, such as the Hedgehog (Hh) pathway and the Notch-Runx pathway [78, 85]. Recently, an *in vivo* chemical screen in zebrafish has identified important roles of the prostaglandin-E2-(PGE2-) Wnt signaling pathway in HSC formation [19, 86], which will be discussed in more detail later. These findings suggest that zebrafish and mammals utilize similar genetic circuitry for regulating HSC formation.

6.2. Hematological Disease Models. Due to the easiness of inspecting blood cell phenotypes in zebrafish embryos, a large number of blood mutants have been isolated in three large-scale genetic screens [11, 14, 87, 88]. Many of these blood mutants have defects in the maturation or iron transport of erythrocytes, and their related phenotypes and orthologous gene mutations have been defined in humans [89–91]. Transgenesis approaches have also been used to create various hematological disease models in zebrafish, of which the majority are blood cancer models [20, 38, 57, 92–96]. In these studies, ectopic expression of human oncogenes resulted in zebrafish phenotypes reminiscent of human leukemia characteristics. In addition, investigators can now perform efficient targeted gene disruption in zebrafish using engineered zinc finger nucleases (ZFNs) and transcription activator-like effector (TALE) nucleases [13, 16, 97–100]. In the future, many of these hematological disease models may be used for chemical suppressor screens. The vast array of research tools available in the zebrafish model combined with *in vivo* chemical screening will prove useful in providing novel insights into the molecular mechanisms and potential therapy for hematological diseases.

7. *In Vivo* Identification of Hematopoietic Stem Cell (HSC) Chemical Modulators

Compounds that can augment HSC formation and function may exert therapeutic benefits to patients undergoing hematopoietic cell transplantation. North et al. performed a chemical screen to identify small molecules regulating HSC formation in zebrafish embryos [19]. In this study, embryos were exposed between 11 and 36 hpf to 2,357 compounds from three chemical libraries of known bioactive compounds. As mentioned above, HSCs are $cmyb^+$ and $runx1^+$ and both transcription factors are indispensable for HSC development. By examining *cmyb* and *runx1* expression using RNA *in situ* hybridization, the authors found 35 compounds that increased HSC numbers and another 47 compounds that decreased them. Based on their known bioactivities, they found that 10 of these compounds affect prostanoid biosynthesis. Prostanoids, including prostaglandins, prostacyclins, and thromboxanes, are lipid mediators that play major roles in inflammation and other physiological responses. The cyclooxygenases (COXs), including COX-1 and COX-2 (also known as prostaglandin G/H synthase 1 and 2), convert arachidonic acid into prostaglandin H2, which can then be metabolized into other prostanoids by additional enzymes [101]. Interestingly, the authors found that while exposure to COX inhibitors such as celecoxib and sulindac reduced *cmyb*/*runx1* expression in the hemogenic aorta, exposure to linoleic acid, a precursor of arachidonic acid, enhanced it. Previously it had been shown that prostaglandin E2 (PGE2) is the major prostanoid produced in zebrafish embryos [102]. Thus, North et al. confirmed the involvement of the prostaglandin pathway in HSC formation by incubating zebrafish embryos with PGE2 or selective inhibitors of COX-1 and COX-2, as well as by genetic knockdown of *ptgs1* and *ptgs2* that encode COX-1 and COX-2 proteins, respectively. Subsequently, the authors investigated the expression patterns of *ptgs1* and *ptgs2* and found that both genes were upregulated at the onset of definitive hematopoiesis. While both genes were expressed in the HSCs, *ptgs1* was also expressed in the neighboring endothelium. These results strongly suggest that COX-1 and COX-2 promote HSC formation through functions in both the HSCs and their

niche. Furthermore, Goessling et al. showed that PGE2 promotes HSC formation by activating the Wnt/β-catenin signaling pathway [86].

In their screen, North et al. *also* found 22 compounds that might regulate HSC formation through their effects on blood flow, such as compounds affecting α- and β-adrenergic receptors, Ca^{2+} or Na^{+}/K^{+} channels, nitric oxide (NO) synthesis, or the angiotensin pathway [103]. They showed that blood flow had a positive impact on *cmyb*/*runx1* expression, suggesting that the hemodynamic force on the endothelium might be an inducing factor for the emergence of HSCs. In addition, the authors found that NO donors could stimulate HSC formation even in the *silent heart* mutant, which does not exhibit blood flow. Using mosaic transplantation experiments, they discovered that NO positively regulated HSC through cell-autonomous signaling.

8. Validation of HSC Chemical Modulators and Their Clinical Potential

Hematopoietic cell transplantation (HCT) is frequently used in the treatment of hematological malignancies. HSCs not only self-renew but also give rise to all blood lineages and can repopulate an entire hematopoietic system. Patients about to receive HCT need to undergo myeloablation and are treated simultaneously with immunosuppressants to prevent transplant rejection. It is essential that the transplanted HSCs effectively and efficiently engraft in the bone marrow. Various methods aiming to enhance the *in vitro* and *in vivo* expansion of stem/progenitor cells and their homing efficiency to bone marrow are currently under intensive investigation [104–107]. The chemical modulators of HSCs identified by North et al. in zebrafish represent another new therapeutic opportunity.

North et al. showed that *ex vivo* exposure of mouse whole bone marrow (WBM) or purified $lin^{-}Sca1^{+}c\text{-}Kit^{+}$ (LSK) cells to dimethyl-prostaglandin E2 (dmPGE2), a long-lasting derivative of PGE2, significantly increased the progenitor cell numbers as measured by spleen colony-forming units at day 12 after transplantation (CFU-S12) in the recipient mice. Using a limiting dilution competitive repopulation analysis, they found that dmPGE2-treated WBM resulted in 4- and 2.3-fold increases of HSCs in the recipients compared to the untreated cells at 12 and 24 weeks, respectively, following the transplants [19]. To define the mechanisms of action of PGE2, Hoggatt et al. *showed* that *ex vivo* exposure to PGE2 promoted HSC homing efficiency, proliferation, and survival during engraftment [108].

Clinically, sources for HCT include bone marrow, mobilized peripheral blood stem cells (MPBSCs), or human cord blood (hCB). Approximately 20% of HCTs in the United States are conducted using hCB [109]. However, recovery after hCB transplant often takes a very long time due to the limited volume of its source. Thus, Goessling et al. went on to show that dmPGE2 could enhance hCB hematopoietic colony formation *in vitro* and its engraftment in xenotransplantation [110]. Interestingly, the authors found that hCB samples treated with dmPGE2 exhibited gene expression patterns reminiscent of the HSCs emerged from a vascular niche [110]. Since hCB contains both HSCs and endothelial cells, the authors postulated that dmPGE2 might promote HSC formation from hemogenic endothelial cells, analogous to the scenario in developing zebrafish embryos. Alternatively, Butler et al. have shown that endothelial cells can provide signals for retaining HSC multipotency [111]. Finally, Goessling et al. provided evidence demonstrating preclinical safety of their regimen in nonhuman primate autologous transplantation [110]. Thus, from its initial discovery using an *in vivo* chemical screen in zebrafish, PGE2 is now entering a Phase I clinical trial.

9. *In Vivo* Identification of Acute Myelogenous Leukemia (AML) Chemical Suppressors

9.1. AML1-ETO and the t(8;21) AML. Our lab has conducted an *in vivo* chemical screen to identify compounds that could reverse the hematopoietic phenotypes of a human leukemia oncogene [20]. AML1-ETO is a fusion gene resulting from t(8; 21)(q21; q22) chromosomal translocation, and it is one of the most common translocation products in AML. In particular, AML1-ETO expression accounts for 40% of AML in the FAB (French-American-British) M2 subtype [112]. These patients can be characterized by overabundance of granulocytic blast cells.

AML-1, also known as Runx-1, is one of two subunits that form a heterodimeric transcription factor called the core binding factor (CBF). The CBF plays many important roles in hematopoiesis by regulating hematopoietic gene expression (for review see [113]). It has been shown that AML1-ETO exerts a dominant-negative effect on CBF function; however, recent studies also suggest that it produces other gain-of-function effects that account for its oncogenicity [114]. Expression of AML1-ETO enhances HSC expansion both *in vitro* and *in vivo* and promotes myelopoiesis while blocking myeloid maturation [115–119]. Despite intensive studies on gene regulation mediated by AML-ETO, to date no effective therapeutic target has been validated *in vivo*. Thus, we postulated that a phenotype-based, nonbiased approach such as *in vivo* chemical screening might uncover potential therapeutics and identify the critical downstream effectors of AML-ETO.

9.2. A Zebrafish Model for AML1-ETO Leukemia. A transgenic zebrafish line Tg(*hsp*:*AML1-ETO*) was established to enable heat-inducible expression of human AML1-ETO [57]. It was found that expression of AML1-ETO in embryonic zebrafish resulted in an accumulation of hematopoietic cells in the posterior blood island [57, 120]. Cytological analysis of the hematopoietic cells isolated from the transgenic embryos showed plentiful immature cells seldom seen in the control samples. In addition, genome-wide expression analysis identified various important similarities between the hematopoietic cells of the transgenic zebrafish and human t(8; 21) leukemia cells [57]. Previously it had been shown that AML1-ETO suppresses erythroid differentiation in human multipotent hematopoietic cells [121]. In the zebrafish model, it was found that AML1-ETO caused the downregulation of *gata1*

and the upregulation of *pu.1* in multipotent hematopoietic progenitors, suggesting a conversion of erythroid to myeloid cell fate. Moreover, the accumulated hematopoietic cells strongly expressed the *myeloperoxidase* (*mpo*) gene, indicative of a granulocytic cell fate. A previous study had shown that AML1-ETO downregulates *c/ebpα*, resulting in a maturation block of the granulocytic cells in human t(8; 21) AML [122]. In the zebrafish model, we also observed a dramatic reduction of *c/ebpα* expression, suggesting that only two days after its expression in zebrafish embryos, AML1-ETO induced an accumulation of granulocytic blast cells resembling the clinical features of human t(8; 21) AML.

9.3. Chemical Screening in the Zebrafish Model of AML-ETO. A library of 2,000 bioactive compounds was screened using the Tg(*hsp*:*AML1-ETO*) zebrafish model [20]. The screening compounds were added to embryos at 12–16 hpf, followed by 1 hour of heat treatment to induce AML1-ETO expression. Fifteen hit compounds were identified by restored *gata1* expression in the transgenic embryos as measured by RNA *in situ* hybridization. We found that some of the compounds affected the heat shock response in zebrafish, preventing AML1-ETO expression. In addition, we identified a histone deacetylase (HDAC) inhibitor sodium valproate as a chemical suppressor of AML1-ETO's effects. HDAC is a transcription corepressor that is known to interact with the ETO moiety of the AML1-ETO protein [123]. It has been shown that recruitment of HDAC is critical for AE's function, and that an HDAC inhibitor trichostatin A (TSA) induces differentiation and apoptosis of a t(8; 21) AML cell line [124]. We have shown previously that TSA also reversed the hematopoietic phenotype of Tg(*hsp*:*AML1-ETO*) zebrafish [57]; therefore, the discovery of sodium valproate validated the biological relevance of the chemical screen performed on the AML1-ETO zebrafish model.

Interestingly, nimesulide, a selective COX-2 inhibitor, was also identified in this screen [20]. Subsequently, we showed that treatments with indomethacin (a nonselective COX inhibitor), NS-398 (a selective COX-2 inhibitor), and nimesulide not only restored *gata1* expression but also inhibited increased expression of *mpo* in the transgenic embryos. Furthermore, we demonstrated that these drugs' effects were on target because they could be reversed by supplementing a downstream metabolite PGE2. Thus, the hematopoietic differentiation defects induced by AML1-ETO *in vivo* can be rescued by inhibiting the COX enzymes.

10. Validation of AML Chemical Suppressors and Their Clinical Potential

Since COX inhibitors scored as hits in our screen, we investigated the genes coding for COX proteins and found that *ptgs2* but not *ptgs1* expression was significantly upregulated in the hematopoietic cells of Tg(*hsp*:*AML1-ETO*) zebrafish [20]. At the time of this discovery, very little was known about the potential contribution of the COX enzymes in AML leukemogenesis, although overexpression of COX-2 had been reported in various types of epithelial tumors, including colorectal carcinoma and breast cancers [125, 126]. Moreover, PGE2 had been shown to promote colon cancer cell growth *via* a β-catenin-dependent signaling pathway [127, 128]. As in zebrafish, we found that AML1-ETO induced *ptgs2* but not *ptgs1* expression in the K562 human myeloid leukemia cell line [20]. AML1-ETO induced the activity of a β-catenin reporter and inhibited erythroid differentiation in these cells, and both effects could be abrogated by NS-398. Subsequently, we found that genetic knockdown of β-catenin rescued AML1-ETO's effects in zebrafish embryos [20]. Thus, AML1-ETO affects hematopoietic differentiation through the COX-2/β-catenin pathway in both zebrafish and human leukemia cells.

Since the publication of these findings, we have obtained strong evidence indicating that AML1-ETO also signals through a COX-2/β-catenin pathway in mouse bone marrow cells (Zhang et al., unpublished results). We have found that COX inhibitors can effectively suppress in vitro serial replating of hematopoietic stem/progenitor cells expressing AML1-ETO as well as AML1-ETO-mediated tumorigenesis in various *in vivo* mouse models (Zhang et al., unpublished results). Two recent studies have also explored the roles of the COX enzymes and β-catenin in leukemia stem cells expressing other leukemia oncogenes [129, 130]. In one of the studies, Wang et al. showed that either the MLL-AF9 fusion oncoprotein or coexpression of Hoxa9 and Meis1a could induce *ptgs1* expression and β-catenin activation. In addition, inhibiting COX activities using indomethacin attenuated leukemia development induced by MLL-AF9 or by coexpression of Hoxa9 and Meis1a oncogenes [129]. In the other study, Steinert et al. found that a nonselective COX inhibitor sulindac prevented β-catenin from being activated and reduced *in vivo* growth of HSCs expressing PML/RARα or PLZF/RARα oncogenes [130].

Collectively, these results suggest that inhibiting the COX enzymes using nonsteroidal anti-inflammatory drugs (NSAIDs) can suppress oncogenic function and β-catenin activation in AML leukemia stem cells. Interestingly, case-based studies have also suggested an inverse relationship between NSAID usage and AML incidence [131, 132]. Although PGE2 can induce β-catenin expression and augment some aspects of HSC function as discussed above, it has been shown that loss of β-catenin does not affect normal hematopoiesis in adult mice [133–136]. At present, a major obstacle for achieving long-term survival of AML patients is relapse. Although chemotherapy can effectively induce remission in the majority of patients, more than 50% of the patients experience relapse within a year after remission [137, 138]. In sum, these results suggest that NSAIDs may impair leukemia stem cell function and thus their clinical efficacy in preventing AML relapse should be explored.

11. Final Considerations for Drug Discovery in Zebrafish

In this paper, we presented two specific studies on hematopoiesis that appropriately exemplify the general utility of embryonic zebrafish and phenotypic *in vivo* chemical

screening in discovering potential new therapeutics. In these cases, the use of an *in vivo* screening platform allowed the identification of compounds that may act in a noncell autonomous fashion such as hemodynamic forces, bypassed the well-known technical difficulties involved in culturing hematopoietic or leukemia stem cells, and also circumvented the obstacles conferred by undruggable targets or unknown disease mechanisms. Both of the studies uncovered novel biological mechanisms as well as strong candidates for clinical therapeutic use. It is important to note that most of the advantageous features of the zebrafish model occur at its embryonic and larval stages. Thus, a disease phenotype under investigation must manifest during these stages in order to be most effectively exploited for chemical screening. Since multitudinous signaling pathways acting together in zebrafish during early development are also likely to play important roles in maintaining homeostasis in adults and may be disrupted or reactivated during disease progression, a surrogate embryonic phenotype can often be very useful for identifying potential disease modulators. For example, compounds that suppress T-cell development in embryonic zebrafish may demonstrate potent inhibitory effects against T-cell leukemia [18]. Overall, drug discovery in zebrafish benefits from the feasibility of high-throughput chemical screening, closer physiological similarities to human than invertebrate screening strategies, and the ability to create complex disease models not achievable *in vitro*. The possibility of detecting a wider range of hematopoietic phenotypes using innovative assays promises an ever-increasing role for zebrafish in future drug discovery processes.

Conflict of Interests

The authors declare no competing financial interests.

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Research Article

Genomic Amplification of an Endogenous Retrovirus in Zebrafish T-Cell Malignancies

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Genomic instability plays a crucial role in oncogenesis. Somatically acquired mutations can disable some genes and inappropriately activate others. In addition, chromosomal rearrangements can amplify, delete, or even fuse genes, altering their functions and contributing to malignant phenotypes. Using array comparative genomic hybridization (aCGH), a technique to detect numeric variations between different DNA samples, we examined genomes from zebrafish (*Danio rerio*) T-cell leukemias of three cancer-prone lines. In all malignancies tested, we identified recurring amplifications of a zebrafish endogenous retrovirus. This retrovirus, ZFERV, was first identified due to high expression of proviral transcripts in thymic tissue from larval and adult fish. We confirmed ZFERV amplifications by quantitative PCR analyses of DNA from wild-type fish tissue and normal and malignant *D. rerio* T cells. We also quantified ZFERV RNA expression and found that normal and neoplastic T cells both produce retrovirally encoded transcripts, but most cancers show dramatically increased transcription. In aggregate, these data imply that ZFERV amplification and transcription may be related to T-cell leukemogenesis. Based on these data and ZFERV's phylogenetic relation to viruses of the murine-leukemia-related virus class of gammaretroviridae, we posit that ZFERV may be oncogenic via an insertional mutagenesis mechanism.

1. Introduction

Zebrafish are an emerging animal model for the study of lymphocytic cancers. A landmark 2003 study first described that transgenic murine *Myc* (*mMyc*) misexpression could induce *D. rerio* T-cell acute lymphoblastic leukemia (T-ALL) [1]. Since that initial report, several other zebrafish ALL models have been described, utilizing transgenic mammalian *TEL-AML1* (human), *NOTCH1* (human), *MYC* (murine and human), and *AKT2* (murine) in similar fashion [2–5]. In addition, we used a phenotypic mutagenesis screen to create three further zebrafish models with heritable T-ALL predisposition [6]. All but one of the eight lines cited above are prone to T-ALL, not B-cell-lineage cancers. Like human T-ALL, *D. rerio* T-ALL often arises in or spreads to the thymus and forms tumors. Hence, these seven zebrafish lines actually more accurately model two related lymphocyte malignancies, T-ALL and T-cell lymphoblastic lymphoma (T-LBL). In fact, *mMyc* zebrafish have even been used to investigate the molecular changes that accompany the transition between T-LBL and T-ALL [7].

Because the molecular origins of T-ALL and T-LBL are not completely understood, these zebrafish models provide opportunities to investigate the genetic underpinnings of these diseases' oncogenesis. In addition, they also facilitate inquiries designed to reveal features associated with T-ALL and T-LBL progression. For example, in the aforementioned study, Feng et al. demonstrated that changes in BCL2, S1P1, and ICAM1 expression were linked to autophagy, intercellular adhesion, and intravascular invasion, thereby governing the T-LBL to T-ALL transition [7]. Similarly, Gutierrez et al. used transgenic zebrafish T-ALL to study the dependence of

MYC-driven cancers upon *Pten* and *Akt* for disease persistence and progression [5].

While these two studies utilized *D. rerio* models to investigate candidate genes of suspected importance to disease progression, zebrafish T-cell cancers can also serve as a means for candidate gene discovery. In our own work, we utilized serial allo-transplantation of *D. rerio* T-ALL as an experimental approach to model clinically aggressive neoplasia [8]. Similar strategies have been employed by other groups, using serially allo-grafted murine T-cell lymphomas or xeno-transplanted human T-ALL into immunodeficient mice [9, 10]. In our study, we performed aCGH to seek acquired genomic changes common to serially passaged *D. rerio* T-ALL and refractory/relapsed human T-ALL. Several candidate genes met this criterion, including *C7orf60* (zebrafish homologue, *zgc*: *153606*), a gene whose amplifications were linked to both accelerated T-ALL progression in fish and inferior outcomes in human T-ALL patients [8].

Although our study concentrated on acquired copy number aberrations (CNAs) shared by zebrafish and human T-ALL, we also identified other genomic amplifications and deletions seen only in *D. rerio* cancers. Amongst these, two recurring copy number gains were observed in every sample, and with further scrutiny we found that both regions corresponded to the same endogenous retrovirus (ERV). This genomically integrated provirus is predicted to have 2–4 integration sites in the zebrafish genome [11], and its ploidy and genomic positions may vary between individual animals, complicating its inquiry. In this paper, we use two independent methodologies to show that this multicopy ERV undergoes further amplification in both normal and neoplastic zebrafish T cells, which could create new and potentially oncogenic integrations. Some cancers showed very high ZFERV copy number, well above that seen in T lymphocytes. We also demonstrate the expression of retrovirally encoded RNAs by both normal and cancerous *D. rerio* T cells, with most malignancies displaying significantly elevated proviral transcription relative to normal T cells. Our findings, and further characterization of this ERV, will be essential to understanding how this biologically active retrovirus impacts normal and malignant zebrafish T lymphocyte biology.

2. Materials and Methods

2.1. Zebrafish Lines and Care. Adult fish from five *D. rerio* lines were analyzed: normal WIK strain *lck*::*EGFP* fish [12], the ENU mutant lines *hulk*, *shrek*, and *oscar the grouch* (*hlk*, *srk*, *otg*; all WIK background) [6], and *rag2*::*MYC-ER* × *lck*::*EGFP* fish (*nacre* × WIK hybrid) [5]. Fish were housed using standard conditions (28.5°C, 14 hr. light/10 hr. dark circadian cycle) in a colony at the University of Utah's zebrafish core facility. For examinations under fluorescent microscopy, fish were anesthetized with 0.02% tricaine methanesulfonate (MS222) and euthanized with ice water prior to dissections. Animals were handled according to NIH guidelines, under an approved protocol (IACUC #08-08005) by the University of Utah Animal Care and Use Committee.

2.2. Dissections and Fluorescence-Activated Cell Sorting (FACS). Zebrafish thymi and GFP$^+$ tumors were dissected, with preparation of single cell suspensions and FACS performed as described previously [6]. BD FACSVantage and FACSAria II SORP (Becton Dickson) instruments were used for FACS. GFP intensity and side- and forward-scatter were gating parameters for GFP$^+$ lymphocyte collections.

2.3. Nucleic Acid Purifications. Genomic DNA for aCGH and qPCR was extracted from FACS-purified GFP$^+$ T cells and matched tailfin tissue using the DNeasy Blood and Tissue Kit (Qiagen) as described previously [8]. Total RNA for qRT-PCR assays was extracted from FACS-purified T lymphocytes and T cell cancers with Trizol (Invitrogen) or the RNeasy Mini-Kit (Qiagen) according to manufacturer instructions. RNA samples were treated with RNase-Free DNase (Qiagen) according to manufacturer instructions prior to qRT-PCR.

2.4. Array Comparative Genomic Hybridization (aCGH). Genomic DNA was labeled with the BioPrime Labeling Kit (Invitrogen), purified, quantified, and hybridized to Zv6-based Zebrafish Genomic Arrays (NimbleGen) as reported previously [8]. Arrays were analyzed using the G2565CA Microarray Scanner System with SureScan High Resolution Technology (Agilent) and normalized using Agilent Feature Extraction software. Copy-number analysis was conducted using the Rank Segmentation algorithm with Nexus Copy Number 5.0 software (BioDiscovery). Detailed descriptions of the aCGH methods used and copy number analyses performed are available in the supplemental sections of the report by Rudner et al. [8].

2.5. Quantitative Polymerase Chain Reactions (qPCR). A LightCycler CFX96 (Bio-Rad) was used for qPCR assays. Briefly, IQ SYBR Green Supermix (Bio-Rad) was used to amplify genomic DNA from various tissue types. Pooled thymocyte DNA (our limiting sample) was spectrophotometrically quantified and then diluted 1 : 100 for use in qPCR. DNA from tailfin tissue and GFP$^+$ tumor cells were diluted to identical concentrations, with 2 μL of each DNA used in reactions with total volumes of 25 μL, and other components added according to manufacturer instructions. All reactions were performed in triplicate. SYBR Green signals were used to derive estimates of relative ZFERV copy number. Since true ZFERV copy number is unknown, values were arbitrarily normalized to 1 copy/haploid genome. Thus, a ZFERV relative copy number equal to 3 indicates three times as many ZFERV copies/genome (e.g., if germline copy number = 3 copies/haploid genome, ZFERV relative copy number = 3 indicates 9 copies/haploid genome). All qPCR results with *pol* and *env* were normalized to *elf2a*, present in 1 copy/*D. rerio* haploid genome. Primers and reaction conditions were as follows:

Forward *pol* primer: CGC-CCC-ACA-CAT-CAC-ATA

Reverse *pol* primer: CAA-CCA-TCA-CAG-AAC-AGA

Forward *env* primer: ATG-TTT-GGG-GAA-TGG-AAG-G

Reverse *env* primer: TTT-GAT-AAG-GAG-GTG-GGT-TTT

Forward *elf2a* primer: TGG-AGG-TGG-AGG-TGA-GAA-CT

Reverse *elf2a* primer: GAG-TGG-TTG-TGT-AAG-CAT-TTC-G

Denaturation: 95°C × 3 minutes

40 cycles:

95°C × 10 seconds
59°C × 40 seconds

Melt curve analysis—55°C–95°C.

2.6. Quantitative Reverse Transcription Polymerase Chain Reactions (qRT-PCR). Total RNA (200 ng/sample) from FACS-purified normal and malignant T cells was assayed with the iScript One-step RT-PCR Kit with SYBR Green (Bio-Rad) using the aforementioned equipment. Reactions were run in triplicate. Results with *pol* and *env* were normalized to *elf2a*, assayed in parallel qRT-PCRs. Expression fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method. Primers and reaction conditions were as follows:

Forward *pol* primer: CAG-CAC-AAA-CGA-AAA-TGG-TCT

Reverse *pol* primer: TGG-CTC-CTC-AGT-GTC-TCC-TT

Forward *env* primer: AGA-GGG-AAA-GGA-TGG-GAT-GT

Reverse *env* primer: TGT-TGG-ATG-TGG-TCT-GGT-CT

Forward *elf2a* primer: ATG-AGA-CAA-TGG-GGA-GAG-CA

Reverse *elf2a* primer: GGA-TGC-GGC-TGG-AGT-TTC

Denaturation: 95°C × 5 minutes

40 cycles:

95°C × 10 seconds
52°C × 10 seconds
72°C × 30 seconds

Melt curve analysis—55°C–95°C.

2.7. Statistical Analyses. The student's *t*-test was used to compare differences in genomic relative copy number or fold changes in RNA expression. *P* values < 0.05 were considered significant.

3. Results and Discussion

We previously performed an ENU-mutagenesis phenotypic screen designed to identify abnormal T-cell phenotypes. Our screen resulted in the discovery of three *D. rerio* lines (*srk*, *hlk*, *otg*) prone to T-cell malignancies, specifically T-ALL and T-LBL [6]. To investigate non-germline acquired genetic changes occurring in these cancers, we used aCGH to compare DNA of neoplastic and normal tissues from individual fish of each of these lines. These experiments revealed several homologous genes that are commonly amplified or deleted in both zebrafish and human T-ALL [8].

In those studies, >98% of *D. rerio* genes with somatically acquired CNAs also had identifiable human counterparts. However, two non-homologous genomic regions unique to zebrafish were also particularly interesting. These loci showed copy number gains in 8/8 zebrafish T-cell cancer genomes relative to DNA of nonmalignant tissues from the same animals (Figure 1). Notably, T-ALLs from all three lines (3/3 *srk*, 3/3 *hlk*, 2/2 *otg*) exhibited copy number gains in both regions, establishing these acquired genomic amplifications as consistent features in T-cell cancers arising from different genetic backgrounds. Our aCGH experiments used a NimbleGen microarray platform constructed from the Zv6 genomic assembly. We subsequently discovered that the probes displaying amplified signals were mistakenly assigned to distinct regions on chromosomes 7 and 14 (hybridization data depicted in Figure 1). However, upon closer inspection these probes actually derive from a single, approximately 11 kb, locus. Intriguingly, this region corresponds to a genomically integrated retroviral element dubbed ZFERV by Shen and Steiner, named as such because it is the first and thus far only described zebrafish endogenous retrovirus [11].

In scrutinizing the six aCGH probe sequences localized to these two chromosomes, we realized they were in fact distributed throughout the ZFERV genome (Figure 2). Collectively, our hybridization results with these 6 probes provide compelling evidence that the entire ZFERV locus is undergoing somatic amplifications in the genomes of zebrafish T-cell cancers. Because our aCGH data is internally normalized by comparing each cancer's DNA to paired non-malignant tailfin DNA from the same fish, our results are protected from possible ZFERV copy number variation (CNV) that might exist between different animals. However, due to ambiguity regarding initial (i.e., germline) ZFERV copy number in individual fish, it is impossible to deduce the absolute number of copies gained by each cancer. Instead, our findings are limited to the conclusion that ZFERV has been amplified, relative to the original number of ZFERV copies, in 8/8 T cell malignancies tested. Moreover, because "normal" ZFERV copy number and genomic locations may vary between fish or between strains, thus far, determining absolute ZFERV copy number prior to oncogenesis has been challenging.

Reinforcing the complexity of this issue, previous *D. rerio* genome builds have displayed ZFERV in multiple locations on each assembly, and also on several different linkage groups (LG 1, 5, 7, 14, 15, 16, 17, and 22). It is inherently difficult to accurately map multicopy loci like ZFERV, and

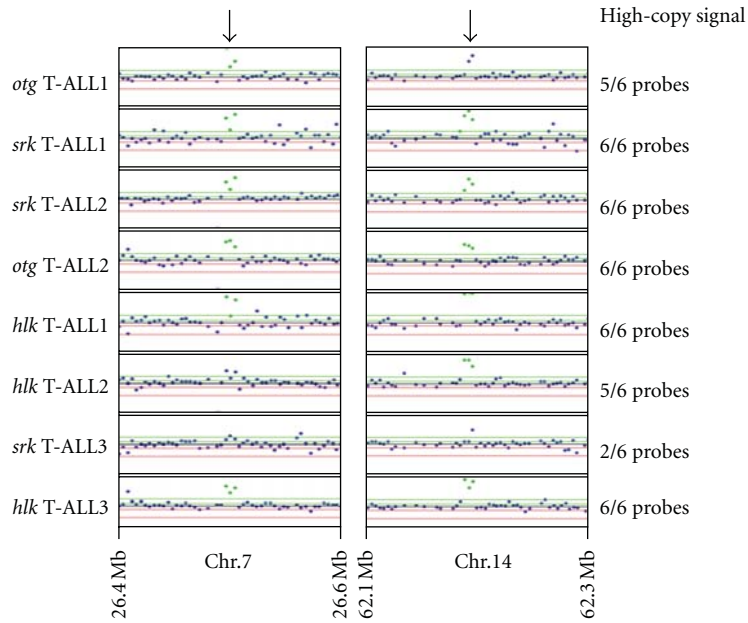

Figure 1: Recurrent amplifications of a small genomic locus in zebrafish T-ALL. Ten kb loci on chromosomes 7 and 14 (Zv6 genomic assembly) show high-copy gains with multiple probes in these regions (black arrows) of 8/8 *D. rerio* T-ALL samples tested. Signal intensities above a "high-copy gain threshold" (upper green line) indicate a greater than 2-fold increase in copy number. Individual probes and their intensities are depicted as blue dots; areas with 3 adjacent probes above the high-copy gain threshold use green dots to denote those probes. Seven cancers exhibited high-copy signals for ≥5/6 probes in this region, while *srk* T-ALL3 had high-copy signals for only 2/6 probes.

this is made even more taxing by its sequence composition. ZFERV harbors several redundant sequence tracks, including 5′ and 3′ long terminal repeats (LTRs) and a 517 bp repeat region (RR) containing 9 consecutive repeat elements (see Figure 2). When compounded with potential variability resulting from strain-specific ZFERV integrations, it is perhaps predictable that ZFERV has not received definitive chromosomal map position(s). Consequently, the current NCBI zebrafish genome actually suppresses ZFERV sequences and curates them so they do not appear on the Zv9 assembly at all.

In the original report describing ZFERV, Shen and Steiner conducted studies to address some of these questions concerning copy number and genomic localization: to prove that ZFERV was integrated into the *D. rerio* germline, they tested sperm DNA from several Tübingen (Tü) fish and verified an integration site common to each of their genomes [11]. Additionally, using Southern blots of Tü genomic DNA, they detected 2–4 bands hybridizing to a ZFERV *env* probe, implying a maximum of four retroviral copies per haploid genome [11]. However, not all Tü fish showed identical hybridization patterns. This could be due to restriction site polymorphisms in the Tü strain but might also suggest that different fish, even from the same strain, can possess different ZFERV copy number and integration sites. Moreover, when Southern hybridizations with an LTR-based probe were performed, 8–10 bands were seen. Most—but not all—of these entities were shared by different Tü fish [11]. As with prior results, this finding might be attributable to variability in ZFERV copy number and genomic position between different fish. Another interpretation that must be considered is that homologous LTRs from other related retroviruses and/or incomplete ZFERV proviral genomes (having ≥1 LTR, but no *env*) would yield a similar experimental outcome.

In spite of these uncertainties, our aCGH data remain convincing as evidence of somatically acquired ZFERV amplifications in *D. rerio* T-cell cancers. None of our aCGH probes correspond to LTR sequences, and 5/6 derive from the retroviral *gag*, *pol*, or *env* genes (Figure 2). Furthermore, even if repeat elements had been used in hybridizations, our method of comparing neoplastic to non-malignant DNA from the same animal is designed to normalize for CNV discrepancies between different fish. Therefore, we conclude that zebrafish T-cell malignancies acquire non-germline ZFERV copies at some point after fertilization, but whether amplifications precede and contribute to oncogenesis is unclear.

Because ZFERV transcription occurs in normal zebrafish T cells [11], we were curious whether normal *D. rerio* T lymphocytes might also have ZFERV copy gains. To determine if retroviral amplifications also occur in nonleukemic T cells, we investigated ZFERV in normal zebrafish T lymphocytes. To emulate our aCGH comparisons, we developed quantitative PCR (qPCR) assays for two ZFERV genomic regions. Using DNA from cancers with gains identified by aCGH, we verified these assays' ability to detect ZFERV copy number gains (data not shown). Next, we employed these qPCRs of amplicons from the *pol* and *env* regions (locations shown in Figure 2) to test genomic DNA from tailfin tissue and FACS-purified T cells of wild-type (WT) adult zebrafish. Thymocytes were obtained from WT WIK

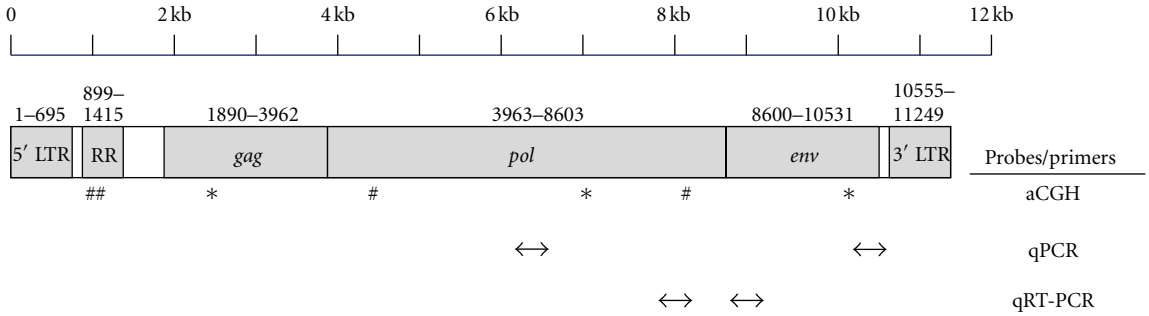

Figure 2: ZFERV Genomic Organization. The ZFERV retrovirus is comprised of two 695 bp long terminal repeats (LTRs), a 517 bp repeat region (RR) containing 9 direct repeats, and ORFs for 3 proteins: *gag*, *pol*, and *env* [11]. The *gag* and *pol* genes share the same reading frame and are predicted to be translated from one transcript by read-through of a stop codon; *env* uses a different reading frame and is probably a distinct transcript [11]. aCGH probe sites are shown beneath the ZFERV genome schematic: probes found on Zv6 chromosomes 7 (*) and 14 (#) are dispersed throughout ZFERV. One aCGH probe had sequences corresponding to the RR (##), and has multiple binding sites in this area. The 5 remaining aCGH probes map to ORFs. Amplicons from qPCR and qRT-PCR assays are also depicted (not shown to scale). The *pol* and *env* qPCR products are 168 and 169 bp, respectively; qRT-PCR products are 225 bp for *pol* and 234 bp for *env*.

D. rerio carrying an *lck*::*EGFP* transgene [12]. Since the zebrafish *lck* promoter is T cell specific, T lymphocytes from this line are GFP$^+$. However, unlike fish with T-ALL or T-LBL, adult (>6 months of age) WT fish have significantly fewer T cells (approximately 5×10^4 GFP$^+$ thymocytes/fish; our unpublished observations). Consequently, we pooled thymic tissue from several WT fish for FACS purifications. We then analyzed amplicons from both ZFERV regions to independently assay copy number differences.

Tailfin DNAs were tested individually or in small groups to ascertain whether there were appreciable germline CNV differences in WIK strain fish (Figure 3, lanes 1–6 and 8–10). As seen in these data, qPCR of *pol* (Figure 3(a)) and *env* (Figure 3(b)) show little deviation between fin DNA from different WIK fish, implying that CNV was minimal in these strain-related animals (lanes 7 and 11). Because copy number was so uniform, this further suggests that ZFERV amplification does not occur in fin tissue. Thus, we conclude that ZFERV status in fin tissue likely represents true germline copy number, and that this level is relatively stable between individual fish.

In contrast, normal T cells pooled from these same WT fish showed significant ZFERV gains relative to tailfin DNA (Figure 3, lanes 12, 13). On average, WIK T cells had 2- to 3-fold as many ZFERV copies as matched tail DNA (compare lane 11 to 14). Since germline copy number is unknown, we cannot deduce the real number of ZFERV copies in these T cells. Nonetheless, if prior data suggesting 2–4 copies/haploid genome are accurate [11], these results indicate normal T cells may average up to 12 copies per haploid genome, or 24 copies/diploid T cell. If correct, this would compute to 16 new ZFERV integrations, on average, in each T cell.

Because we used T lymphocytes pooled from several WIK fish in these studies, we cannot definitively conclude whether all animals' T cells bore evidence of ZFERV amplification. It is possible that only one or a few fish have ZFERV gains, with DNA from those fish skewing the average upward. However, even in one fish, T lymphocytes constitute a nonclonal population. It is possible—perhaps even likely—that ZFERV copy number varies on a cell-to-cell basis. ZFERV amplifications may occur in T cells themselves; alternatively, they might take place earlier along the hematopoietic stem cell/T cell progenitor differentiation spectrum. We have not tested precursor populations, as these are impossible to obtain in *D. rerio* owing to the dearth of antibodies to cell surface receptors. Irrespective of its precise timing, we conclude that thymocytes acquire additional genomic ZFERV copies at some point after fertilization, exactly like those detected in our aCGH analyses of zebrafish T-cell cancers.

Notably, there is precedent proving that ZFERV is active in zebrafish T cells. This retrovirus was originally discovered from a thymic cDNA library, after adult *D. rerio* thymus had been subtracted against 2-day postfertilization (dpf) larval fish, which have not yet developed T lymphocytes [11]. This study identified 43 clones hybridizing to only adult thymic cDNA. Of these, 21 clones also showed thymus-specific staining in 7-dpf *in situ* hybridizations (ISH). After sequencing, Shen and Steiner recognized that all 21 clones derived from various segments of the ZFERV genome [11]. So, not only was ZFERV transcribed by both 7-dpf and adult thymocytes, its expression in these cells was significantly higher than in other tissues by these two methodologies. Subsequent ISH experiments in 4-dpf, 5-dpf, and 3-month-old juvenile fish, as well as Northern blotting of RNA from adult fish thymocytes, confirmed these findings [11]. Together, these prior studies and our own new findings demonstrate that ZFERV is highly transcribed by larval and adult *D. rerio* thymocytes and that ZFERV amplifications occur in the genomes of normal and malignant zebrafish T cells.

To further expand our understanding of these phenomena, we next compared ZFERV amplifications in cancer-prone thymocytes and neoplastic T cells to WT T cells. For these experiments, we used our qPCR assays to compare ZFERV copy number in two other T-cell malignancy predisposed lines, *hlk* and *MYC-ER*. Both of these lines are prone to T-LBL and T-ALL, allowing ZFERV quantification of their germlines, their "premalignant" T lymphocytes,

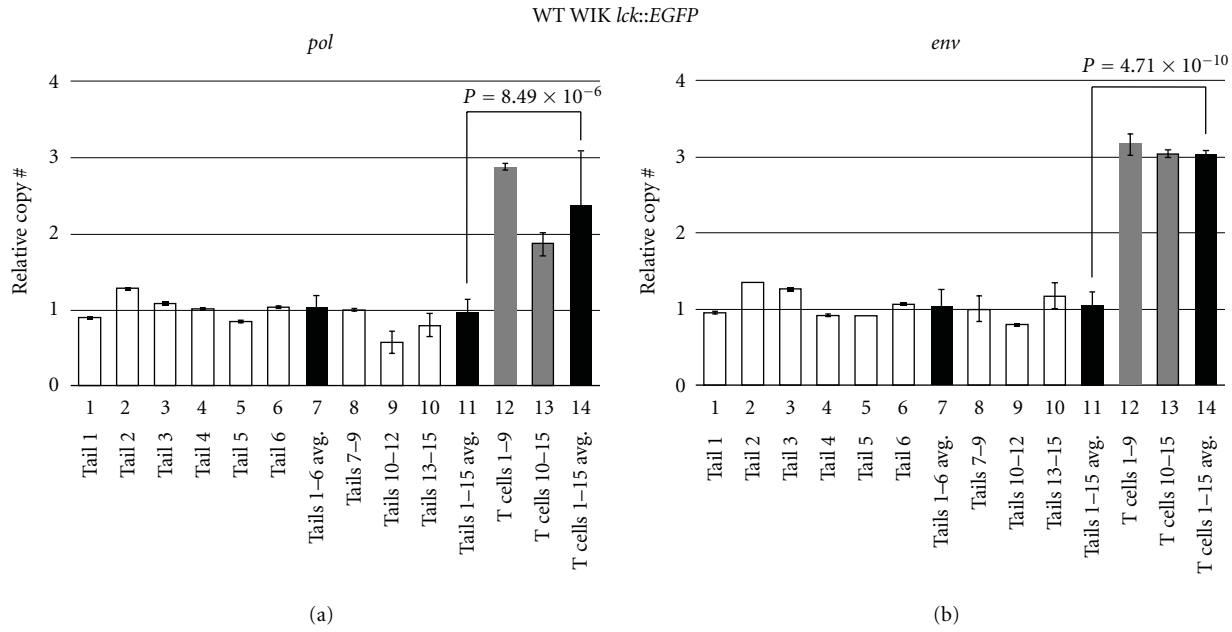

Figure 3: ZFERV amplifications in WT WIK *D. rerio* T cells. Genomic DNA from 15 WIK *lck::EGFP* fish was analyzed by qPCR of the ZFERV *pol* (a) and *env* (b) genes. White bars depict results from tail DNA of individual fish (lanes 1–6) or groups of 3 fish (lanes 8–10). Black bars show calculated means of 6 singly tested tails (lane 7) or all 15 tails (lane 11). "Tails 7–9" sample (lane 8) was arbitrarily assigned copy number equal to 1, and this DNA was used as the reference standard for all subsequent qPCRs. T cells pooled from 9 or 6 WT fish (gray bars) had 2- to 3-fold gains in ZFERV. Mean copy number was higher in T cells than tailfin DNA for the 15 fish cohort (lane 11 versus 14). Zebrafish *elf2a* (1 copy/haploid genome) qPCR was used to normalize *pol* and *env* results (not shown). Water-only template controls lacked detectable product (not shown). Reactions were performed in triplicate, and error bars show standard deviations (*env* qPCR of tails 2 and 5 had standard deviations too small to be seen).

and their malignant T cells. All *MYC* transgenic fish have hypertrophic thymi, likely reflecting abnormal T-cell proliferation and physiology. In contrast, *hlk* fish carry an unidentified mutation, display normal-appearing thymi, and the molecular basis for their cancer predisposition is unknown. T-ALL or T-LBL afflicts roughly 35% of *hlk* homozygotes by one year [6], reflecting a requirement for additional mutations to promote malignant transformation [8]. By comparison, WT *lck*::*EGFP* fish rarely develop T-cell cancers (<0.1%, our unpublished observations) and have normal T-cell development and physiology [12]. Thus, using these samples we could investigate whether normal, abnormal, and neoplastic T cells all exhibited similar degrees of ZFERV amplification.

As in earlier experiments, we examined tailfins from individual fish to ascertain ZFERV germline variability. Tails from single *hlk* and *MYC-ER* fish (Figures 4 and 5, lanes 3–8) demonstrated consistent copy number between animals. Moreover, both *hlk* and *MYC-ER* tails had ZFERV CNV similar to the WT WIK line (compare lane 1 to other white bars in Figures 4 and 5). Based on these results, identical for both the *pol* and *env* regions, we conclude that all 3 lines have approximately equivalent germline copies of ZFERV. In pooled premalignant T cells (i.e., thymocytes from *hlk* and *MYC-ER* fish lacking tumors or other non-thymic GFP), genomic ZFERV was again elevated relative to tailfin DNA from the same fish (Figures 4 and 5, compare gray bars in lanes 10-11 to white bars in lanes 3–8). Overall, mean T-cell ZFERV copy number was roughly 3-fold above germline in WT, 4-fold higher in *hlk*, and 5-fold increased in *MYC-ER* (compare lane 9 to 12 in both figures). Since WT, *hlk*, and *MYC-ER* thymocytes all showed approximately equivalent gains, we infer that genomic integration is not appreciably enhanced in T lymphocytes of either cancer-prone genotype. Thus, while retroviral amplification is clearly a common feature of all *D. rerio* T cells, cancer predisposition probably does not directly originate from increased susceptibility to ZFERV integration, as these events evidently transpire in normal T cells regularly. However, it is plausible that cancer predisposing mutations and ZFERV copy number gains may cooperate to promote malignant transformation of T cells, as amplifications were uniformly present in every T-ALL sample examined by aCGH.

To investigate how WT and cancer-prone T cells compare to actual neoplasias in the *hlk* and *MYC-ER* lines, we also analyzed malignancies from these same genetic backgrounds. We performed *pol* and *env* qPCRs on 3 *hlk* and 5 *MYC-ER* fish, each of which had large thymic tumors and/or extensive GFP^+ disease in extra-thymic areas (Figures 4 and 5). Tail DNA from these 8 fish all had similar germline ZFERV content to previously tested tailfin samples (compare lanes 13–15 in Figure 4 and lanes 13–17 in Figure 5 to other white bars in both figures). Like *hlk* T lymphocytes, *hlk* cancers had ZFERV amplification (Figure 4, lanes 16–18). However, gains were similar in magnitude to those seen in *hlk* premalignant T cells (compare lane 12 versus 19). In *MYC-ER* cancers,

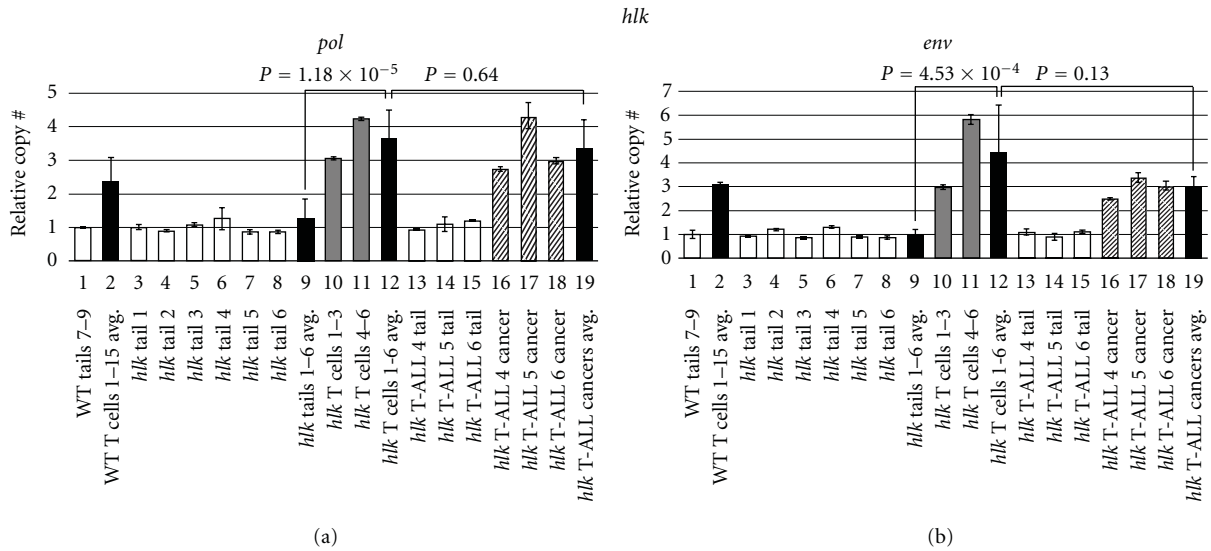

Figure 4: ZFERV amplifications in *hlk* zebrafish. DNA from 6 *hlk* fish with normal phenotype and 3 with GFP$^+$ cancers was tested by qPCR of *pol* (a) and *env* (b). White bars show tail DNA of individual normal (lanes 3–8) or T-ALL$^+$ (lanes 13–15) fish. All were statistically similar to each other and to WT Tails 7–9 (lane 1). Pooled T cells from *hlk* fish without T-ALL (gray bars) had ZFERV gains comparable to normal WIK T cells (lane 2). Mean copy number was higher in *hlk* T cells than tails (lane 9 versus 12) in the same animals. Diagonally striped bars show amplifications in neoplastic T cells of 3 *hlk* fish (lanes 16–18). Mean copy gains were similar in non-malignant and malignant *hlk* T cells (lane 12 versus 19). Other details are as described in the legend to Figure 3.

ZFERV gains were also detected (Figure 5, lanes 18–22). As in *hlk*, benign and malignant *MYC-ER* T cells did not show appreciable copy number differences (compare lane 12 to 23).

We also tested 2 other malignancies by qPCR. In one WT *lck*::*EGFP* fish, we noticed a large GFP$^+$ thymic tumor. In our experience, the spontaneous occurrence of T-cell cancer in WT fish is exceedingly rare, so we used this opportunity to investigate whether ZFERV amplification accompanied this event. Tail DNA indicated this animal had normal ZFERV germline content (Figure 6, compare lane 1 versus 9), and cancerous T cells from this fish showed approximately 5.5-fold higher copy number (lane 10). This degree of amplification is roughly twice that seen in normal WIK T cells and more closely resembled typical copies in *MYC-ER* T cells and cancers (compare lane 10 to lanes 4, 6, and 8). However, since this result reflects only one tumor, no general conclusions can be drawn about retroviral amplification in the rare cancers of WT fish. Lastly, in one additional *hlk* cancer, we found remarkably high ZFERV levels, showing 25- to 30-fold amplification above germline (lanes 11 and 12). This degree of copy number gain is nearly ten times higher than the other 9 T-ALLs we examined by qPCR, or the 8 tested previously by aCGH. Nonetheless, this infrequent scenario clearly demonstrates that ZFERV can parasitize the zebrafish genome in striking fashion, as this cancer likely harbors as many as 50–100 newly acquired retroviral copies.

Taken together, we conclude that virtually all MYC-driven, *hlk*-, *srk*-, and *otg*-induced, or even spontaneous zebrafish T-cell cancers have ZFERV amplifications. However, since nearly all benign, cancer-prone, and malignant T cells show similar genomic levels, the absolute amount of ZFERV amplification does not appear to be an important oncogenic determinant. This is not surprising, as it is likely that the site rather than the number of integrations is the crucial factor. To pursue this premise, one could identify new loci where ZFERV has integrated into cancer genomes, with the hypothesis that these might lie near or within proto-oncogenes or tumor suppressors. We have initiated such studies, and they are currently in progress. As an adjunct, we chose to investigate transcription of ZFERV-derived RNAs. We reasoned that integrations into transcriptionally permissive genomic sites might be accompanied by increased ZFERV RNA expression, perhaps signifying "active" proviral copies. While these insertions might not denote sites where oncogenes or tumor suppressor reside, it could serve as a proxy for ZFERV promoter potency in the genome overall. If so, this predicts that cancers would have higher ZFERV transcription than normal T cells and perhaps premalignant T lymphocytes as well.

To conduct these studies, we developed quantitative Reverse Transcription-Polymerase Chain Reactions (qRT-PCR) of the ZFERV *pol* and *env* genes (amplicon locations shown in Figure 2). As for qPCR, we used pooled normal T cells from WT WIK fish as our reference. Recall that even normal T lymphocytes highly express ZFERV transcripts [11], so these RNAs are already plentiful in the cells used as our standard. Results for *pol* and *env* were highly reproducible between two pooled T-cell samples from different groups of WT fish, and this value was arbitrarily assigned an expression level of 1 (Figure 7, lanes 1 and 2). By comparison, pooled pre-cancerous T cells from *hlk* fish exhibited approximately 6-fold and 7-fold enhanced *pol* and *env* transcription, respectively (lane 3, white bar). Likewise, pooled premalignant T cells from *MYC-ER* fish also had higher ZFERV transcripts (lane 9; *pol*: 9-fold increase,

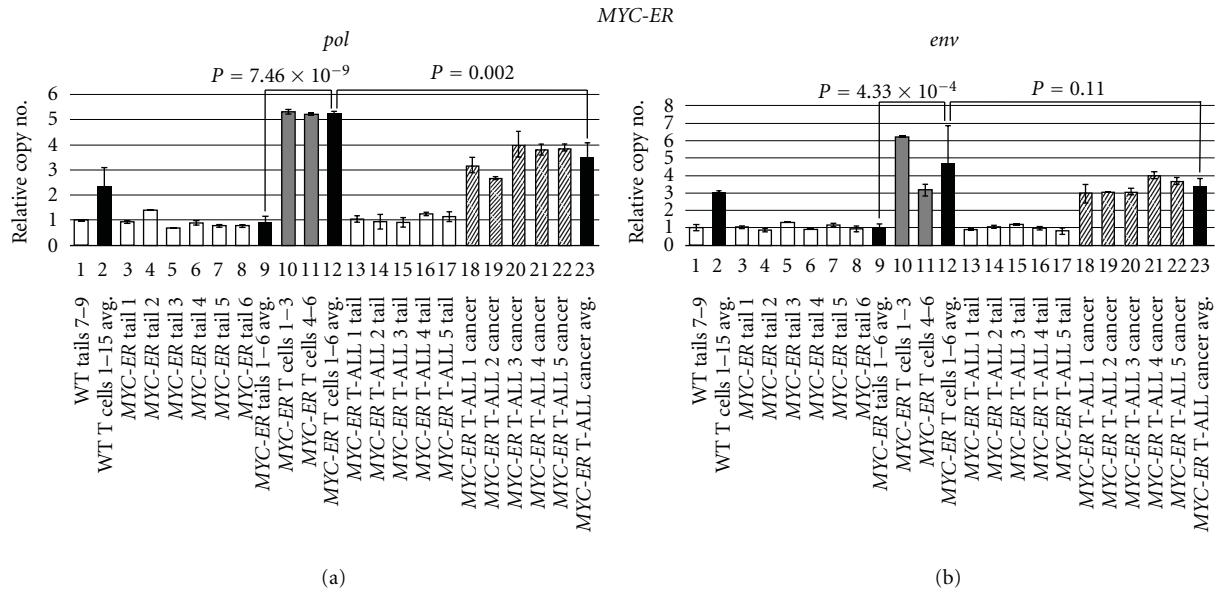

FIGURE 5: ZFERV amplifications in *MYC-ER* zebrafish. Phenotypically normal ($n = 6$) or T-ALL$^+$ ($n = 5$) *MYC-ER* fish were tested by qPCR of *pol* (a) and *env* (b). White bars display tail DNA from single normal (lanes 3–8) or diseased (lanes 13–17) fish. *MYC-ER* tails had similar copy number to each other (compare lanes 3–8 and 13–17) and to WT Tails 7–9 (lane 1). T cells pooled from groups of 3 normal *MYC-ER* fish (gray bars) showed ZFERV amplification; higher gains were seen in *MYC-ER* than WT T cells (lane 2 versus 12; P values 5.86×10^{-4} for *pol*, 0.15 for *env*). T cells showed 4- to 5-fold higher ZFERV copy than matched tails (lane 9 versus 12). Diagonally hatched bars depict amplifications in T-ALL cells from 5 *MYC-ER* fish (lanes 18–22). Cancer ZFERV levels were well above paired tails (compare lanes 13–17 to 18–22). Slightly lower gains were seen in cancerous than non-malignant *MYC-ER* T cells (lane 12 versus 23); this reached statistical confidence for *pol*, but not *env*. Other details are as listed in Figure 3's legend.

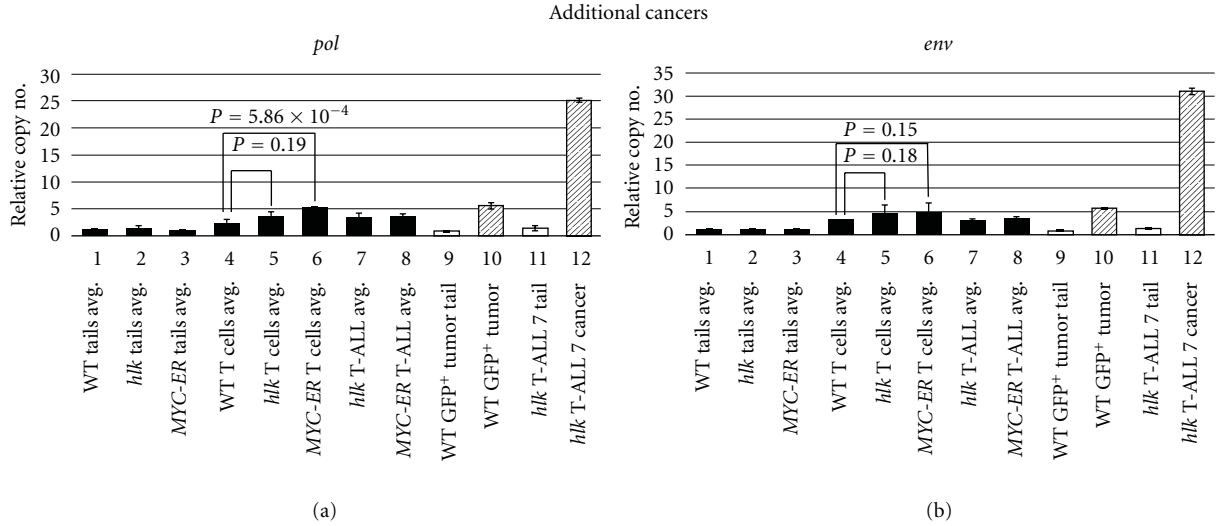

FIGURE 6: ZFERV amplifications in two other *D. rerio* T-cell cancers. One WT WIK fish spontaneously developed a GFP$^+$ thymic tumor and was tested by qPCR of *pol* (a) and *env* (b). Average copy numbers of other samples tested previously are shown as black bars. Germline ZFERV copy number in this fish (lane 9) was similar to the 15 WIK fish already examined (lane 1). This tumor showed 5.5-fold amplification (lane 10, diagonal bar), similar to non-malignant T cells and cancers from WT, *hlk*, and *MYC-ER* fish (lanes 4–8). One other *hlk* T-ALL exhibited high-level, 25- to 30-fold gains (lane 12), although its germline copy number (lane 11) was comparable to other fish (lanes 1–3).

env: 2.5-fold increase). So, while ZFERV genomic amplification did not differ impressively between WT and cancer-prone T cells (3- to 5-fold; Figure 6), expression of retroviral transcripts was more pronounced in T cells from both cancer-prone genotypes.

We also examined T-cell cancers from both lines ($n = 10$; 4 *hlk*, 6 *MYC-ER*). In *hlk* malignancies, all 4 cancers (lanes 4–7, gray bars) showed increased *pol* and *env* compared to normal T cells. One cancer (*hlk* T-ALL 4; lane 4) resembled premalignant *hlk* T cells in its transcriptional profile. This same tumor had also been tested by qPCR and showed comparable ZFERV amplification to non-malignant *hlk* T cells (see Figure 4, lanes 12 and 16). So, in this instance, copy number mirrored ZFERV expression. Three other *hlk* cancers

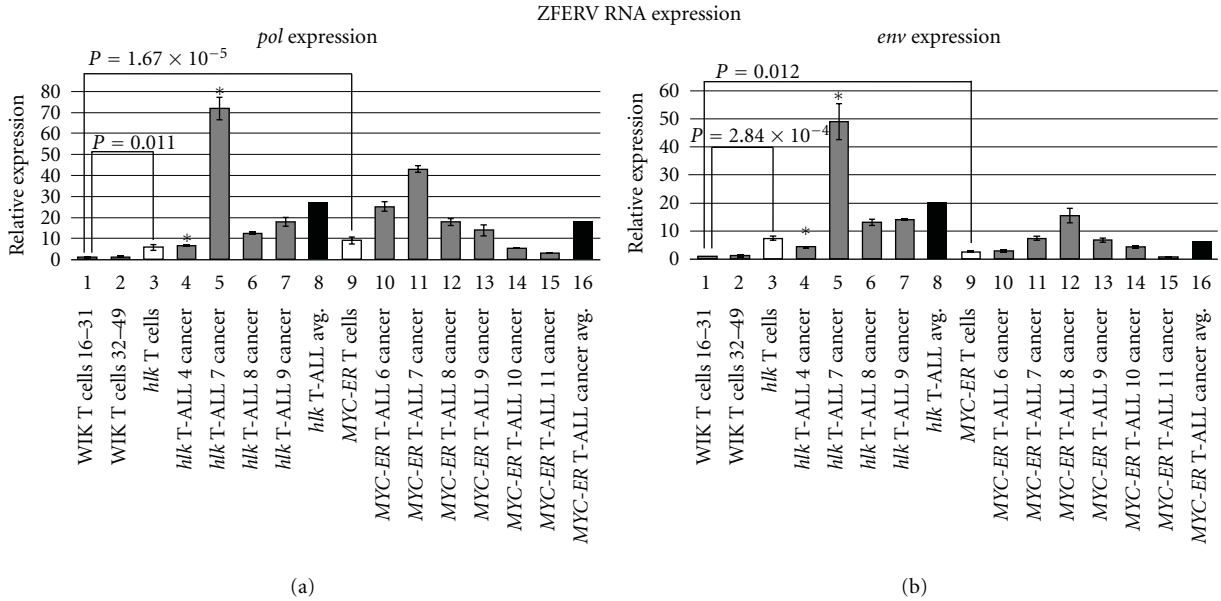

Figure 7: ZFERV gene expression by normal and abnormal *D. rerio* T cells. Total RNA was tested by qRT-PCR of the ZFERV *pol* (a) and *env* (b) genes. Normal T cell RNA pooled from WIK fish ($n = 16$ and 18; lanes 1, 2) were used as control, and the "WIK T cells 16–31" sample was arbitrarily set to an expression value = 1. Premalignant T lymphocytes from *hlk* ($n = 6$) and *MYC-ER* ($n = 3$) fish had higher expression than WT fish (white bars; lanes 3, 9), and this higher transcription reached statistical significance. Individual cancers from *hlk* ($n = 4$; lanes 4–7) and *MYC-ER* ($n = 6$; lanes 10–15) fish are depicted with gray bars. Cancer cells from these fish invariably showed higher *pol* and *env* transcripts than T cells from WT fish, and nearly always had elevated RNA expression relative to normal T cells from these same two lines. Mean expression of *pol* and *env* in malignant T cells (black bars; lanes 8, 16) exceeded both WT and premalignant T cell transcript levels. Two cancers highlighted by asterisks (*hlk* T-ALL 4, *hlk* T-ALL 7; lanes 4, 5) were also tested for genomic copy number by qPCR. The *hlk* T-ALL 4 cancer had ZFERV copy number similar to *hlk* premalignant T cells (see Figure 4), and its *pol* and *env* expression also resembled *hlk* T cells. Cells from the *hlk* T-ALL 7 sample had high-level genomic ZFERV gains (see Figure 6), and likewise demonstrated dramatically increased ZFERV transcription.

(Figure 7, lanes 5–7) had greater *pol* and *env* transcription than *hlk* premalignant T cells, with at least 2-fold increases in both transcripts. One of these (*hlk* T-ALL 7; lane 5) had markedly higher levels, with 13-fold *pol* upregulation and 7-fold higher *env* than non-malignant *hlk* T cells (lane 3). The *hlk* T-ALL 7 sample was also analyzed by qPCR and exhibited high copy gains (Figure 6, lane 12), providing a second example that correlated genomic copy number to ZFERV transcriptional activity. Overall, mean transcription was 5-fold greater for *pol* and 3-fold higher for *env* in *hlk* cancers than their pre-cancerous T lymphocytes (compare lane 3 versus 8), although the *hlk* T-ALL 7 cancer skews this result somewhat. That notwithstanding, every *hlk* cancer showed ≥4-fold upregulation of both transcripts relative to WT thymocytes, proving that higher ZFERV expression does coincide with malignancy.

Similar findings were also obtained in 6 *MYC-ER* cancers (lanes 10–15, gray bars). Although transcript levels varied in individual cancers, mean *pol* expression was 2-fold increased and *env* was 3-fold higher in all six malignancies compared to *MYC-ER* premalignant T cells (compare lane 9 versus 16). Expression of *pol* and *env* by the same tumor usually followed the same trend. However, some cancers did have discordant transcription of these two genes. Despite these disparities, *MYC-ER* cancers averaged 18- and 7-fold higher *pol* and *env*, respectively, than normal T cells from WT fish, further implicating ZFERV in zebrafish T-cell oncogenesis.

Though ZFERV copy number and transcriptional activity correlated in the two cancers where we evaluated both genomic and expression data, variation between *pol* and *env* in the same tumor requires another explanation. Unlike ZFERV *gag-pol*, which is thought to be transcribed as a single RNA, *pol* and *env* come from distinct transcripts [11]. Thus, these genes could be differentially regulated. In addition, other factors may impact overall ZFERV transcription. As noted previously, certain integration sites might foster retroviral activity. In addition, cancers with very high copy number might be expected to have commensurate RNA levels, and our limited data support this. Another potential factor regulating transcription pertains to normal patterns of ZFERV expression in T lymphocytes. While it is known that *D. rerio* T cells normally make ZFERV RNA ([11] and this work), it is not known if all T-lineage cells do, or rather if only some T lymphocyte developmental stages have active ZFERV. Since T-ALL can exhibit differentiation arrests at multiple maturational stages [13, 14], it is possible that individual cancers with differing arrest points might also demonstrate different ZFERV transcription patterns. Unfortunately, the lack of antibody reagents able to recognize zebrafish T cell surface markers currently limit testing of this latter hypothesis.

Despite these limitations, our findings indicate that both genomic amplification and transcription of ZFERV may impact normal *D. rerio* T-cell biology and oncogenesis. In

particular, our results bolster the notion that new retroviral integrations could be pathologic on the molecular level. Stably integrated retroviral elements are common in vertebrate genomes, with nearly 10% of the human genome comprised of ERVs or their derivatives [15]. However, most ERVs are inert due to their accrual of point mutations and partial deletions. ZFERV is atypical in that its genes apparently retain unmutated ORFs. Moreover, these genes are robustly transcribed by *D. rerio* T cells as verified by ISH, Northern blotting, and qRT-PCR ([11] and this paper). The abundance of ZFERV RNA in T lymphocytes is perhaps not surprising, as ZFERV's LTR was the most potent promoter among several transcriptional regulatory sequences assayed in a carp (*Cyprinus carpio*) epithelial cell line, including the oft-used CMV promoter [16].

Rather, ZFERV's apparent T-cell specificity may be the more intriguing finding. Shen and Steiner identified putative binding sites for the lymphoid transcription factors Ikaros and Tcf3 (E47) in the ZFERV LTR, but also for other factors (FOS/JUN, C/EBP, STAT, NF-κB, and others) that are more general activators of transcription [11]. Indeed, the sequencing of ZFERV-derived transcripts by EST projects from several other tissue types suggests that non-T-cells may transcribe ZFERV also [11]. Whether this finding reflects low-level ZFERV transcription by other cell types, or low-level T-cell contamination in these tissues, is not clear. In either case, the atypical persistence of intact ZFERV ORFs, and their transcriptional activity in zebrafish T cells, raises the question of whether ZFERV proteins might serve a functional purpose. Selective pressure would normally favor mutations disabling a potentially genotoxic retrovirus. Instead, we hypothesize that ZFERV may in fact serve some important biologic role, accounting for its paradoxical maintenance as an active retrovirus in the zebrafish genome.

ZFERV's apparent absence in the genomes of other *Danio* genera [11] implies that its entry into zebrafish is fairly recent in evolutionary terms, but ZFERV sequences have been identified from several different strains, suggesting that its integration is pervasive in the species. It is not known whether ZFERV is present in all *D. rerio*, and to our knowledge, this question has not been investigated. To date, the closest relative to ZFERV is an exogenous piscine retrovirus, SSSV. Curiously, this virus is linked to swim bladder leiomyosarcomas in Atlantic salmon, and like our results with ZFERV, these tumors show high copy number proviral SSSV integration [17].

Besides its close relation to SSSV, ZFERV also shares sequence conservation and similar genomic structure with gammaretroviridae of the murine leukemia virus (MLV) class [11, 17]. MLV-related retroviruses are known to be oncogenic by insertional mutagenesis [18], and the determinants governing their preferred integration sites have been the subject of intense scientific scrutiny [19–21]. Although an obvious ZFERV homologue has not been identified in humans, other MLV-related sequences have been detected in human cell lines. However, it appears that these retroviral sequences may have been acquired by human cells during xenografting into murine recipients or result from reagent contamination by murine DNA [22, 23]. In addition, a long ORF on human chromosome 14 bears high homology to ZFERV's *env*, and upstream sequences contain a short *gag-pol* element [24]. So, ZFERV-related retroviruses are evidently integrated in the human genome as well. Incorporating all these circumstantial data, it becomes plausible that ZFERV integrations—like SSSV and MLV—may not only be oncogenic in zebrafish, but might also have relevance for human biology in general.

4. Conclusions

Nearly a decade ago, Shen and Steiner discovered a zebrafish endogenous retrovirus, ZFERV, based on its high transcriptional activity in larval and adult *D. rerio* thymocytes [11]. Their work suggested that multiple copies of ZFERV existed in the zebrafish genome, and since that time, the loci where ZFERV resides still have not been definitively assigned. These difficulties are probably attributable to the fact that this multicopy locus may vary in copy number and genomic positioning in different fish. Amidst this backdrop, we have found that ZFERV copy number is increased still further in every *D. rerio* T-cell malignancy we examined from 4 different genetic lines.

Somewhat surprisingly, our results demonstrate that ZFERV amplification is not unique to cancerous T cells. Rather, copy number gains also occur in T lymphocytes of WT *D. rerio*, the same cells where ZFERV transcription was first identified. Moreover, ZFERV copy number appears to be fairly consistent amongst normal, premalignant, and malignant T cells (Figure 6, lanes 4–8), although individual cancers can occasionally show even higher levels of ZFERV in their genomes. It is possible that individual normal T cells have similar variability in ZFERV copy number, but this has not been experimentally addressed.

As seen with genomic amplifications, ZFERV transcription occurs within normal, pre-cancerous, and neoplastic T cells. Our results suggest that expression of ZFERV RNAs is higher in cancer samples, but we do not recognize a consistent trend from one cancer to the next. Nonetheless, these commonalties between normal and malignant T lymphocytes imply that ZFERV activation and amplification may be a normal feature of zebrafish T cell biology, with no pathologic consequence. Still, ZFERV's abundant transcription, apparently functional ORFs, and ability to undergo genomic amplification all allude to its oncogenic potential. Compounded with mutations like *hlk*, *srk*, and *otg* that confer malignancy predisposition, ZFERV may help promote T-cell transformation. Given that the closest phylogenetic relatives of ZFERV are an exogenous piscine retrovirus linked to sarcomagenesis and MLV-class retroviruses that are leukemogenic via genomic integration, it is tempting to speculate that ZFERV may contribute to cellular immortalization by similar mechanisms.

Certainly, ZFERV integrations in crucial genomic sites could have transformative properties. For example, integration within a tumor suppressor gene might render it unable to generate its normal protein product, thereby ablating function. Conversely, integrations into the promoter or

enhancer regions of proto-oncogenes might augment their transcription. Since ZFERV appears to be specifically and highly expressed by thymocytes, this scenario could be analogous to the translocation of proto-oncogenes into the T-cell receptor loci, which are well described in T-ALL [25, 26]. However, proof of this hypothesis will require identification of somatically acquired ZFERV integrations at these genomic sites. At this point, the possibility that ZFERV amplifications may contribute to T-cell oncogenesis remains an open question that will require further investigation to resolve decisively.

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Review Article

Hydrogen Peroxide in Inflammation: Messenger, Guide, and Assassin

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Starting as a model for developmental genetics, embryology, and organogenesis, the zebrafish has become increasingly popular as a model organism for numerous areas of biology and biomedicine over the last decades. Within haematology, this includes studies on blood cell development and function and the intricate regulatory mechanisms within vertebrate immunity. Here, we review recent studies on the immediate mechanisms mounting an inflammatory response by *in vivo* analyses using the zebrafish. These recently revealed novel roles of the reactive oxygen species hydrogen peroxide that have changed our view on the initiation of a granulocytic inflammatory response.

1. Introduction

The innate immune system comprises the cells and mechanisms that defend the host from infection by other organisms or damage to tissue integrity, in a nonspecific manner. This means that the cells of the innate system recognise and respond to pathogens and trauma in a generic way, but unlike the adaptive immune system, it does not confer long-lasting or protective immunity to the host. The innate immune system provides an immediate defence. A typical vertebrate immune response depends on the orchestrated motility and activity of various haematopoietic compartments and their interactions that ultimately control the magnitude of the response [1–3]. Inflammation is one of the first responses of the immune system to infection or irritation. Stimulated by factors released from injured cells, it serves to establish a physical barrier against the spread of infection. This further promotes healing of any damaged tissue following the clearance of pathogens or cell debris. Molecules produced during inflammation sensitise pain receptors, cause localised vasodilatation of blood vessels, and attract phagocytes, especially neutrophils and macrophages, which then trigger other parts of the immune system.

Failure to initiate a response allows uncontrolled proliferation of invading microorganisms and severe tissue damage that may become fatal. Failure to resolve an immune response can also cause severe tissue damage, due to persistent degranulation, and may lead to chronic inflammation, which ceases to be beneficial to the host. Overall, inflammation is now recognised as a central feature of prevalent pathologies, such as atherosclerosis, cancer, asthma, thyroiditis, inflammatory bowel disease, autoimmune disease, as well as Alzheimer's and Parkinson's disease [4–6]. Hence, the regulation of an inflammatory response is an active field of research. New players or novel functions of old players continue to be identified and we are only beginning to understand their specific function at the corresponding level during inflammation. Hydrogen peroxide is an example of a molecule with a long known function for pathogen clearance in inflammation. Here, we discuss how recent work using the zebrafish model has revealed a pivotal role of hydrogen peroxide in mounting an inflammatory response.

2. Cellular Lifecycle of Hydrogen Peroxide

Hydrogen peroxide belongs to a group of chemically reactive molecules known as reactive oxygen species (ROS) that arise through oxidative metabolism. ROS comprise oxygen derived small molecules such as the oxygen radicals:

superoxide, hydroxyl, peroxyl, and alkoxyl; or the nonradicals: hypochlorous acid, ozone, singlet oxygen, and the current topic in focus, hydrogen peroxide [7]. ROS generation can either occur as a by-product of cellular metabolism (e.g., in mitochondria through autoxidation of respiratory chain components) or it can be created by enzymes with the primary function of ROS generation [8]. Enzymes capable of rapidly increasing local H_2O_2 levels include the family of NADPH oxidases [7] and other oxidases such as xanthine oxidase [9] and 5-lipoxygenase [10]. The mammalian NADPH oxidase family encompasses 7 members, which are NOX1-5 and DUOX1-2. To date, a single isoform of *duox* and four *nox* genes (*nox1, 2, 4, 5*) have been identified in the zebrafish genome [11]. Each member is capable of converting NADPH to $NADP^+$ and then transporting the freed electrons across membranes. DUOX enzymes are capable of direct hydrogen peroxide production, while NOXes1-5 produce superoxide, which is rapidly converted to H_2O_2 by a separate superoxide dismutase or occurs spontaneously [12]. H_2O_2 may subsequently be utilised by peroxidase, such as thyroperoxidase, to produce thyroid hormones or myeloperoxidase and lactoperoxidase to generate more potent ROS. However, if not consumed, high concentrations of H_2O_2 may result in DNA damage and modifications of proteins, lipids and other molecules [13]. Thus, to avoid H_2O_2-mediated deleterious effects, excess H_2O_2 is usually rapidly catalysed or reduced by various antioxidant enzymes: such as glutathione peroxidase and catalase [14].

3. Functional Activities of H_2O_2

H_2O_2 is also involved in many regulatory cellular events including the activation of transcription factors, cell proliferation, and apoptosis [8]. H_2O_2 produced from the mitochondrial electron transport chain has been shown to play a role in haematopoietic cell differentiation and cell division in flies [15, 16]. NADPH oxidase generated H_2O_2can affect cardiac differentiation [17], vascularisation [18], and angiogenesis [19]. In targeting cysteine and methionine residues of protein kinases and phosphatases, H_2O_2 is capable of modulating a number of principal signalling cascades including ERK, JNK, p38, MAPK, and PI3K/Akt [20, 21].

3.1. Inflammation-Related Functions

3.1.1. Respiratory Burst. The classical physiological role attributed to H_2O_2 is its capability to induce bacterial killing [12]. NOX2 is the enzyme responsible for phagocyte respiratory burst responses and is expressed in neutrophils, eosinophils, monocytes/macrophages, as well as nonphagocytic cells such as fibroblasts, cardiomyocytes, haematopoietic stem cells, and endothelial cells [7]. Under resting conditions neutrophil NOX2 resides in secondary granules, which upon activation of neutrophils fuse with phagosomal as well as plasma membranes [22].

The NADPH-oxidase-mediated respiratory burst response of neutrophils generates two superoxide anions by transporting two electrons from one NADPH across the membrane to the extracellular or intra-phagosomal space. Superoxide is further converted into hydrogen peroxide either through spontaneous dismutation, which involves the consumption of two protons, or facilitated by the catalytic activity of superoxide dismutase. Hydrogen peroxide alone and in conjunction with the amplification activity of myeloperoxidase (MPO) is responsible for bacterial killing [23, 24]. MPO, which is abundantly present in phagocyte granules, catalyses the conversion of halides and pseudohalides such as Cl^-, I^-, Br^-, and SCN^- to form hypohalous acids or pseudohypohalous acids. HOCl, however, is the primary MPO product in neutrophils responsible for bacterial killing.

3.1.2. Hydrogen Peroxide Mounting an Inflammatory Response. Recent advances accomplished by utilising the model organism zebrafish greatly expanded our view of H_2O_2 mediated cellular activities. The optical transparency of zebrafish larvae offers the unique advantage of real-time monitoring an immune response in a whole animal context. This is in contrast to *in vitro* studies and/or end-point analyses of stained tissues. Additionally, a recently developed genetically encoded H_2O_2 sensor provided an elegant solution for investigating the role of hydrogen peroxide dynamics during an immune response *in vivo* [25].

The previous view on the critical mechanisms in immediate inflammation focused on the activity of damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). Tissue damage results in the release of intracellular DAMPs usually hidden from the immune system (i.e., ATP, uric acid, lipids, DNA, nuclear proteins) or extracellular DAMPs released through degradation of extracellular matrix upon tissue injury (i.e., hyaluronan, byglycan, heparan sulfate). The receiving cell senses these signals through 5 different types of pattern recognition receptors (PRRs). Activation of these receptors in turn activates downstream NFkB, MAPK, or type I interferon-signalling pathways that are important for inflammatory and antimicrobial responses. The significance of DAMPs, PAMPs, and PRRs is comprehensively reviewed elsewhere [26, 27]. However, the mechanisms for immediate immune cell recruitment were not well defined.

Recently, Niethammer et al. described for the first time that wounded epithelium of zebrafish larvae produces a tissue-scale gradient of H_2O_2 mediating leukocyte recruitment [28]. This finding was in contrast to the prevalent view that leukocytes undergoing an oxidative burst response were the only source of H_2O_2 at a site of trauma or infection [29]. The authors employed the genetically encoded ratiometric HyPer sensor to visualise H_2O_2 *in vivo* and in real time. HyPer consists of the bacterial H_2O_2-sensitive transcription factor, OxyR, fused to a circularly permutated yellow fluorescent protein (YFP). Cysteine oxidation of OxyR induces a conformational change in the YFP that increases emission excited at 500 nm and decreases emission excited at 420 nm. This change is rapidly reversible within the reducing cytoplasmic environment, which allows dynamic monitoring of the intracellular hydrogen peroxide concentration [30].

Tailfin transection on zebrafish larvae induced a rapid increase in H_2O_2 levels extending approximately 100–200 μm from the wound margin as a decreasing concentration gradient. Furthermore, gradient formation preceded leukocyte arrival at the scene and H_2O_2 levels started to decrease again with accumulation of immune cells. Generation of the gradient as well as leukocyte recruitment was dependent on the activity of Duox in epithelial cells. Both, genetic knockdown of Duox and chemical inhibition of oxidase activity abolished gradient formation and significantly decreased absolute numbers of leukocytes at the wound margin, without affecting general cellular motility. These findings were corroborated by a study in drosophila focusing on prioritising competing signals by migrating macrophages [31] emphasising the crucial role of the tissue scale gradient of H_2O_2 for leukocyte attraction.

A study, also using zebrafish larvae, demonstrated that newly oncogene-transformed cells and their neighbours attracted leukocytes through H_2O_2 signalling. Utilising the H_2O_2-indicating dye, acetyl-pentafluorobenzene sulphonyl fluorescein, and 5,5-dimethyl-l-pyrroline N-oxide (DMPO) that reports a history of ROS exposure, it was shown that H_2O_2 was stochastically and momentarily produced around V12RAS expressing cells in the epidermis. Like wounded epithelial cells, transformed cells generated H_2O_2 in a Duox dependent manner, highlighting parallels between oncogene-transformed cells and mechanical induced injury initiation of the host inflammatory response [32].

3.1.3. Hydrogen Peroxide as a Signalling Molecule in Inflammation. Functional roles of H_2O_2 during inflammation have been observed previously. Mechanistically, hydrogen peroxide can modulate protein function by reversible chemical modification of protein thiols, which can result in conformational changes affecting DNA binding, enzymatic activity, multimerisation, or protein complex formation. For example, the NFkB/Rel family, key regulatory molecules in the transcription of many genes involved in inflammation, is a well-known redox-sensitive transcription factor family [33]. H_2O_2-induced activation of NFkB, which includes tyrosine phosphorylation of IkB and activation of IKK by H_2O_2 has been reported [34, 35]. Moreover, H_2O_2 can activate the release of high mobility group 1 protein from macrophages resulting in amplification of proinflammatory stimuli [36] or modulate leukocyte adhesion molecule expression and leukocyte endothelial adhesion [29]. VCAM-1, an endothelial scaffold on which leukocytes migrate, can activate signals in endothelial cells required for VCAM-1-dependent leukocyte migration. Leukocyte binding to VCAM-1 stimulates NOX2 in endothelial cells, resulting in the generation of H_2O_2, which locally activates matrix metalloproteinases (MMPs). These MMPs in turn degrade matrix and endothelial cell surface receptors in cell junctions facilitating leukocyte transendothelial migration [37, 38].

These examples show how H_2O_2 can act as an intracellular or local signalling molecule but long-distance intercellular mechanisms of H_2O_2-mediated leukocyte recruitment were less well defined.

The open question of how leukocytes may receive the signal to initiate directional migration was recently addressed in another elegant study using the zebrafish model by Yoo et al. [39]. They have identified the SRC family kinase (SFK) Lyn as a redox sensor in neutrophils that detects hydrogen peroxide emanating from wounds and guiding their migration. Yoo and colleagues were able to provide direct evidence for punctate SFK activation at the leading edge of neutrophils in response to wounding. Through the knockdown of Duox, which is responsible for H_2O_2 production at the wound margin, they have explored the role of H_2O_2 in SFK activation. Duox knockdown prevented SFK phosphorylation indicating that activation of neutrophil SFKs may be dependent on the presence of hydrogen peroxide levels at wounds. Further evidence suggesting that SFKs can act as a redox sensor was provided by utilisation of SFK inhibitors that resulted in impairment of early neutrophil accumulation, while having no effect on epithelial hydrogen peroxide bursts [39].

Profiling SFK family members in zebrafish myeloid cells identified the Lyn kinase as a promising candidate acting as the redox sensor in neutrophils and macrophages. Morpholino knockdown of Lyn impaired directional migration of neutrophils to a tailfin wound in zebrafish larvae.

Further *in vitro* investigation revealed that hydrogen peroxide directly activates Lyn through the oxidation of Cys466, leading to downstream signalling, for example, Erk activation. This *in vitro* evidence was elegantly confirmed *in vivo* using a combination of genetic knockdown of Lyn and neutrophil-specific transgenic reconstitution of a Cys466 mutant or wild-type Lyn-GFP fusion.

In conclusion, these two sophisticated studies demonstrated a novel role of H_2O_2 as mediator of immediate inflammation and revealed aspects of the mechanisms resulting in leukocyte recruitment to a site of trauma (Figure 1). Evidence is accumulating that H_2O_2 signalling to phagocytes is a widely conserved mechanism present not only in zebrafish [28, 32, 39] but also flies [31] and mammals [39, 40].

4. Outlook

The discovery of a new biological mechanism opens up a new line of research and poses numerous new questions to address. The most obvious being: How is Duox activated in epithelial cells upon wounding and how is the H_2O_2 gradient resolved? One hypothesis would place calcium as the immediate injury signal to the wounded cell in order to produce hydrogen peroxide through Duox. Physical disruption of plasma membranes results in an uncontrolled influx of calcium [41]. Giving credence to this hypothesis, evidence exists showing that DUOX activation by calcium regulates H_2O_2 generation [42].

In order to avoid excess tissue damage and persistent granulocyte recruitment/retention, the presence of the hydrogen peroxide gradient must be tightly regulated. Regulation could occur on the enzymatic level in terms of H_2O_2 production as well as on the molecular level in terms of H_2O_2

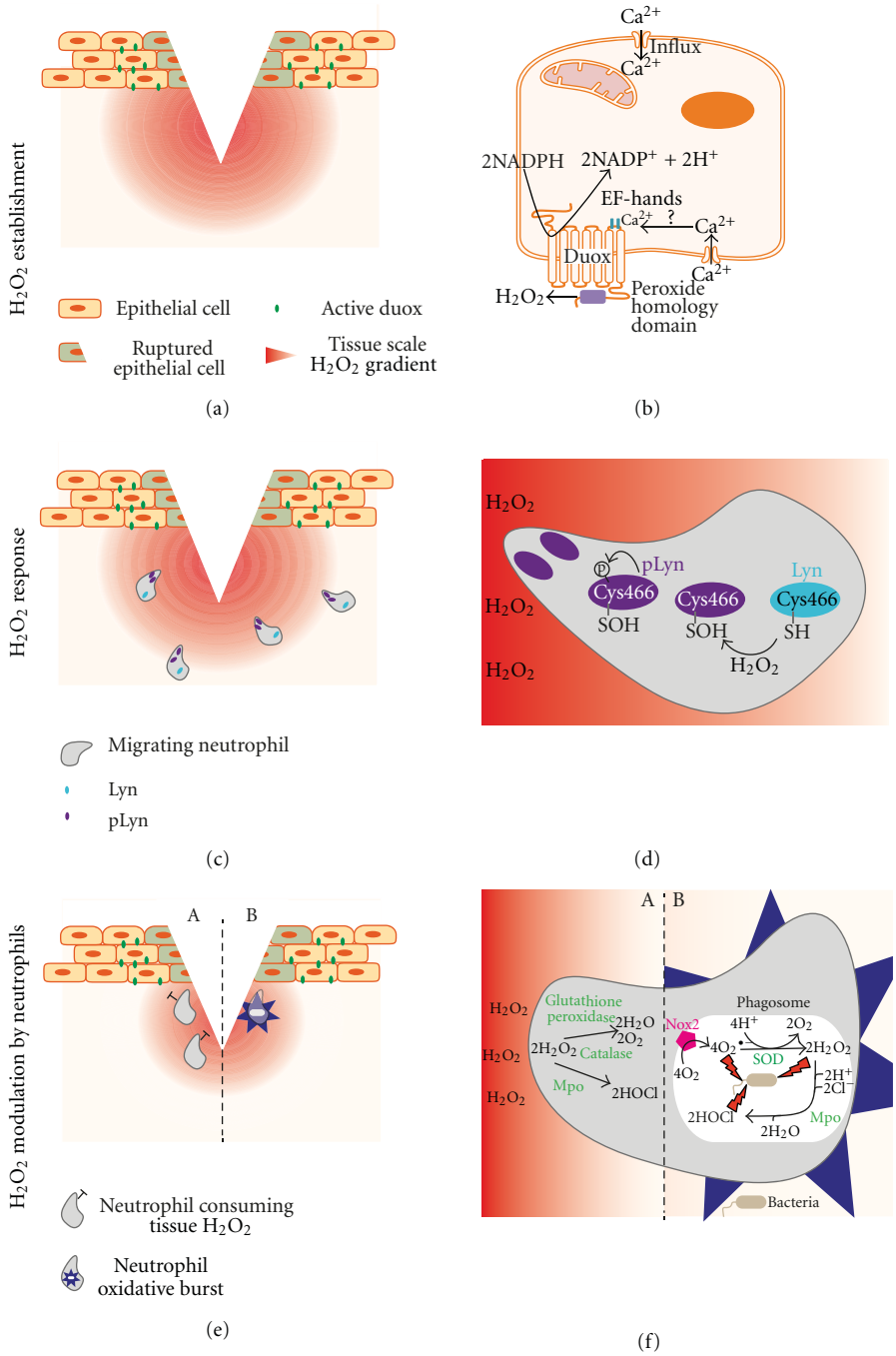

Figure 1: The role of hydrogen peroxide during the inflammatory response. (a) Upon tissue injury/trauma, epithelial cells adjacent to damaged cells activate the NADPH oxidase, Duox. Duox generates and establishes a localised tissue scale gradient of hydrogen peroxide, (b) Potential cellular events that result in Duox activation in epithelial cells. Disruption of epithelial cell membranes by mechanical trauma could lead to an increased influx of calcium in adjacent cells. Calcium binding to the EF-hand domain of Duox (residing in plasma membranes of epithelial cells), may initiate generation of hydrogen peroxide. (c) A tissue scale gradient of hydrogen peroxide acts as the first attraction signal for leukocytes. (d) Neutrophils sense hydrogen peroxide emanating from the wound partly through Lyn, a Src family kinase. Oxidation of Cys466 activates Lyn, resulting in autophosphorylation (pLyn) and punctate appearance of pLyn at the neutrophil leading edge is observed. (e) At the site of injury, neutrophils may alter hydrogen peroxide levels, both by consuming epithelial-derived hydrogen peroxide (A) or by local production of hydrogen peroxide through oxidative bursts (B). (f) Antioxidants, such as glutathione peroxidase and catalase could catalyse the decomposition of hydrogen peroxide into oxygen and water, while myeloperoxidase (Mpo) may consume hydrogen peroxide to produce hypochlorous acid (A). Neutrophils are equipped with multiple mechanisms to kill foreign organisms, one of them being the generation of ROS. Upon activation, phagosomal Nox2 generates superoxide, which is further converted into hydrogen peroxide by superoxide dismutase (SOD). Hydrogen peroxide alone and in conjunction with hypochlorous acid, generated by myeloperoxidase and other ROS exert bactericidal functions (B).

stability. Oxidase activity results in membrane depolarisation due to the electrogenic properties of the enzymes to the point of NADPH oxidase inhibition. Prolonged H_2O_2 production depletes the NADPH pools, which may automatically result in cessation of H_2O_2 generation. Alternatively or in addition, neutrophil MPO could be responsible for the decrease in hydrogen peroxide levels upon arrival at the wound [24].

This mechanism suggests new approaches to therapeutically modulate both the onset of the cellular inflammatory response and its resolution, particularly as it involves a small, relatively unstable signalling molecule and is dependent on multiple enzymatic steps amenable to pharmacologic intervention.

Authors' Contribution

C. Wittman and P. Chockley contributed equally to this paper.

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