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The Zebrafish as a Model Organism to Study Development of the Immune System

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Abbreviations

WISH	whole mount *in situ* hybridization
hpf	hour post fertilization
dpf	day post fertilization
ENU	ethylnitrosourea
NITR	Novel Immune-Type Receptor
*	indicates movies, to be found at: http://www-alt.pasteur.fr/~herbomel/

I. Introduction

Over the past 25 years the field of immunology has greatly benefited from the advances in molecular biology. For example two seminal techniques, Southern blotting and DNA sequencing were employed shortly after their discovery by S. Tonegawa (Tonegawa, 1983) and others to unravel one of the great secrets of biology that had mystified legions of immunologists: the generation of somatic diversity in antigen receptors. Transgenic mice (Le Meur *et al.*, 1985; Yamamura *et al.*, 1985) and targeted gene inactivation by homologous recombination (Zijlstra *et al.*, 1989) or conditional inactivation (Gu *et al.*, 1994; reviewed in Rajewsky *et al.*, 1996) were successfully employed for analysis of genes with pivotal roles in the mammalian immune system. Furthermore, the study of mice and patients with immune deficiencies has greatly enhanced our understanding of the molecular processes involved in developmental aspects of the immune system (reviewed

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in Fischer and Malissen, 1998; Fischer, 2001). However, mammalian immunology is handicapped by the fact that the earliest steps in the ontogeny of the immune system occur in-utero, and are therefore difficult to study in an *in vivo* system. Prominent among these early steps are thymic organogenesis and T-cell lymphopoiesis, crucial interdependent processes that establish a functional vertebrate immune system. Current understanding of vertebrate thymic development during embryogenesis remains incomplete and would benefit from novel approaches. Despite much innovative work, immunologists have been hesitant to use novel model systems to study these earliest steps in immune ontogeny.

In this chapter, we propose to use the zebrafish (*Danio rerio*) as a model organism for immunology as an alternative to study humans or mice. Studies in several species of teleosts have demonstrated presence of all major blood lineages. As teleosts do not have bone marrow, blood cells are produced in the kidney (Willett *et al*., 1999). The immune system of teleosts has been previously explored in the channel catfish (*Ictalurus punctatus*), in particular by the groups of L. W. Clem and N. W. Miller (Miller *et al*., 1985, 1987; Ellsaesser *et al*., 1988; Shen *et al*., 2002) and in rainbow trout (*Oncorhynchus mykiss*) by J. D. Hansen (Hansen, 1997; Hansen and Strassburger, 2000; Hansen and La Patra, 2002) and others. Given their large size and abundance of specific cell types and tissues, catfish and trout have become extensively used for cell biologic studies involved in infection and immunity. These studies established that teleosts have T cells, B cells, antigen presenting cells, and natural killer cells and can mount antibody-mediated and cellular immune responses to infections. Recently, efforts were initiated to establish the rainbow trout as a genomic model "to address areas such as the evolution of the immune system and duplicated genes" (Thorgaard *et al*., 2002).

Despite its small size and comparative paucity of molecular reagents for immunological research, the zebrafish has many advantages over the other vertebrates in studying the immune system. Several lines of evidence suggest that zebrafish have a complete set of genes required for the establishment of a fully functional adaptive immune system. We and others have cloned genes involved in early T-cell development, including *ikaros*, *GATA-3*, *Rag-1*, *Rag-2*, and *lck* (Willett *et al*., 1997, 2001; Trede *et al*., 2001). Recently, the genomic area encompassing the zebrafish TCR alpha locus has been fully sequenced (C.T.A. *et al*., unpublished). Sequence analysis revealed a similar organization of the variable (V) segments to those in humans and mice. Furthermore, the high degree of conservation of the heptamer and nonamer sequences in all species of jawed vertebrates as well as the length of the spacer in the recombination signal sequences strongly suggests conservation of the mechanism of the V(D)J recombination machinery. Zebrafish have a thymic organ, which remains bilateral in the third pharyngeal pouch (Willett *et al*.,

1999; Lam *et al*., 2002). Expression of early T cell genes can be observed in the bilateral thymi starting on day 3 post fertilization (pf) (Willett *et al*., 1997, 1999; Trede and Zon, 1998; Schorpp *et al*., 2000; Trede *et al*., 2001; Lam *et al*., 2002). Zebrafish also have B cells as evidenced by expression of VH genes in the kidney, the bone marrow equivalent of the zebrafish (Danilova and Steiner, 2002). Recently, zebrafish B cells have been reported to first arise in the pancreas (Danilova and Steiner, 2002). Genes encoding class I and II major histocompatibility (MHC) molecules have been isolated from the zebrafish genome (Ono *et al*., 1992; Sultmann *et al*., 1993; Takeuchi *et al*., 1995) and the presence of macrophages has been documented by whole mount *in situ* hybridization (WISH) and by differential interference contrast (DIC) video microscopy (Herbomel *et al*., 1999, 2001). Taken together, these data strongly suggest that zebrafish can mount innate and adaptive immune responses to pathogens much like higher vertebrates.

The zebrafish provides a unique vertebrate model system for the analysis of developmental processes due to the transparency of the larvae, the short generation time and the ease with which mutations can be created and detected by various types of screens. Standard ethylnitrosourea (ENU)-based large-scale screens have identified a large number of mutants affecting early development and organogenesis (Haffter *et al*., 1996). Gynogenetic diploid embryos can be obtained thus permitting the expeditious detection of heterozygous individuals (Beattie *et al*., 1999). Zebrafish produce a large number of embryos and can be mated every week, facilitating the swift accumulation of meiotic recombinants for the purpose of positional cloning of mutant genes.

Several tools are available for mapping and positional cloning in the zebrafish. These include a dense map of sequence-specific length polymorphisms (SSLP), an increasing number of expressed sequence tags (ESTs) mapped on genetic and radiation hybrid panels, and a variety of genomic libraries. Furthermore, the zebrafish genome has been sequenced in its entirety and a high degree of synteny to the human and pufferfish (*Fugu rubripes*) genomes has become apparent. These reagents make the zebrafish an extremely versatile genetic system for the generation of mutant phenotypes and cloning of the corresponding genes. Given these advantages the zebrafish is an excellent genetic system to study vertebrate immunology, and to define novel factors that participate in the development of the immune system.

Zebrafish researchers receive frequent inquiries from biologists in various fields, including immunology, because during homology, searches of the gene of interest, zebrafish ESTs are found to be highly represented in the Genbank database. A number of recent publications have promoted the zebrafish as a powerful vertebrate model organism where, for example, transient gene

inactivation using morpholinos can be done speedily (see special issue of Genesis, volume 30, July 2001). This raises the expectation of many researchers outside the zebrafish field that quick answers can be found for their biologic questions using this model organism.

In this chapter we intend to introduce the immunologically inclined reader to the structural background and developmental aspects of the zebrafish immune system. We propose that the zebrafish system offers many opportunities to explore vertebrate immunology. For example, forward genetic and expression screens can uncover novel genes required for immune cell development and function. We also point to the limitations of the zebrafish system, where relatively few traditional reagents (such as specific antibodies directed against surface molecules) are available, and where functional properties of the various immune cells are at the early stages of investigation. Furthermore, if one considers that the entire kidney (the bone marrow equivalent of the zebrafish) contains only about 10^6 cells, 40% of which are mature red cells, the number of cells of a particular hematopoietic lineage that can be obtained per fish is rather limited. However, the ready availability of a large number of fish can circumvent this problem.

This chapter provides an overview of the current knowledge accumulated—much of it still unpublished—in the following aspects of zebrafish immunology.

Section II introduces fish innate immunity with an emphasis on the early origin of effector cells. Here we point to the power of DIC video microscopy in determining cell trafficking and behaviors to gain insight into potential functions of early macrophages. A separate subsection (Section II.C) is devoted to a novel family of immune type receptors (NITRs), which have been characterized extensively in zebrafish. This exciting novel finding has multiple implications, which are discussed in detail. The origins of adaptive immunity with an emphasis on primary and secondary lymphoid organs in fishes, as well as their antigen receptors are outlined in Section III. This section, which also treats the specific zebrafish immune system, points to several immunologic peculiarities, such as absence of lymph nodes and conventional class switching encountered in fishes. There is a paucity of reagents available for the study of zebrafish immune cells. For example, antibodies directed to immune surface receptors, which have been generated against phylogenetically related catfish molecules, do not cross-react in zebrafish and multiple attempts at raising antibodies against zebrafish blood cells have failed in our laboratories. Section IV discusses these shortcomings and offers possible alternative imaging and cell isolation techniques. A detailed account of the various types of mutagenesis screens that can be accomplished in zebrafish to obtain mutant phenotypes is the subject of Section V, followed by a description of lymphoid screens carried out to date (Section VI). The emerging field of reverse genetic

approaches in zebrafish, which allow for transient gene knockdowns as well as permanent gene inactivation, is exposed in Section VII. An alternative screening method combining the power of genomics and early development of the zebrafish to identify genes involved in a particular biologic process is detailed in Section VIII. Here, cDNA libraries from various stages of development, or from specific blood cell lineages, can be used to identify genes expressed in the tissue of interest throughout early development. Genomic analysis exploring syntenic relationships and functional assays involving gene knockdown experiments can make this approach a powerful tool for detecting immune genes and probing their function. Finally we provide a draft of a hands-on manual of how to proceed as a newcomer in the field of zebrafish genomics with a focused interest in mind (Section IX).

As zebrafish immunology is still in its infancy and published literature on this subject to date remains rather scant, we include in this chapter a number of unpublished results to demonstrate the power and potential of the zebrafish system. We hope that this contribution will entice stimulating discussions and will be instrumental in forging fruitful collaborations between established immunologists and scientists in the emerging field of zebrafish immunology.

II. Innate Immunity of Teleosts

A. The Components of Innate Immunity of Bony Fish

1. Leukocytes

Teleosts have monocytes, granulocytes and tissue macrophages. Monocytes and macrophages appear similar to those of mammals in terms of ultrastructure and cytochemistry, and phagocytic and secretory abilities (Secombes and Fletcher, 1992; Beattie *et al*., 1999; Neumann *et al*., 2001). In addition, fish have pigmented macrophages, called melanomacrophages, that are found as aggregates in lymphoid tissues (Herraez and Zapata, 1991). On the other hand, there has been much debate about granulocyte typology. The classes of granulocytes that could be discerned seem to vary among different fish species, and establishing their correspondence to mammalian types is not straightforward, partly because of insufficient functional studies (Rowley *et al*., 1988; Press *et al*., 1998). Among cyprinids, the taxon to which zebrafish belong, two species have been mainly studied, carp and goldfish (Press *et al*., 1998; Barreda *et al*., 2000). In both, and in zebrafish (Jagadeeswaran *et al*., 1999; Bennett *et al*., 2001; Lieschke *et al*., 2001), the head kidney, blood and spleen were found to contain neutrophilic/heterophilic and eosinophilic granulocytes. Neutrophils (>90% of blood granulocytes) have an eccentric,

pluri-lobed nucleus, and two kinds of granules: many cigar-shaped granules, ca. 0.4 μm in length, the mature form of which have a crystalline, striated axial core structure, and less numerous, round and homogeneous granules. Eosinophils have an eccentric nucleus attached to the plasma membrane, and are packed with larger, roughly spherical granules (0.7 μm mean diameter). Basophils were not detected in zebrafish, but were found in carp, while in goldfish their presence has been disputed (Rowley *et al*., 1988).

Tissue mast cells have been detected in various fish species including cyprinids (Reite, 1998), but not yet in zebrafish (Lieschke *et al*., 2001). In the striped bass (perciforms) they were shown to produce potent broad-spectrum peptide antibiotics called piscidins (Silphaduang and Noga, 2001).

2. *Complement*

Fish have a highly developed complement system, with the three known—lectin-dependent, alternative, and classical (antibody-dependent)—pathways of complement C3 activation, followed by the common lytic pathway leading to self-assembly of the membrane attack complex. Most of the 25 proteins of the system found in mammals arose by successive duplications from four primordial genes, and this led to the emergence of distinct activation pathways (Zarkadis *et al*., 2001). Some of these duplications happened after the fish/tetrapod divergence. Thus, fish have no factor B/C2 dichotomy, but a factor B that functions in both the alternative and the classical pathways. On the other hand, fish have developed multiple isoforms of C3, the central factor of the system, and of factor B. These isoforms are the products of different genes, and differ in structure and functional characteristics. Furthermore the genes are quite polymorphic, and can differ vastly among fish species. Therefore, several authors have suggested that the low-affinity antibody response in fish (see Section III.D) may have been compensated by the diversification of innate immune mechanisms such as the complement system. In support of this notion, whereas classical pathway titers in fish serum are similar to those found in mammals, alternative pathway titers are 5–10 times higher in fish (Zarkadis *et al*., 2001).

The number of complement genes differs among the various fish species. Carp have five C3 and three factor B genes, the gilthead sea bream, a diploid fish, has five C3, while zebrafish have three C3 and two factor B genes (Gongora *et al*., 1998; Nakao *et al*., 2000; Zarkadis *et al*., 2001). The C3 isoforms display often widely different affinities for various microbial cell walls. Strikingly, in the carp, a histidine residue at the catalytic site of C3, so far thought to be required for the covalent binding of C3 to biological surfaces (the key-point of the whole system), is substituted in three out of the five C3 isoforms by a serine or a glutamine. The serine-substituted isoform actually

showed a three-fold increased hemolytic activity (Nakao *et al*., 2000). Complement C3-dependent phagocytosis has been described in several fish species (Secombes and Fletcher, 1992), but a phagocyte CR3 receptor has not yet been characterized at the molecular level.

3. Inflammatory Processes

As in mammals, upon wounding or local infection, the first leukocytes that operate in fish are tissue macrophages, and possibly mast cells. Then if signals are released to launch a systemic inflammatory process, the first inflammatory cells to arrive at the site of injury are neutrophils, detected from 6 h to 4 days, followed about a day later by monocytes that then differentiate into macrophages (Ellis, 2001; Neumann *et al*., 2001). The major proteins and corresponding genes involved in inflammation and its resolution in mammals have recently been found in fish: TNF-α, NF-κB, COX2, IL-1, IL-8 and other C-C and C-X-C chemokines (Engelsma *et al*., 2001; Secombes *et al*., 2001). Several signaling pathways appear conserved. For instance, bacterial LPS induce IL-1, TNF-α, and iNOS transcription in macrophages by an NF-κB dependent mechanism, and sequences that would be typical NF-κB response elements in mammals are found in the carp iNOS gene promoter. Besides peptide chemokines, eicosanoids, particularly lipoxins, produced by fish macrophages, appear to be potent chemoattractants for fish leukocytes (Sharp *et al*., 1992). Functional studies strongly suggest that both monocytes/macrophages and neutrophils possess receptors for lectins, complement and immunoglobulins. Recently an Fc receptor gene was cloned in the carp (Fujiki *et al*., 2000).

In terms of microbicidal weapons, the two main phagocytes, macrophages and neutrophilic granulocytes, produce reactive oxygen intermediates and H_2O_2 through a well-documented inducible respiratory burst, that seems to involve a membrane-bound phagocyte (NADPH) oxidase similar to that of mammals (Secombes and Fletcher, 1992; Neumann *et al*., 2001). Upon infection, fish macrophages, as their mammalian homologs, express inducible nitric oxide synthase (iNOS, see above), leading to increased production of NO and reactive nitrogen intermediates. One originality found in goldfish and carp, relative to mammals, is that contact of the macrophage with the microbes appears sufficient to trigger induction of iNOS, with no need for exogenous cytokine signals such as IFN-γ. Peptides derived from the proteolysis of transferrin appear to be involved in this induction of iNOS (Neumann *et al*., 2001).

In mammalian monocytes and neutrophils, myeloperoxidase uses the H_2O_2 produced by the respiratory burst to catalyze the formation in the phagosome of hypochlorous acid. In fish, monocytes, neutrophils, eosinophils, and macrophages in some species (e.g., goldfish) were found to be peroxidase-positive

(Rowley *et al*., 1988; Belcourt *et al*., 1995). The first fish myeloid peroxidase gene cloned, in zebrafish, suggests that the diversification of myeloid peroxidases found in mammals (three members: myelo-, lacto- and eosinophil peroxidase) occurred after the fish/tetrapod divergence (Bennett *et al*., 2001; Lieschke *et al*., 2001). Comparison of the zebrafish and fugu genome sequences should tell us whether teleosts have independently diversified their myeloid peroxidases.

4. Connection of the Innate and Adaptive Immune Systems

Several studies have shown that fish macrophages can activate T and B lymphocytes. Consistently, teleost fish have polymorphic class I and class II MHC genes, and overall, the latter were found to be expressed primarily in lymphoid tissues (Sultmann *et al*., 1993; Rodrigues *et al*., 1995; Press *et al*., 1998). However, in mammals, it has recently become clear that a distinct set of myeloid cells, the dendritic leukocytes, and not macrophages, are able to prime naive T lymphocytes. In fish, the potential distinction between macrophages and dendritic leukocytes has not yet been approached. It will certainly be a major question in fish immunology for the near future.

5. Non-specific, non-phagocytic cytotoxic cells (NCC)

Teleosts have non-specific or natural-cytotoxic cells (NCC) that appear to function as mammalian natural killer (NK) cells (Jaso-Friedmann *et al*., 2001). They can kill allogeneic and xenogeneic tumor cells, virus-infected cells, and protozoan parasites. Unlike mammalian NK cells, they are small, agranular lymphocytes, which display constitutively a spectrum of cytotoxicities that lies between that of naive and cytokine-activated NK cells. In addition, leukocytes other than NCC appear to have cytotoxic capacity. In the channel catfish, normal peripheral blood leukocytes negative for a well-defined NCC cell surface marker showed potent cytotoxic activity against allogeneic and virus-infected autologous cells (Yoshida *et al*., 1995; Hogan *et al*., 1996). No marker is presently available to characterize these non-NCC cytotoxic cells. Section IV revisits zebrafish NCC, and discusses novel surface receptors instrumental in their characterization.

B. The Zebrafish Innate Immune System

One of the prime strengths of the zebrafish is its optical clarity, which allows direct inspection during early development. For this reason the zebrafish has been a particularly useful model for the developmental biologist. The main contribution of zebrafish to the understanding of vertebrate innate immunity has therefore come from the opportunity to study early developmental stages of the cells that compose the innate immune system, which were hardly accessible in more classical vertebrate model species. These studies revealed

that the embryo already had a fully operational, macrophage-based immune system. By the time this book is published, the availability of the zebrafish genome sequence will have provided a much more comprehensive account of which of the numerous molecular components relevant to the innate immune system known in mammals are also found in fish. Thus, the first contribution of zebrafish to innate immunity was to reveal the origin and functional characteristics of a lineage of early macrophages that arises well before any other leukocytes, and even precedes the emergence of dedicated hematopoietic organs.

1. The Early Macrophage Lineage

Macrophages are an ancient cell type, older than blood itself, as initially identified by Metchnikoff over a century ago. Metchnikoff, then a comparative embryologist, was wondering about the function of mesoderm, and found that the phylogenetic origin of mesoderm coincides with the origin of phagocytes, which he named macrophages (Metchnikoff, 1892). From this, Metchnikoff then developed his "phagocytic theory of inflammation" that founded immunology.

It is common knowledge that in mammalian or avian ontogeny the first leukocytes to arise are macrophages. Their appearance on the yolk sac coincides with development of the first erythroid blood islands. 10–15 years ago, a few pioneering teams went further, providing evidence that these early macrophages differentiated along a pathway that bypassed monocytic intermediates and granulocyte precursors (Takahashi *et al*., 1989, 1996; Sorokin *et al*., 1992a,b; Cuadros *et al*., 1992, 1993). The work of these groups collectively showed that from the yolk sac, these primitive macrophages quickly invaded the embryo's mesenchyme. Invasion was especially prominent in the head, and from there they entered the still unvascularized, developing organs, starting with the brain (see also Kurz and Christ, 1998; Knabe and Kuhn, 1999; Lichanska *et al*., 1999). Mitotic figures were seen in these early tissue macrophages, again at odds with "classical," monocyte-derived macrophages, which are thought to be mostly post-mitotic cells. These findings contradicted the dogma that in ontogeny, the first tissue macrophages were derived from circulating monocytes, extravasating from blood vessels into tissues to become macrophages, as occurs during inflammation in adults (reviewed in Sorokin *et al*., 1992a and Takahashi *et al*., 1996).

In the zebrafish embryo, analogous early macrophages were detected, and could be studied in detail by combining fluorescent cell tracing and video-enhanced DIC microscopy in live embryos, with WISH in fixed embryos. They were found to differentiate in the yolk sac before the onset of blood circulation, and from there to invade cephalic mesenchyme, followed by brain,

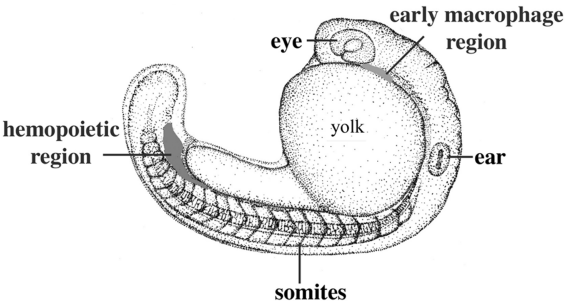


FIG. 1. Hematopoietic regions in the zebrafish embryo. Two hematopoietic regions can be distinguished in the lateral plate mesoderm of the zebrafish embryo at the 22-somite stage: the rostral region gives rise to the "early macrophages"; the caudal hematopoietic region gives rise to embryonic erythroblasts and probably also to the progenitors of all definitive blood cell lineages, which will colonize the definitive hemopoietic organs, thymus and head kidney, 2–4 days later.

retina, and epidermis, in a pattern remarkably similar to that described in mammals and birds (Herbomel *et al*., 1999, 2001).

The yolk sac, where the early macrophages differentiate, has a very simple histological structure. It is essentially one giant yolk cell (actually a thin "yolk syncytial layer" enclosing a large yolk mass), covered by a thin "skin," made of two closely juxtaposed thin cell monolayers, the epidermis and a protective "periderm." Early macrophages are initially found in the free space between the yolk cell and skin, most often spread out on or tethered to the basal side of the overlying epidermis (see Fig. 1). Thus, using video-enhanced DIC microscopy, these cells can be imaged and followed *in vivo* at high magnification (see movie 4*). Furthermore, the zebrafish displays a highly convenient peculiarity among teleosts. At the start of blood circulation, the venous blood, as it arrives on the yolk sac, is no longer contained in a blood vessel, but flows freely over the surface of the yolk cell, to be collected in the single atrium of the zebrafish heart. Thus many of the early macrophages present on the yolk sac are actually standing in the blood flow, and their interaction with other blood cells or with intravenously injected microbes can be imaged conveniently. In mammals and birds, the origin of early macrophage precursors before they differentiate in the yolk sac is not known. In the zebrafish, their embryologic origin could be traced back to gastrulation. The surprising discovery was that unlike all other hemopoietic lineages in amniotes—and probably also in zebrafish—their precursors did not originate from the *caudal* lateral plate mesoderm, but from the rostral-most lateral mesoderm, just anterior to the cardiac field in the developing head region (Herbomel *et al*., 1999). This rostral site of origin of early macrophages is not specific to the zebrafish. In Xenopus, early macrophages were recently found

to arise from the same region (Smith *et al*., 2002). It is worth noting here that in *Drosophila*, embryonic macrophages also arise from the cephalic mesoderm (Tepass *et al*., 1994). Hence the production of early macrophages directly from cephalic lateral mesoderm in vertebrate ontogeny might be considered as a case of ontogeny recapitulating phylogeny—since the developmentally later, elaborate system of pluripotent stem cells giving rise to all leukocytes within a dedicated hematopoietic stroma (often centered on macrophages) was probably discovered in vertebrates. It is tempting to suggest that a similar site of origin will also be found for mammalian and avian early macrophages.

In zebrafish, this small region of rostral-most lateral mesoderm (about 50 cells) from which the early macrophages originate also gives rise to endothelial cells, that will notably make up the carotids (Herbomel *et al*., 1999). It thus conforms to the rule derived from mammalian and avian studies that hematopoietic lateral plate mesoderm is always also vasculogenic (Pardanaud *et al*., 1996).

In the rostral territory, vascular and macrophage precursors segregate from each other during the final convergence of this rostral-most lateral mesoderm to the midline, beneath the paraxial mesoderm and neural tube at approximately 14–16 hpf. Several genes coding for transcription factors (TFs) were found to be turned on specifically in mesodermal macrophage precursors just before or during this short period. Two of these code for the zebrafish homologs of PU.1 and C/EBP-β, both key transcription factors for the commitment of hematopoietic cells to a myeloid fate in mammals (Lieschke *et al*., 2002; P.H., B. Thisse and C. Thisse, unpublished results). DIC video-microscopy reveals that macrophage precursors, still with mesenchymal morphology, subsequently almost reverse the direction of migration and emigrate, now as single cells, to the neighboring, anterior aspect of the yolk sac (Herbomel *et al*., 1999). During this emigration they express other TFs of the C/EBP family (Lyons *et al*., 2001a,b), and in addition now start transcribing genes coding for markers of leukocyte or macrophage differentiation. Among these are L-plastin, a leukocyte-specific actin-bundling protein likely involved in the amoeboid motility that characterizes leukocytes, lysozyme C, and the M-CSF (CSF-1) receptor—an important marker of the macrophage lineage in mammals (Herbomel *et al*., 1999, 2001; Liu and Wen, 2002). In Xenopus embryo, two other genes were recently found to also mark the early macrophage population: POX2, a new member of the myeloid peroxidase gene family, and LURP-1, a novel and likely secreted member of the Ly-6 family (Smith *et al*., 2002).

Once on the anterior aspect of the yolk sac, the macrophage precursors evolve into "pre-macrophages" that are characterized as round cells of homogeneous size (12 μm) with little cytoplasm, and a consistent mini-blebbing

behavior observed by DIC video-microscopy (Herbomel *et al*., 1999; Movie 6*). These pre-macrophages differentiate into "wandering" immature macrophages, which will become competent for phagocytosis in the next few hours (see below). These "pre-" and "young" early macrophages are likely homologous to the "primitive" and "fetal" macrophages described by Takahashi *et al*. (Takahashi *et al*., 1989) in the mouse yolk sac. Thus, early macrophages arise rapidly from the rostral-most lateral mesoderm, after only one morphologically distinguishable intermediate, the "pre-macrophage." Such a direct process makes physiologic sense, for a population of wandering macrophages is thus produced in only a few hours and is now ready to remodel tissues and to fight pathogens, prior to the onset of adaptive immunity (see Section III.B).

The finding that cells of a special early macrophage lineage are the first resident macrophages in the tissues, and that they constitute autonomous immune sentries before any other leukocytes are produced (see below), raises a number of questions.

How similar are they from definitive monocyte-derived macrophages? Are they involved in developmental processes? Do they persist in the adult, as suggested by data obtained in rodents (reviewed in Takahashi *et al*., 1996; see also Ginsel, 1993; Leenen and Campbell, 1993), to constitute a significant, self-renewing fraction of tissue macrophages and/or dendritic leukocytes, possibly endowed with a distinct reactivity and functional connection to the other cells of the adult immune system?

A recent study of goldfish macrophages (Barreda *et al*., 2000) suggests that a rapid macrophage differentiation pathway bypassing monocytes may also be operative in adults, arising from blast-like progenitors in the head kidney, and coexisting with the better known pathway involving monocytes.

a. Functional Characteristics of Early Macrophages. In the yolk sac, early macrophages become phagocytic, ingesting apoptotic erythroblasts as well as injected microbes (see below), at 23–26 hpf, coinciding with the onset of blood circulation (P.H., unpublished data). Subsequently, their deployment in embryonic tissues is accompanied by tissue- and stage-specific variations in gene expression patterns and levels of endocytic activity (Herbomel *et al*., 2001; B. Thisse and C. Thisse, unpublished data), thus starting to delineate local differentiation patterns, a typical feature of tissue macrophages in mammals (Leenen and Campbell, 1993; Gordon, 1995). Some are transient, and are associated with a defined tissue-remodeling task, such as the elimination of the remnants of the hatching gland after hatching has occurred, others are permanent. The most striking example for the latter is a sudden phenotypic transition that exclusively affects all brain and retinal macrophages

at 55 hpf, regardless of how much time they have already spent in this tissue. The resulting cells were termed "early microglial cells" (Herbomel *et al*., 2001). They are highly endocytic, and notably express high levels of apolipoprotein E (apoE). While apoE is a typical secretion product of many tissue macrophages in adult mammals, in zebrafish embryos, the early microglial cells are the only macrophages that express transcripts of the gene by WISH.

DIC video-microscopy revealed that once in the epithelial tissues (brain, epidermis), most early macrophages wander restlessly among epithelial cells, showing a remarkable ability to wind through tissues as dense as the neuroepithelium of the developing brain (Herbomel *et al*., 2001; Movies 1 and 2*). So far, in mammals, the movement of macrophages had largely been considered in the context of inflammation, and towards inflammatory foci. The live observations in the zebrafish embryo suggest that resident macrophages may well be constantly "patrolling" in vertebrate tissues at steady state—conceivably for immune surveillance, and more broadly as guardians of homeostasis (Gordon, 1995).

b. Functional Relevance of the M-CSF Receptor. As in mammals, the zebrafish has an M-CSF receptor gene (*c-fms*) that is expressed in all early macrophages (Herbomel *et al*., 2001). In the *panther* mutant, a loss-of-function defect in *fms* (Parichy *et al*., 2000) early macrophages differentiate and behave normally in the yolk sac. This suggests that the *fms* gene is dispensable in terms of proliferation, survival, motility, and endocytic or phagocytic abilities in early macrophages. However, they subsequently fail to invade embryonic tissues, and remain in the yolk sac and in the blood (Herbomel *et al*., 2001). This suggests that the tissues that normally become colonized attract macrophages actively, by secreting M-CSF as a chemoattractant. A similar finding was recently reported in *Drosophila* embryos, where VEGF-R deficient macrophages are specified but fail to migrate properly (Cho *et al*., 2002).

Another great asset of the zebrafish is the wide temperature range under which zebrafish embryos and adults can live (20–33°C), making it possible to screen for temperature-sensitive mutants. Thus a thermo-sensitive M-CSF receptor (*fms*) mutant was recently obtained (Parichy and Turner, 2003). It will likely be useful for studying monocyte/macrophage differentiation, physiology and contribution to immunity in embryos, fry and adults.

c. Autonomous Immune Function of Early Macrophages. When massive amounts of live gram+ (*B. subtilis*) or gram− (*E. coli*) bacteria are injected in the bloodstream of zebrafish embryos, they are quickly phagocytosed

and killed by the early macrophages. It was shown by DIC video-microscopy that three hours after injection, there were no more free bacteria, and still a few hours later, no more traces of bacteria were seen in the macrophages. In addition, the macrophages now show increased ruffling and also start phagocytosing live, apparently healthy erythroblasts—often catching them in the middle of their mitosis (Herbomel *et al*., 1999; Movie 8*). Thus the infection triggered a type of macrophage activation that is not rapidly downregulated and remains to be defined at the molecular level.

All microorganisms tested so far were readily phagocytosed: live gram− and gram + bacteria, and yeast (*Saccharomyces cerevisiae*) (P.H., unpublished data). This suggests that early macrophages have pattern-recognition receptors for antibody-independent phagocytosis of most microbes. Their capacity to phagocytose live yeast suggests they possess at least a mannose/fucose receptor akin to that of mammals. Serum proteins, such as complement may well play an adjunct role for killing or opsonizing the microbes. However, in adults these proteins are produced by the liver, and the macrophage-based immune system of the embryo is operational before the development of a functional liver. One putative source of complement in the embryo would be the yolk syncytial layer, which assumes many aspects of the role of an embryonic liver, releasing nutrients into the blood, and thus quite possibly also defense-oriented proteins. Another potential source of opsonizing complement factors could be the macrophages themselves (Ezekowitz *et al*., 1985).

To further study the functionality of early macrophages, bacteria were injected into the hindbrain ventricle, a closed cavity remote from the blood. This experiment was carried out at 21 hpf, a developmental stage that precedes vascularization and seeding of the head with early macrophages. Five hours after the injection, many macrophages had entered the hindbrain ventricle and cleared the bacteria (Herbomel *et al*., 1999).

Because of the initial lack of resident leukocytes of any kind at the infection site, the 24 hpf zebrafish embryo offers the unique opportunity to directly assess the chemoattractive nature of microbes in an entire live organism—a question that is not easily accessible in mammalian systems.

So what attracts the macrophages to the infected brain ventricle? One possibility is that the bacteria in the ventricle release some molecules small enough to permeate the ventricle walls, reach the macrophages ≥ 100 microns away and chemoattract them (N-formylated peptides would be possible candidates). Alternatively, microbial molecules are first detected by the neuroepithelial and/or meningeal walls of the ventricle, which then secrete chemokines that are sensed by the distant macrophages. The latter would imply that vertebrate epithelial tissues are equipped with microbe sensing, possibly Toll-like receptors and chemokine signaling abilities early in their

ontogeny, even before they are fully differentiated. Interestingly, all macrophages in the embryo acquire an activated morphology (abundant ruffling) and behavior upon infection of the hindbrain ventricle. This is true not only for macrophages that home to the infected brain ventricle but also for those in the blood flow that now start phagocytosing apparently healthy erythroblasts (Herbomel *et al*., 1999). Such concerted behavioral change in activation suggests cytokine-mediated communication within the macrophage population throughout the organism.

d. Attraction of Early Macrophages to Wounds. Infliction of an aseptic wound anywhere in the embryo as early as 40 hpf (and possibly earlier) attracts *fms* + early macrophages to the wound site within the next four hours. Macrophages are still found gathered at the wound site 35 h later (P.H., B. Thisse and C. Thisse, unpublished data). The initial absence of leukocytes at several of the tested wound sites introduces a novel situation compared to mammalian systems. In this setting the macrophages must have been attracted to the wound by signals emanating from the dead or dying cells themselves, possibly relayed or amplified by healthy neighboring cells in the epidermis. Despite the intense scientific interest in apoptosis, it is still not known if dying cells can directly chemoattract macrophages *in vivo*. The zebrafish embryo could be instrumental in elucidating this point.

2. *Granulocytes*

The next type of immune cells that arise during zebrafish development are neutrophilic granulocytes. Immature neutrophils are first reliably detected by E.M. at 48 hpf, 24 h after the onset of blood circulation and early macrophage phagocytic functions. The initial site of appearance is the caudal/axial vein and the mesenchyme surrounding it—in the tail proper and more rostral along the ventral trunk (see Fig. 1) (Willett *et al*., 1999; Lieschke *et al*., 2001). Their detection in the vein suggests that some neutrophils must be circulating at that time in the blood, though they likely represent a small minority relative to the early macrophages, and even to the thrombocytes, that appear around 36 hpf (Gregory and Jagadeeswaran, 2002). Since lymphocytes will appear even later (see Section III.B.2), the four cell types appear developmentally in the same order as in mammalian embryos. The initial location of the neutrophilic granulocytes in the mesenchyme around the caudal vein suggests that they, as well as the thrombocytes, arise from the hematopoietic stroma that is steadily expanding at this stage in between the caudal artery and caudal vein, and has been presumed to be equivalent to the mammalian AGM region (Thompson *et al*., 1998). When a wound is introduced in the caudal area of the embryo/larva, i.e., close to the site where neutrophils arise, these neutrophilic

granulocytes home to the wound within minutes (M. Redd, personal communication) to hours (Lieschke *et al*., 2001). Whether they can respond to microbial infections and are phagocytic remains to be tested.

The second type of granulocytes found in adult zebrafish, eosinophils, were not detected up to five dpf (Lieschke *et al*., 2001). It is therefore quite possible that their generation is delayed and initiates in the definitive hematopoietic organ, the head kidney. The head kidney is also the site where neutrophilic granulocytes will become the most numerous myeloid leukocytes, and where monocytes likely arise for the first time.

3. *Impact of Zebrafish on Understanding Innate Immunity*

Notwithstanding much accumulated knowledge about the in-and-out of our immune system, relatively, little is known about the repertoire of behaviors by which leukocytes residing in the tissues at steady state control microorganisms on a daily basis. In particular it is unclear how resident macrophages contain infections, most of the time successfully and silently, i.e., without launching a systemic immune response. The constant activity of these resident leukocytes scattered in the tissues, and the basis of their variable efficiency at controlling a given microorganism—among different individuals, or species—are difficult to approach in mammals.

To explore further the potential of the zebrafish model along these lines, the establishment of transgenic zebrafish expressing different fluorochromes under the control of promoters specific for the various types of leukocytes will be instrumental. By working at progressively later stages of development, the various cellular components that will make up the mature immune system can be implemented one by one, a prospect also inaccessible in mammals. To take full advantage of the system, zebrafish lines will have to be generated that remain transparent into adulthood—as was successfully done in the medaka fish (Wakamatsu *et al*., 2001).

C. Natural Killer Cells, Natural Cytotoxicity and the Possible Role of Novel Immune-Type Receptor Genes

Whereas zebrafish Ig and TCR genes are easily identified as orthologs of their mammalian counterparts (see Sections III.B and III.C) and can be used to identify B and T cells respectively, the identity of NK-like receptors in zebrafish remains in question. This observation, compounded with the apparent absence of a morphological equivalent to NK cells in bony fish makes the study of natural killing in zebrafish challenging, but highly intriguing. Nevertheless, several studies have demonstrated that fish possess at least three types of cytotoxic cells. Non-specific cytotoxic cells (NCC) which primarily populate the pronephros (the hematopoietic head

kidney), have been described in catfish, trout (Evans *et al*., 1984; Greenlee *et al*., 1991) and other fish species (Shen *et al*., 2002) and have been referred to as "NK-like" although their morphology contrasts with that of mammalian NK cells (NCC are small and agranular). NCC have been reported to recognize and kill a wide variety of target cells including allogeneic and xenogeneic tumor cells (Jaso-Friedmann *et al*., 1997). An undefined population of cells within the peripheral blood leukocytes (PBL) of catfish have been shown to kill allogeneic target cells, but fail to recognize xenogeneic targets (Yoshida *et al*., 1995). In addition, a population of cells isolated from the pronephros in carp (*C. carpio*) kill a profile of xenogeneic target cells that differs from the profile recognized by NCCs (Kurata *et al*., 1995); these cells have been morphologically characterized as neutrophilic granulocytes, and thus are distinct from NCC. Zebrafish spleens and kidneys possess similar populations of cytotoxic cells that also can recognize and lyse specific mammalian tumor target cells (J.A.Y., S. Wei and J. Djeu, unpublished observations).

NK activity in mammals also is associated with a number of different cell lineages (McQueen and Parham, 2002) and recent advances in understanding the molecular biology of NK cell function in man and mouse have identified diverse receptor gene families that are associated with the recognition of different ligands. Mammalian NK receptors can be classified into two broad categories based on the structure of their extracellular domains: (1) immunoglobulin (Ig) domain containing receptors [primarily encoded at the leukocyte receptor complex (LRC)] and (2) C-type lectin (CTL) receptors [primarily encoded at the natural killer complex (NKC)] (Barten *et al*., 2001), both of which include activating and inhibitory forms. Despite overall differences in extracellular structure, mammalian NK receptors share common modes of intracellular signaling; activating signaling leads to target cell lysis and inhibitory signaling represents a means to control misdirected lysis. Activating signaling is based on the association of NK receptors with adaptor proteins (e.g., DAP12, CD3ζ), that typically possess cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs), although other adaptor proteins function through alternative pathways (e.g., DAP10). This protein–protein interaction involves the association of a positively charged residue in the transmembrane region of an NK receptor with a negatively charged residue in the adaptor molecule. Inhibitory signaling is based on the presence of cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs) in certain (non-activating) NK receptor molecules. It is becoming clear that NK cells utilize both activating and inhibitory receptors to maintain a signaling balance; a shift towards the activating pathway can override the inhibitory pathway and lead to target destruction (Ravetch and Lanier, 2000; Cerwenka and Lanier, 2001).

Although gene families resembling either the LRC or the NKC encoded receptors have not been reported in species at or below the phylogenetic level of bony fish, Novel Immune-Type Receptors (NITRs), which have been characterized in pufferfish, zebrafish, catfish and trout (Strong *et al*., 1999; Yoder *et al*., 2001, 2002a; Hawke *et al*., 2001), encode receptors that share overall structural characteristics with certain innate Ig-type NK receptors (KIR-type) but possess diversified variable (V) domains reminiscent of the adaptive immune genes. NITRs may be the bony fish equivalent to NK receptors (Litman *et al*., 2001). Interestingly, a single copy CTL-type receptor maps next to an NITR locus in trout, which may provide a link between the Ig-type and CTL-type of NK receptors (Yoder *et al*., 2002a).

Most NITRs identified to date, including those identified in zebrafish, possess two extracellular Ig domains, a transmembrane region and a cytoplasmic tail containing ITIMs (Strong *et al*., 1999; Litman *et al*., 2001; Yoder *et al*., 2001, 2002b). The N terminal Ig ectodomain of the prototypic NITR is of the V-type and contains a contiguous Ig/TCR-like joining (J) region motif; the C terminal ectodomain shares characteristics of both V- and C2-type domains. The presence of authentic V domains with contiguous J-like sequences is a hallmark of NITR genes in multiple fish species (Strong *et al*., 1999; Yoder *et al*., 2001, 2002a; Hawke *et al*., 2001; Litman *et al*., 2001) and distinguishes them from other Ig-like innate receptors (e.g., KIRs which possess C2 domains). As discussed elsewhere in this review, highly diversified T cell receptor and immunoglobulin gene families have been identified in zebrafish (see Sections III.B and III.C), which strongly argues that the NITRs do not supplant the function of genes of the adaptive immune system. Furthermore, radiation hybrid mapping data indicate that the identified zebrafish TCR, MHC and Ig loci are on different chromosomes from the NITR gene cluster (Yoder *et al*., 2001; reviewed in Yoder *et al*., 2002b).

Basic questions exist about the complexity, extent of diversity and transcriptional regulation of NITR genes in zebrafish. The Zebrafish Genome Project (ZGP; being conducted at the Wellcome Trust Sanger Institute) has prioritized the characterization of the NITR gene cluster and, while not complete, already has resulted in the identification of new families of NITR genes. Taken together with earlier studies, several conclusions can be drawn from the emerging genomic DNA dataset as regards the structure of NITRs and the organization of the NITR gene cluster in zebrafish: (1) the NITRs are highly diverse; seven families of V regions have been identified (Yoder *et al*., 2001; J. Y., unpublished observations); (2) sequence data acquired to date are consistent with the possibility of a single gene cluster as at least four NITR gene families map to a single genomic region based on STS mapping using

radiation hybrid panels (Yoder *et al*., 2001); (3) >100 NITR genes, including polymorphic variants, currently have been identified; (4) the vast majority of these possess ITIM-containing cytoplasmic tails; however, the type 5 (*nitr*5) genes possess an ITAM in the cytoplasmic tail (see below); (5) efforts to determine if the map position of zebrafish NITRs (LG7) share conserved synteny with the human 19q13 LRC are inconclusive (owing to disrupted synteny) (Yoder and Litman, 2000; Yoder *et al*., 2001); (6) emerging data from the ZGP, which is based on PAC sequences of pooled "inbred" AB line embryos, have defined extensive allelic polymorphism in the polygenic NITRs, resembling that seen with the KIRs (Uhrberg *et al*., 1997; Vilches and Parham, 2002), and are consistent with RFLP analyses of zebrafish genomic DNA employing NITR family-specific probes (J.A.Y. and G.W.L., unpublished observations); and (7) NITRs encoding only one (vs two) V-type ectodomains have been identified (Hawke *et al*., 2001; Yoder *et al*., 2001).

Based on earlier work with pufferfish and catfish, the most unexpected findings relate to the receptors encoded by the *nitr*5 genes, which may possess a novel activating mechanism in an IgSF-related putative NK-type gene product. The *nitr*5 genes encode two extracellular Ig domains (the N-terminal ectodomain is of the V-type and the C-terminal ectodomain is of the V/C2 or I (intermediate) type), a transmembrane domain that has a neutral charge and a cytoplasmic tail that contains an ITAM. A current analysis of the Zebrafish Genome Project database has identified six different *nitr*5 genes ("alleles"), and the RNA splice junctions have been confirmed through analysis of corresponding cDNAs (J. Y. and G. W. L., unpublished observations). It has not escaped our notice that the presence of an ITAM within the cytoplasmic region of an NITR may allow for direct activating function, independent of an adaptor protein. Such a mechanism is in contrast with the two-protein complexes utilized in the mammalian NK system. In this regard Nitr5 receptors are distinguished from any known activating component of the NK system in mammals. Nitr5 receptors might fulfill an activating function that is coupled with inhibitory functions of the ITIM-containing Nitrs 1–4, 6, and 7.

Although the ligand binding of NITRs is presently uncharacterized, it has been possible to make a number of observations regarding the cytoplasmic signaling and developmental expression of NITRs, which may facilitate understanding their function. Specifically: (1) studies using vaccinia-transfected, FLAG-tagged Nitr3.1 indicate that epitope-crosslinking results in ITIM-mediated inhibition of both the phosphorylation of MAP kinase and NK cytotoxicity; (2) the earliest NITR expression in somatic cells can be detected at seven dpf, with the exception of *nitr*3, which can be detected at 72 hpf; in addition, maternal stores of *nitr*3 mRNA have been observed (J.A.Y. and G.W.L., unpublished observations); and (3) embryonic expression

of *nitr*3 appears to be downregulated (disrupted) in a zebrafish mutant line (H75) that displays delayed hematopoietic development (J. A. Y. and N. S. T., unpublished observations).

The hypothesis that the NITR genes may represent another variant form of NK-related receptors (Litman *et al*., 2001) is based on the general organizational features of NITRs, as well as the patterned sequence diversity in the V ectodomains, which is consistent with ligand binding. Furthermore, the polygenic and polymorphic character of NITRs, the preponderance of inhibitory to activating forms and the close similarity in the ectodomains of some activating and inhibitory forms of NITRs are characteristic of certain NK receptors (Litman *et al*., 2001). Specifically, the variable nature of the N-terminal ectodomains, character of the transmembrane and cytoplasmic regions, as well as the polygenic and polymorphic nature of NITRs, collectively have led us to draw analogies between these genes and KIRs (Litman *et al*., 2001). Despite the presence of diversified V region, NITRs resemble KIRs more closely than do the C-type lectins (e.g., Ly49) that mediate NK function in mouse. Although NKp30 and NKp44 are V domain-containing NK receptors (Pessino *et al*., 1998; Cantoni *et al*., 1999; Pende *et al*., 1999), they are single copy as opposed to the highly diversified multigenic families encoding the NITRs.

The studies conducted to date with the various families of NITR genes in zebrafish have paved the way for future research aimed at (1) identifying additional NITRs as well as related genes, (2) defining the ligands recognized by NITRs, and (3) characterizing the functional role NITRs may play in the natural killing of tumor cells. The ability to produce transgenic zebrafish and perform mutagenesis screens makes zebrafish amenable to studies addressing the role of NITRs in the development of the immune system. The continued investigations into the forms and functions of the NITRS may contribute to a better understanding of how NK receptors function in mammals and likely will shed light on the evolutionary pathway(s) that have led to the unusual diversity found in mammalian NK receptors.

III. Ontogeny of Adaptive Immunity from Fishes to Mammals

As mentioned above, the zebrafish as a model system for immunology is a relative latecomer. Thus, it is prudent to review the abundant literature on comparative vertebrate immunity when considering the zebrafish adaptive immune system. In particular, it should be realized that the components of the adaptive immune system, namely the immunoglobulin (Ig), T cell receptor (TCR) and major histocompatibility complex (MHC), are not necessarily identical to those of mouse and human (where they have been described most

extensively) and may exhibit notable differences between various vertebrate lineages (Du Pasquier and Flajnik, 1998). These differences primarily involve variations in genomic organization of the respective gene families, and manifest in presumptive alterations in the manner in which these genes are regulated. In addition, there are poignant differences in the types of hematolymphoid tissues within and among the various vertebrate classes, including the primary sites of production of T and B cells. This section is not meant to be a comprehensive treatise of the evolution and phylogeny of the adaptive immune system; rather, we review what is known about adaptive immunity amongst the poikilothermic (cold-blooded) vertebrates and highlight significant departure points from other systems. Where known, data on primary and secondary lymphoid organs, as well as the Ig and TCR systems of zebrafish are presented and discussed.

A. Thymic Development is Conserved Throughout the Evolution of Jawed Vertebrates

1. Phylogeny of Thymic Organogenesis

The phylogeny of extant chordate taxa, all of which have been examined, to some degree, with respect to the adaptive immune system, is outlined in Fig. 2. It is important to note that although lymphocyte-like cells are seen in protovertebrate taxa such as urochordates and cephalochordates, it was not until the emergence of the gnathostomes (jawed vertebrates), roughly 500 million years ago, that the presence of Ig, TCR and MHC are first observed (Du Pasquier and Flajnik, 1998; Litman *et al*., 1999). During evolution the development of the jaw in vertebrates coincides with the emergence of adaptive immunity and the thymic organ. The thymus is the centerpiece of the adaptive immune system because it provides the environment for generation and selection of large numbers of antigen-specific T cells. The appearance of the thymic organ in evolution therefore marks an important milestone in the ability to fight infections with a specific cell- and/or antibody-mediated immune response. The phylogenetically oldest vertebrates, lamprey and hagfish, are jawless and therefore lack a thymus and evidence of secondary lymphoid organs. It was long held that these agnathan species of fish rely solely on granulocytes (Rowley *et al*., 1988) and C3-like complement (Nonaka, 2000; Zarkadis *et al*., 2001) for protection against infections. Support for this notion comes from the fact that the pivotal components of adaptive immunity, the T- and B-cell receptors for antigen, as well as MHC molecules have not been identified in this class of fishes (Klein *et al*., 1999). However, lymphocyte-like cells were detected in these jawless fishes, particularly in the intestinal tract (Kampmeier, 1969; Cohen, 1977; Marchalonis, 1977). Recently, the demonstration of the presence of members of the

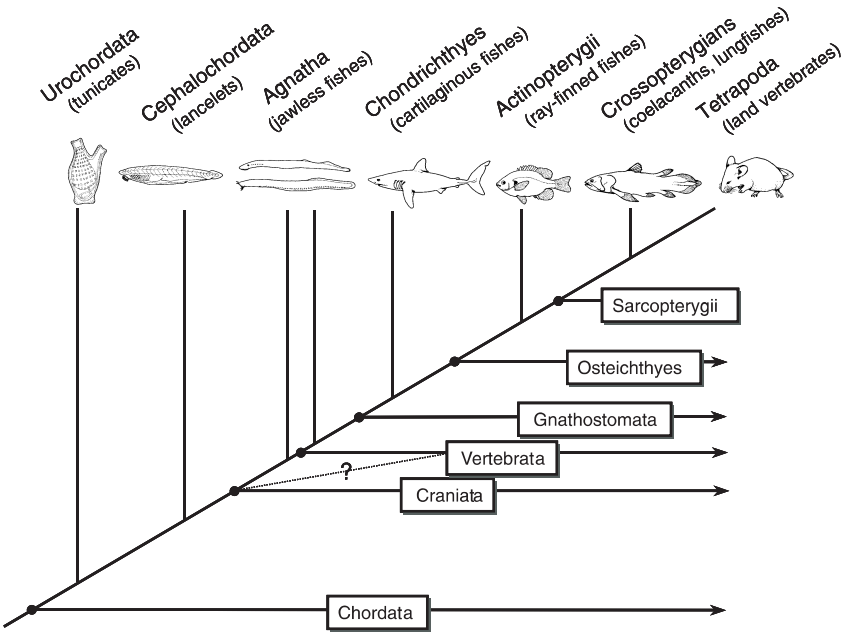


FIG. 2. Inter-relationships of the phylum Chordata. This figure was modified from Nelson (1984) and Zapata and Amemiya (2000). Taxa are shown above the tips of the cladogram and well-defined monophyletic assemblages are listed below the cladogram. The zebrafish, *Danio rerio*, is a member of the teleost (bony) fish lineage. This cladogram merely provides a framework for the major extant chordate clades. With regard to the immune system, salient features of immunological import are listed briefly in Table I. The adaptive immune system appears to be a gnathostome (jawed vertebrate) invention, corresponding possibly to the co-option and utilization of RAG-based somatic diversification in rearranging genes (Du Pasquier and Flajnik, 1998; Plasterk, 1998; Litman *et al.*, 1999; Harsen and McBlane, 2000). It is important to note that the Agnatha, a group that includes the extant cyclostomes (M) Myxini (hagfishes) and (P) Petromyzontiformes (lampreys), is thought to be a "paraphyletic" group. Several advanced anatomical characters exist in lampreys that are absent in hagfishes, although the presence or absence of neural crest cells (which, in part, define the Vertebrata) has not been adequately assessed in hagfishes due to the paucity of embryos. Lastly, lungfish are placed with the coelacanth as they both are "lobe-finned" fishes; however, lungfish are not technically "crossopterygians."

Ikaros (Mayer *et al.*, 2002b) and *Spi* family (Shintani *et al.*, 2000) of DNA-binding molecules in the lamprey genome and their expression in lymphocyte-like cells in the gastrointestinal tract bolsters the notion that lymphocytes may predate the development of the thymus. The functionality of these lymphocyte-like cells remains to be elucidated as no obvious homologs of Ig superfamily genes involved in immune recognition or lymphocyte differentiation were identified in a screen of over 8000 ESTs (Uinuk-Ool *et al.*, 2002; Mayer *et al.*, 2002a).

It has been speculated that with the appearance of the jaw dietary changes as well as increased injuries and infections occurred, creating selective pressure for the development of an organ of high output of immune cells with a diverse and anticipatory repertoire (Matsunaga and Rahman, 2001). Thus the cartilaginous (chondrichthyes) and bony fishes (osteichthyes) are the first vertebrates in which a thymus can be identified. In both classes of jawed fishes the thymus is a bilateral organ located close to the gill cavity. Amongst teleosts, the bony fish division possesses thymi that remain in continuity with the pharyngeal epithelium. Furthermore, there is debate as to whether the teleost thymus is compartmentalized into cortex and medulla (Romano *et al*., 1999a,b; Lam *et al*., 2002), or if it lacks a clear demarcation (Castillo *et al*., 1990; Zapata *et al*., 1996, p. 359; Willett *et al*., 1997). In non-teleostean fishes the thymus appears to be composed of a clearly distinguishable cortex and medulla (Zapata and Cooper, 1990). Gene expression patterns (see below) and the capacity to mount T cell dependent immune responses, such as specific antibody production (Palm *et al*., 1998; Espelid *et al*., 2001), graft rejection and GvHD (Nakanishi and Ototake, 1999) suggest that there is no functional difference between the teleost thymus and that of other fishes or mammals. The compartmentalized thymus is a constant feature of higher vertebrates from frogs to birds and mammals, and also includes sharks and skates.

2. Ontogeny

Thymic development from lower vertebrates to mammals requires the contribution of tissues from all three embryonic germlayers: ectoderm, mesoderm and endoderm (see Fig. 3). Two steps in this process can be distinguished-formation of the thymic rudiment and the interaction between T cells and thymic epithelial cells (TECs) (excellent reviews in Manley, 2000; Rodewald, 2003). Common to all vertebrates is the first step in the formation of the thymic anlage. It initiates with the migration of neurectodermally derived neural crest cells from the sixth hindbrain rhombomere to the pharyngeal area. It has been proposed that interaction of neural crest cell-derived mesenchyme with endodermally derived epithelial cells of the pharyngeal pouches is crucial for thymic development (Anderson *et al*., 1993; Shinohara and Honjo, 1997; Suniara *et al*., 2000). This concept was first proposed when the seminal experiments of Bockman and Kirby (Bockman and Kirby, 1984) demonstrated that ablation of the sixth hindbrain rhombomere in chicks leads to the absence of the thymus. Positional information indispensable for proper neural crest cell migration is provided by the homeobox gene *Hoxa-3* and the paired box gene *Pax-3*. Inactivation of these genes leads to defects in thymic and cardiac development similar to those observed in the human DiGeorge syndrome.

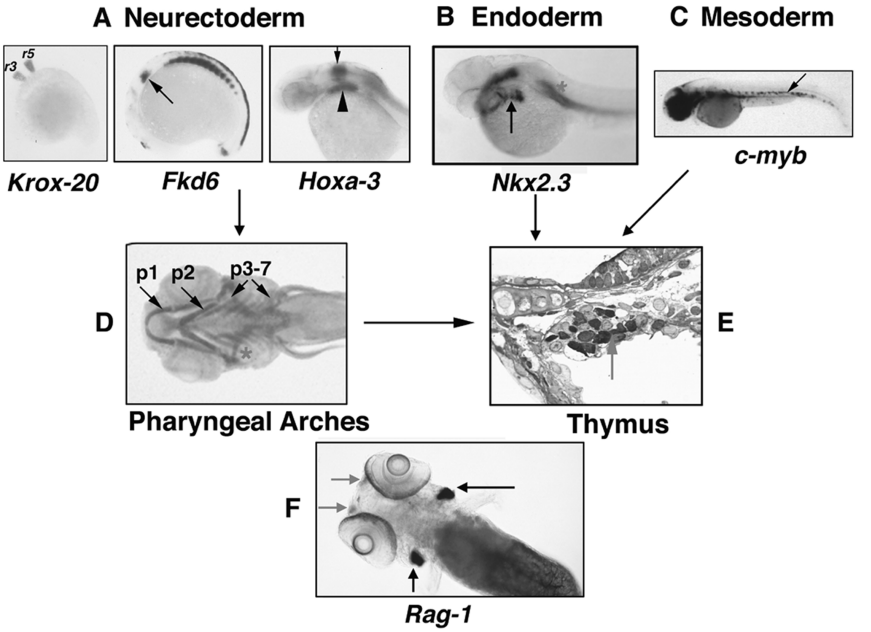


FIG. 3. Expression of genes involved in zebrafish T cell lymphopoiesis and thymic organogenesis. A. The hindbrain rhombomeres (left panel) from which neural crest cells arise are stained with *Krox-20* (third and fifth rhombomere, r3 and r5). Neural crest cell (NCC) expression of *forkhead 6* (*fkd6*) in 15-somite (a developmental stage, approximately 20 hpf, at which 15 body segments can be counted) wild-type embryo is shown in the middle panel. Arrow indicates the third, postotic (behind the ear vesicle) stream of neural crest that invades the third and fourth pharyngeal pouches. In d2 wild-type embryos (right panel) *Hoxa-3* is expressed in rhombomeres 5 and 6 (arrow) and pharyngeal endoderm (arrowhead). B. Expression of *Nkx2.3* in the pharyngeal endoderm in d2 wild-type larva. Expression is seen in pharyngeal pouches (arrow) and in anterior gut (asterisk). C. Mesodermally derived hematopoietic progenitor cells, stained with *c-myb* (arrow), presumably arise from the dorsal aorta in zebrafish embryos at 36 hpf. Pro-T cells probably originate from this area and invade the thymic rudiment. D. Alcion blue staining of skeletal elements of the head region in a d7 wild-type zebrafish shows arches p1 to p7. Asterisk indicates the third pharyngeal pouch, where thymus originates. E. Thymus (grey arrow) of d7 wild-type zebrafish with heterogeneous cell populations, consisting of lymphoblasts and thymic epithelial cell. F. Expression of *Rag-1* in d8 wild-type embryo is seen in the olfactory pit (grey horizontal arrows) and bilateral thymi (arrows). (Reproduced with modifications from Trede *et al.* (2001) with permission.)

A number of genes are expressed at the critical developmental time point (E9.5–10.5) for neural crest cell–endoderm interaction in the pharyngeal pouches between arches 3, 4, and 6. For example, inactivation of the T box gene *Tbx-1*, as well as *Pax-1* and *Pax-9* similarly lead to defects in the formation of proper pharyngeal arch and artery architecture and result in a

DiGeorge-like phenotype of variable severity. These results emphasize the importance of pharyngeal endoderm in the formation of the thymic rudiment. *Hoxa-3* is expressed in both migrating neural crest cells and endoderm (Manley and Capecchi, 1995) (see Fig. 3) and the absence of this gene in both tissues might be responsible for the severe thymic defects seen in *Hoxa-3−/−* mice (Su and Manley, 2000; reviewed in Manley, 2000). Furthermore, attempts have been made at trying to place the above genes in a hierarchical order. For example, an interaction of Hoxa-3 and Pax-1 is suggested by the severity of thymic defects in double-mutant *Hoxa-3+/− Pax-1−/−* mice (Su and Manley, 2000). A picture emerges where Hoxa-3 is placed "upstream" of Pax-1 (Su and Manley, 2000; reviewed in Manley, 2000) and Pax-9 (Peters *et al*., 1998; Hetzer-Egger *et al*., 2002) in a pathway to form the endodermal basis of the thymic rudiment (Su and Manley, 2000; reviewed in Manley, 2000). A functional *whn* gene is indispensable for TEC differentiation. This is borne out in studies with the *whn*-deficient nude mouse, which exhibits differentiation arrest and lack of migration of T cell precursors into the thymic rudiment (Nehls *et al*., 1996). Whether the *whn* gene product acts in a separate pathway from the Hoxa-3/Pax-1 pathway (Su and Manley, 2000) or is placed between Hoxa-3 and Pax-9 as recently suggested by Tom Boehm's group (Hetzer-Egger *et al*., 2002) remains an open question.

3. Thymic Organogenesis in Zebrafish

a. Formation of the Thymic Rudiment. Based on the above data obtained in chicks and mammals we obtained molecular markers to probe evolution of the thymic rudiment during zebrafish development (Fig. 3). Neural crest cells contributing to thymic organogenesis are expected to arise from hindbrain rhombomeres 5 and 6 and migrate in the post-otic stream to the pharyngeal area. *Krox-20* (red dye) is expressed in hindbrain rhombomeres 3 and 5 (Fig. 3A, left panel) and is useful as a landmark to identify the region of the hindbrain of interest. Prior to and during migration, neural crest cells express the transcription factor *forkhead 6* (*Fkd6*, Fig. 3A, middle panel). In agreement with data from mice, *Hoxa-3* is expressed in the fifth and sixth rhombomere and also stains pharyngeal endoderm (Fig. 3A right panel) (Su and Manley, 2000). *Nkx2.3*, a marker of pharyngeal endoderm, is expressed throughout pouches two to six (Fig. 3B). Similarly to mammals, formation of pharyngeal arches in zebrafish (Fig. 3D) is critically dependent on interaction of neural crest cells and pharyngeal epithelium and endoderm. For example, the zebrafish mutant sucker (*suc*) has defects in the four most anterior pharyngeal arches (Piotrowski and Nusslein-Volhard, 2000). Cloning of the defective *suc* gene identified a mutation in the *endothelin-1* (*et-1*) gene,

which is expressed in a central core of arch paraxial mesoderm and in arch epithelia, both surface ectoderm and pharyngeal endoderm, but not in skeletogenic neural crest (Miller *et al*., 2000). Miller *et al*. demonstrated that *suc/et-1* functions nonautonomously in neural crest cells, and is thus required in the environment of post migratory neural crest cells to specify ventral arch fates. These results confirm and extend previous findings observed in the mouse knockout of *et-1* which results in a disruption of pharyngeal arch architecture and cardiovascular defects reminiscent of the human CATCH 22 syndrome (Kurihara *et al*., 1994). An identical phenotype is caused by targeted inactivation of the Endothelin Converting Enzyme-1 (ECE-1) necessary for proteolytic activation of Endothelin-1 in mouse embryos (Yanagisawa *et al*., 1998). Finally, disruption of the Endothelin-A Receptor (ETAR), which is expressed by the neural crest-derived ectomesenchymal cells of pharyngeal arches and cardiac outflow tissues, causes a phenocopy of the *et-1* and ECE-1 knockouts (Clouthier *et al*., 1998). A pathway similar to the et-1–ETAR interaction has to date not been described for the more caudal pharyngeal arches.

In contrast to mammals, the zebrafish thymi remain in continuity with the pharyngeal endoderm. The thymic rudiment arises between pharyngeal arches 3–5, in the third and fourth pharyngeal pouch (our own observations and Lam *et al*., 2002). It can first be detected between 54 and 60 hpf as a small outgrowth of pharyngeal epithelium (Willett *et al*., 1997, 1999). At this stage, the thymic rudiment consists of two layers of epithelial cells surrounded by a basement membrane (Willett *et al*., 1999) and is devoid of lymphocytes. In mouse, the prospective TECs express Whn/Foxn1, the gene disrupted in the nude mouse (Nehls *et al*., 1996), which is absolutely required for further epithelial differentiation into thymic tissue. The initially reported zebrafish *whn/Foxn1* homolog (Schlake *et al*., 1997) is expressed in the developing eye and in brain structures (our own observations and Schorpp *et al*., 2002), as well as in a few scattered epithelial cells in the adult thymus. However, this gene was never expressed during early thymus development, initiating a search for the true zebrafish ortholog of *whn/Foxn-1*. Recently, a second zebrafish *whn* gene (designated *whnb*) was cloned by the group of Tom Boehm (Schorpp *et al*., 2002). The *whnb* gene appears to be the true orthologue of *Foxn1* as it is expressed in TECs on day 3 of development and is furthermore more closely related to the mammalian *Foxn1* gene by phylogenetic analysis and synteny (Schorpp *et al*., 2002). The original zebrafish *whn* gene (now designated *whna*) is now thought to be the orthologue of *Foxn4* (Schorpp *et al*., 2002).

T cell precursors in mammals are derived from hematopoietic stem cells of mesodermal origin in the yolk sac (Yoder *et al*., 1997) and aorta-gonad-mesonephros (AGM) region (Cumano *et al*., 1996, 2000). There is

evidence to suggest that the AGM equivalent in zebrafish is the dorsal aorta (Thompson *et al*., 1998). For example, markers of early hematopoietic differentiation, such as *c-myb*, are expressed in the dorsal aorta between 36 and 48 hpf (Thompson *et al*., 1998) (Fig. 3C). It is therefore possible that T cell precursors in the zebrafish are derived from progenitor cells in the ventral wall of the dorsal aorta. There are currently no markers available to corroborate the presence of T cell precursors in the dorsal aorta. Furthermore, transplantation or *in vitro* differentiation experiments are only beginning to be explored in zebrafish. Ultrastructural analysis (Willett *et al*., 1999) identifies the first T cell precursors in the thymic rudiment at 65 hpf and WISH at 72 hpf (Willett *et al*., 1999; and our own observation). This leads to a bilateral staining pattern with lymphoid probes such as *Rag-1* (see Fig. 3F). A section of a day (d) 7 wild-type thymus displaying a heterogeneous population of cells of epithelial and hematopoietic origin is shown in Fig. 3E.

In summary, genes required for the formation of the thymic rudiment in chicks and mammals are expressed in zebrafish in the correct temporo-spatial frame. This suggests that early thymic development is conserved throughout vertebrate evolution.

b. Thymus Development Beyond the Larval Stage. Subsequent to its initial formation during the first week of development, the zebrafish thymus undergoes a morphologic transformation, which has been examined by detailed morphometric analysis (Lam *et al*., 2002). From its original pouch-like shape the thymus rapidly grows into a cone-like structure between weeks two and six of life. At week three a demarcation into thymic cortex and medulla is first discernible (Lam *et al*., 2002; Schorpp *et al*., 2002) evidenced by absence of Rag-staining in the medulla, paucity of TCR-alpha in the cortex, which remains in close proximity to the pharyngeal cavity (Schorpp *et al*., 2002) and strong TCR-alpha expression in the medulla. Furthermore, the cortex is far more cellular than the medulla, in keeping with findings in mammals, where positive selection occurs predominantly in the cortex, while negative selection is effected at the cortico-medullary zone. In addition, septa-like structures were observed in the 6-week-old thymus at the cortico-medullary junction. *Whnb* is expressed in epithelial cells of both, cortex and medulla (Schorpp *et al*., 2002).

Lam *et al*. also described involution of the zebrafish thymus as measured by a decrease in thymic volume as a consequence of atrophy and replacement with connective tissue. The initiation of this process coincides with sexual maturity, a phenomenon also known to occur in other fishes (Chilmonczyk, 1992) and mammals (Turner, 1994).

B. T Cell Development

1. T Cell Receptor Genes in Teleosts

Unlike the immunoglobulins (see below), much less is known regarding the TCRs of primitive vertebrates. The higher molecular evolutionary rate of TCRs precludes routine cross-hybridization screening approaches and the vast majority of studies have required highly degenerate PCR strategies as a means to isolate the respective TCR genes (Rast *et al*., 1995; Hawke *et al*., 1996, 1999; De Guerra and Charlemagne, 1997; Wilson *et al*., 1998; Haire *et al*., 2000). While scant data are available regarding the TCRs in primitive vertebrates, it is presumed that all four classes of TCRs (α, β, γ, δ) will be identified since they have been found in the skates, a taxon which represents the most basal vertebrate that possesses an adaptive immune system (Rast *et al*., 1997; Litman *et al*., 1999).

Thus far, TCRα genes have been isolated from the zebrafish (Haire *et al*., 2000) and their expression patterns are consistent with early development of the T cell compartment (Trede *et al*., 2001; Lam *et al*., 2002). As an alternative to the PCR based strategy, we have taken a genomic sequencing approach in order to identify and characterize zebrafish TCRα genes. Using probes to both Vα and Cα we constructed a PAC contig encompassing the entire TCRα locus (T. Ota *et al*., unpublished). Partial annotations are listed for GenBank nos. AL591481, AL591511 and AL592550. Thus far the sequencing data suggest that the locus contains over 150 non-allelic Vα genes and that these can be classified into at least 86 different V families. Characterization of the expression patterns of many of these families is in progress (L. Steiner, unpublished). In terms of genomic organization, it appears that the zebrafish locus is very similar to that of the freshwater pufferfish, *Tetraodon nigroviridis*, whose TCRα locus has been sequenced in its entirety (Fischer *et al*., 2002). The linkage of TCRδ genes to the TCRα locus in zebrafish has not yet been established, however, it is predicted to be tightly linked as in the pufferfish. The gene complexity of the zebrafish TCRα locus, however, is considerably greater than that found in either *Tetraodon* or *Fugu* (Fischer *et al*., 2002; *Fugu* genome supplementary online materials: www.sciencemag.org/cgi/content/full/1072104/DC1).

2. T Cell Ontogeny in Zebrafish

In mouse, T cell precursors can be found on embryonic day 9.5 (E9.5) in the yolk sac (Yoder *et al*., 1997) and on E10.5 in the AGM region (Cumano *et al*., 1996; 2000). As mentioned above (Section III.A.3.b) the anatomic region corresponding to the AGM region in zebrafish may be the dorsal aorta circa 36 h of development. Data to corroborate this line of reasoning come from gene expression studies, demonstrating expression of markers of

hematopoietic progenitors, such as *c-myb*, in the dorsal aorta (Thompson *et al*., 1998). In the mouse, T lineage-committed (Thy-1^{+}c-kit^{low}) cells have been identified in fetal blood circulation (Rodewald *et al*., 1994). This type of analysis in the zebrafish awaits identification of the Thy-1 and c-kit orthologs and/or availability of antibodies directed against the corresponding surface proteins. Transplantation of hematopoietic precursors from various stages of development derived from transgenic fish expressing green fluorescent protein (GFP) under the control of the *GATA-1* or *LMO2* promoters into *Rag-1* deficient recipients (see Sections IV.B and VII.A) could be instrumental for the identification of the origin of pro-T cells in zebrafish. Identifying GFP positive cells in the thymus of the *Rag-1* deficient recipients, followed by FACS sorting and morphologic as well as gene-expression analysis would be a straightforward assay in this system.

By ultramicroscopy and gene expression studies T cell-specific genes can first be detected at 72 h in the thymus, making it the first zebrafish organ to become lymphoid. Analysis of T cell development by WISH during the first week of wild-type zebrafish development is shown in Fig. 4. By analogy with mammals, T cells are expected to express genes such as *Ikaros*, *GATA-3*, *Rag-1*, *lck* and *TCRα* during early development. *Ikaros* belongs to the family of zinc-finger transcription factors that act early in lymphoid development (Georgopoulos *et al*., 1994) and is expressed in the brain and thymus (Willett *et al*., 2001). *GATA-3*, which acts at the level of the T cell progenitor is expressed in brain, thymus and pharyngeal endoderm. *Rag-1* (Fig. 4) and *Rag-2* (not shown) are expressed in the thymus and the olfactory placode throughout the lifespan of zebrafish. The presence of *Rag-1* and *Rag-2* in the olfactory placode of zebrafish (Jessen *et al*., 1999, 2001) prior to the onset of thymic population with T cells is intriguing, but so far gene expression studies (our own observations) and histologic analysis (Jessen *et al*., 2001) have failed to demonstrate the presence of T cell precursors in this organ. For example, the src kinase p56*lck* is exclusively expressed in the thymus (Fig. 4). The thymus is permissive for the development of non-T cells belonging to the myeloid and B cell lineages (Barcena *et al*., 1994). In the absence of standard T cell markers of differentiation in zebrafish, such as CD4 and CD8, or B cell markers such as CD19, it is not possible to attribute the lymphoid cells in the thymus to the T or B cell lineage definitively. However, the expression of *GATA-3* and *TCRα*, combined with the absence of *Pax-5* expression (Fig. 4, bottom panel), in the zebrafish thymus strongly argues for a T cell identity of these cells. Furthermore, B cells of higher vertebrates develop primarily in the bone marrow. The pronephros/kidney of the zebrafish, which is the larval and adult site of hematopoiesis in this organism, is devoid of *Rag-1* expression until 21 days of development (Willett *et al*., 1999). The paucity of available reagents in the zebrafish, such as antibodies directed against T cell surface markers, or

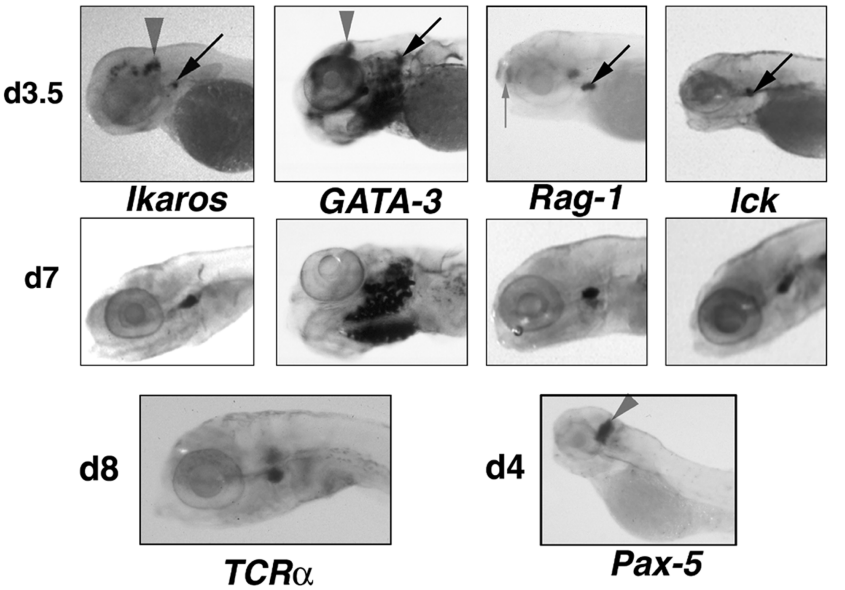


FIG. 4. Expression of lymphoid genes in zebrafish larvae. The top row shows whole mount *in situ* hybridization (WISH) analysis of day (d) 3.5 wild-type zebrafish larvae with the indicated probes. A grey arrowhead indicates expression of *Ikaros* and *GATA-3* in brain, black arrows indicate thymic expression with all probes, and a grey vertical arrow indicates *Rag-1* expression in the olfactory pit. Expression of the indicated probes is shown in the middle row in d7 wild-type larvae. The bottom row shows expression of *TCRα* and *Pax-5* in d8 and d4 wild-type larvae, respectively. The grey arrowhead indicates expression of *Pax-5* at the midbrain/hindbrain boundary. Given the transparency and positioning of the larvae, contralateral thymic staining at d3.5 with the *Rag-1* and at d8 with the *TCRα* probes is seen. (Reproduced with modifications from Trede *et al.* (2001) with permission.)

genetic probes for markers of T cell activation and differentiation, currently renders a detailed analysis of T cell development difficult. We are in the process of creating a zebrafish lymphoid library (see Section III.A.1), which will provide some of the reagents necessary to gain new insights into zebrafish lymphopoiesis.

C. B Cell Development in Teleosts

1. Immunoglobulin Genes in Teleosts

Among the receptor molecules of the adaptive immune system, immunoglobulins have been the most extensively studied among vertebrates. This is primarily due to the fact that Ig gene probes will cross-hybridize across

wide phylogenetic distances, a situation not possible for MHC or TCR genes. Indeed, the first shark Ig V_H genes were isolated via cross-hybridization with a mouse V_H segment (V_H-S107) known to be specific for phosphorylcholine (Litman *et al*., 1985). Thus, using heterologous gene probes it has been possible to isolate and characterize IgH and IgL genes from myriad vertebrate species both at the cDNA and genomic levels. This has resulted in a fairly comprehensive survey, albeit with some taxon sampling biases, of Ig genes across the vertebrate radiation. Table I lists features of the Ig system from the major lineages of vertebrates and highlights salient similarities and differences among and within the group.

While the overall structure and function of immunoglobulin molecules *per se* are highly similar across all gnathostomes, the genomic organization of immunoglobulin genes can be quite disparate. That is, the V, D (heavy chain only), J and C components that comprise Ig genes are highly similar between vertebrate lineages. The genomic structure of the Ig families, however, can be radically different. This is evinced by the different genomic organizations observed within and among the vertebrates. The two general forms observed are the "clustered" arrangement first seen in the IgH genes of cartilaginous fishes and the "translocon" arrangement described for mouse and human (see Table I). The translocon arrangement is the organization found in mouse and human and consists of a long, extended locus comprised of tandem segments that are recombined during B cell development (Vn-Dn-Jn-Cn for the heavy chain, where n can be anywhere from 1 to >100). In contrast, the clustered arrangement is fundamentally different in that each cluster contains essentially one or a few respective segments, e.g., (V-D-J-C)n, where the cluster is reiterated throughout the genome (Du Pasquier and Flajnik, 1998; Litman *et al*., 1999; Bengten *et al*., 2000). In both situations, translocon and clustered, RAG-driven recombination between the V, (D) and J segments are required.

Another salient feature of humoral immunity in lower vertebrates is that the tissues associated with development and differentiation of B lymphocytes vary between major lineages (Table I). For example, in fishes (including cartilaginous fishes) there is no bone marrow; B lymphocyte development thus occurs at other sites such as kidney, spleen and elsewhere. The bone marrow can thus be considered a "derived" tissue within vertebrates that has been co-opted for B lymphocyte development (B cell receptor production) in the more recent phylogenetic lineages (Table I). With zebrafish, we need to be cognizant of these sorts of differences if we are to use this system as an immunological model. Most recently, it was suggested that the pancreas is the primary site of early B cell development and Ig expression in the zebrafish (see Section III.C.2), a surprising finding given that the pancreas has never been recruited for this function in other vertebrates.

TABLE 1. IMMUNOGLOBULINS AND ASSOCIATED FEATURES AMONG THE VERTEBRATES.[1]

Vertebrate taxon	Adaptive immunity	Bone marrow equivalents[2]	Ig isotypes	Gene organization[3]	Germinal centers	Class switching
cyclostomes	no	kidney, protovertebral arch (fat column), intestine	none	n/a	n/a	n/a
cartilaginous fishes	yes	epigonal organ, Leydig organ, spleen, kidney, gonads	IgM, IgNAR, IgW; three or more different light chain isotypes	clustered for both heavy and light chain loci	no	no
teleost fishes	yes	head kidney, spleen, pancreas, gonads, heart	IgM, IgD; two or three light chain isotypes	translocon for heavy chain; clustered for light chain	no	no
lobe-finned fishes	yes	liver, kidney, spleen, gonads, spiral valve in lungfish; not known in coelacanth	IgM, two other isotypes known in lungfish; light chain isotypes unknown	coelacanth has an IgM organization intermediate between clustered and translocon	no	?
amphibians	yes	bone marrow, liver, kidney	IgM, IgY, IgX; two or three different light chain isotypes	translocon	no	yes
reptiles	yes	bone marrow, other?	IgM, others?	translocon	no	yes?
birds	yes	Bursa of Fabricius	IgM, IgY, IgG; two light chain isotypes	translocon	yes	yes
mammals	yes	bone marrow	IgM, IgD, IgG, IgA, IgE; two light chain isotypes (κ, λ)	translocon	yes	yes

[1]Data taken and/or summarized from numerous sources including: (Amemiya *et al*., 1993, Aparicio *et al*., 2002, Bengten *et al*., 2000, Turchin and Hsu, 1996, Marchalonis *et al*., 1998, Zapata and Cooper, 1990, Danilova and Steiner, 2002, Zapata and Amemiya, 2000, Litman *et al*., 1993, Du Pasquier and Flajnik, 1998, Flajnik, 2002, Warr, 1997). This table is a broad generalization only and is not meant to be all-encompassing.

[2]"Bone marrow equivalents" refers to the histological observation of hematolymphoid tissue resembling bone marrow, the site of mammalian B-cells. Note, some of the tissues listed in this column may actually represent secondary sites of development of B-cells.

[3]The mammalian type, extended locus with multiple tandem segments is referred to as the "translocon" organization; the shark organization whereby individual segmental elements are organized in discrete units (e.g., V-D-J-C for the heavy chain) is referred to as the "clustered" organization (Litman *et al*., 1999, Bengten *et al*., 2000).

Immunoglobulin Ig V_H and Cμ genes have been isolated from the zebrafish and are similar in inferred amino acid sequences to those of other vertebrate species (Danilova *et al*., 2000). Preliminary data from genomic mapping and sequencing studies of zebrafish IgH-containing PAC clones suggests that the zebrafish, as predicted, has a translocon organization of its heavy chain locus (T. Ota, unpublished). Isolation of its light chain genes as well as genomic characterization of its other heavy chain isotype (IgD) (Wilson *et al*., 1998; Bengten *et al*., 2000) is in progress. It is predicted, however, that the results will be unremarkable based on the genome sequence of *Fugu* (Aparicio *et al*., 2002), a comparatively closely related teleost fish.

An area of fundamental importance in humoral immunity is the regulation of gene expression of the Ig genes. Considerable progress has been made in understanding the IgH enhancer in the channel catfish (Magor *et al*., 1994, 1997; Cioffi *et al*., 2001). It is important to note that the IgH locus in the catfish exhibits a translocon organization similar to that of mammals, yet its major IgH enhancer is in a vastly different locale. In the mouse and human, this enhancer is found in the intron between the J_H region and the first exon of Cμ1. In the catfish, however, the strongest IgH enhancer has been functionally localized to a region near the second transmembrane exon (Magor *et al*., 1994; Warr, 1995). Using the same mammalian cell culture-based system for assaying enhancer activity, B. Magor and colleagues (Univ. Alberta) have shown that the major zebrafish enhancer is not localized to the same region as in either the mouse or catfish (B. Magor, personal communication). This is somewhat surprising given the close phylogenetic relationship of the zebrafish and catfish within the Ostariophysans (Nelson, 1984). It will be interesting to determine whether the zebrafish system has enhancers and other *cis*-regulatory elements that specifically drive IgM expression in the pancreas.

2. B Cell Development Initiates in the Zebrafish Pancreas

Detection of T cells by WISH using probes such as *Rag-1* (see Section III.B.2) starting at 72 hpf is facilitated by the very superficial position of the thymus. B cell development is more difficult to examine by WISH given the central location of the pronephros, the site where lymphocytes are produced starting at three weeks post fertilization (Willett *et al*., 1999). At that stage of development WISH is unable to detect transcripts except for the most superficial regions of the juvenile zebrafish. Transcripts of *Rag-1* as well as cμ were detectable on sections in adult zebrafish in the kidney and intestine (Danilova and Steiner, 2002), consistent with other teleost species (Zapata and Cooper, 1990; Zapata *et al*., 1996; Fournier-Betz *et al*., 2000; Zapata and Amemiya, 2000) and higher vertebrates, where B cells are produced in the bone marrow and are also found in the intestine. Intestinal B lymphocytes in

teleosts are found in the loosely organized gut associated lymphoid tissue (Picchietti *et al*., 1997; Fournier-Betz *et al*., 2000; Zapata and Amemiya, 2000). Surprisingly, Danilova and Steiner also found co-expression of *Rag-1* and cμ in the pancreas of adult zebrafish. Tracing this gene expression pattern back in ontogeny, the pancreas was shown to be the initial site of B cell development in zebrafish larvae, initiating as early as 10 dpf by sectioning and 4 dpf by WISH (Danilova and Steiner, 2002). By RT-PCR, membrane IgM was first expressed on day 7 pf. Although double-stains with pancreas-specific (e.g., insulin) and lymphocyte-specific probes (e.g., *Rag-1*, *Ig*μ) were not done, these findings provide the first suggestion of initial B cell development in pancreatic tissue and open the way for future studies. For example, it will be interesting to study other teleosts and higher vertebrates for a role of the pancreas in B cell development. As the developing B cells are presumably not produced *in situ* in the pancreas, but are rather derived from hematopoietic stem cells, it will be interesting to determine the soluble factors secreted by the developing pancreas, which attract B cell progenitors to the organ. Furthermore, zebrafish mutants affecting pancreas development can be studied to ascertain the necessity of this organ for physiologic B cell development. Analogous experiments can be carried out by transient inhibition of pancreatic development using morpholinos (Huang *et al*., 2001; Yee *et al*., 2001) followed by analysis of this delay on B cell development.

D. Secondary Lymphoid Organs

1. The Spleen is the Major Secondary Lymphoid Organ in Teleosts

Primary lymphoid organs are the sites where T and B cell production occurs. Clusters of lymphoid cells can first be observed in lampreys (Zapata *et al*., 1981; Hagen *et al*., 1983) and in the Atlantic hagfish in the "central mass" of the pronephros (Zapata *et al*., 1984; Zapata and Amemiya, 2000), which is the site of bone marrow hematopoiesis in many lower vertebrates. However, there are no distinct secondary lymphoid organs in these primitive vertebrates (Zapata *et al*., 1996; Zapata and Amemiya, 2000). It is only with the advent of modern fishes that primary and secondary lymphoid organs are truly discernible. In chondrichthyes and osteichthyes B cells are produced in different organs that represent sites of hematopoiesis. These can range from the meninges to the gonads, but in most species involve the kidney (reviewed in Zapata and Amemiya, 2000), while T cells are always produced in the thymus (see above).

Secondary lymphoid organs in vertebrates serve as trapping and processing devices for antigen. It is here that antigen-presenting cells communicate with T cells, and where T cell–B cell interactions take place. The major secondary lymphoid organ consistently found in fishes is the spleen. The mammalian

spleen is compartmentalized into areas of erythroid predominance (red pulp) and lymphoid follicles (white pulp). At the border between red and white pulp is the marginal zone, where antigen trapping occurs and cells transit in and out of the white pulp. Upon exposure to antigen, B–T cell cooperation leads to activation of both cell types. Activated B cells along with the antigen-specific T helper cells migrate to the primary follicles of the spleen, where the B cells form distinct structures, the germinal centers. It is here that B cell repertoire selection occurs through isotype class switching and somatic hypermutation-mediated affinity maturation of the B cell receptor for antigen (BCR). In birds and rabbits gene conversion in secondary lymphoid organs is the major event that confers diversification to the Ig repertoire. There appears to be no direct correlation between germinal center formation and efficient affinity maturation. For example in birds, which form germinal centers during secondary immune response, affinity maturation is rather poor (Du Pasquier *et al*., 1998).

In contrast to higher vertebrates, the marginal zone separating the splenic white and red pulp is not fully developed in fishes. White pulp, consisting of lymphocytes, APCs and plasma cells are intermingled with red pulp (Zapata and Amemiya, 2000). Following antigen stimulation white pulp in teleosts increases in size. However, germinal centers are conspicuously absent in ectotherms, including fishes. Class switching is a process that has its earliest evolutionary roots in amphibians and does not occur in fishes. On the other hand, somatic hypermutation has been clearly demonstrated in lower vertebrates, including fishes. However, affinity maturation of immunoglobulins is inferior in fishes compared to higher vertebrates and the anatomic site where this process occurs in ectotherms is unknown.

The three processes affecting diversification of the immunoglobulin repertoire, somatic hypermutation, class switching and gene conversion (found in birds and rabbits) depends on the activity of a recently discovered enzyme, activation-induced cytidine deaminase (AID). The presence of this enzyme has been documented and its activity studied in mammals. Given the evidence for hypermutation of the shark heavy and in particular light chains (Lee *et al*., 2002b), AID has been presumed to be present in lower jawed vertebrates (see above). It is conceivable that the mechanism of hypermutation evolutionarily precedes adaptive immunity (Flajnik, 2002). Verification of this hypothesis awaits cloning of the AID gene and study of its function in agnathan vertebrates.

The gut is the other anatomic site where lymphocytes are consistently found in fishes. Lymphoid aggregates are found in the lamina propria of the teleostian gut, but they are not encapsulated and hence do not represent true, isolated lymphoid organs. These aggregates represent mostly Ig positive

plasma cells (Rombout *et al*., 1993). DLT15+ T cells were also found in the intestine of the sea bass (*Dicentrarchus labrax*) (Picchietti *et al*., 1997; Romano *et al*., 1997) and probably represent a majority of the Ig negative intraepithelial lymphocytes (Rombout *et al*., 1993).

2. *Secondary Lymphoid Tissues in Zebrafish*

As mentioned above, teleosts do not have lymph nodes. This makes the spleen the major secondary lymphoid organ that can be traced throughout vertebrate phylogeny (reviewed in Zapata and Amemiya, 2000). In mammals, spleen development has been shown to be critically dependent on the homeobox gene Hox-11 (Roberts *et al*., 1994). During zebrafish development, Tom Look's group first identified the putative splenic primordium by WISH using the *Hox-11/Tlx-1* probe (Langenau *et al*., 2002). Figure 5 shows developmental expression of *Hox-11/Tlx-1* in wild-type zebrafish larvae. We first detected evidence of *Hox-11* as an asymmetric focus of expression on the left anterior gut, in the region of the putative spleen primordium on day 4 pf. Over the next several days, signal intensity increased in this area. Cross-sections confirm the identity of this focus of expression with an organ on the left side of the larva in proximity to the gut (Fig. 5, T. Palomero, D. Langenau, A. Ferrando, J. Kanki and Tom Look, personal communication).

In adult zebrafish the spleen is a highly cellular organ. Danilova and Steiner found predominantly erythrocytes in the adult spleen and no significant expression of either *Rag-1* or *Igμ* (Danilova and Steiner, 2002). Figure 7 shows a FACS profile of cells populating the spleen and confirms the erythroid predominance of the teleostian spleen. However, there is a subpopulation of lymphocytes, which can be distinguished by light scatter characteristics (see Section IV.A). These cells presumably represent mature lymphocytes, which do not rearrange their receptors for antigen (TCR and BCR, respectively), explaining the absence of Rag-expression, which we also confirmed in *Rag-2*-GFP transgenics (Fig. 7C). Germinal centers are not found in teleost spleen and class switching does not occur due to the absence of isotypes other than IgM and an IgD equivalent (see Section III.C.1). However, evidence for somatic hypermutation is found in teleost B cells (reviewed in Flajnik, 2002). We have therefore conducted a search for a zebrafish homolog of AID. A stretch of sequence homology to human AID was found in the zebrafish database Sanger Center. Further examination of the genetic region surrounding the sequence homology revealed the entire ZF AID gene encompassing 5700 bp. Several lines of evidence support the identity of zebrafish AID as the true ortholog of human AID. There is a high degree of sequence homology between zebrafish AID with human (60% identity at the protein level) and mouse AID (64% identity at the protein level). Compared with human AID,

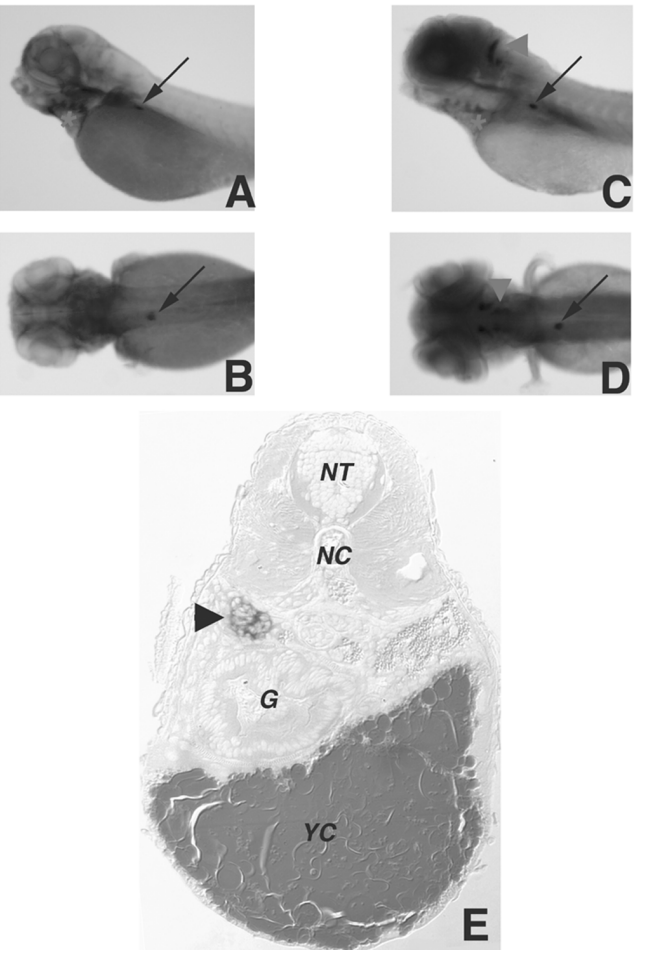


FIG. 5. Expression of Hox-11 in Zebrafish Larvae. A–D. Hox-11 expression in wild-type zebrafish. WISH analysis reveals Hox-11 in pharyngeal endoderm (asterisks) in side view on d4 (A) and 6 (C), brain in dorsal view on d6 (D), and in the left anterior gut (arrows). E. Cross-section of d4 wild-type zebrafish larva after WISH. Black arrowhead indicates Hox-11 expression on left side in proximity of gut (G). YC = yolk cell, NC = notochord, NT = neural tube. (Cross-section courtesy of T. Palomero, D. Langenau, A. Ferrando, J. Kanki, T. Look.)

zebrafish AID has conserved intron-exon boundaries for exons 1–5. Expression studies are under way. We were able to amplify a correctly spliced AID transcript from day 2 embryos. This implies that zebrafish AID has a role beyond B cell antigen receptor maturation, as B cells are first detected in the zebrafish pancreas at 4 dpf (Danilova and Steiner, 2002) and

predicts that AID may also be found to be expressed in more primitive vertebrates, where it could fulfill a role in DNA metabolism.

The gut is a site of lymphoid activity in zebrafish, although no distinct secondary lymphoid structures, such as Peyer's patches, can be identified histologically. Thus, *Igμ* and *Rag-1* positive cells were detected in the lamina propria of the straight part of the intestine (Danilova and Steiner, 2002) reminiscent of *Rag-1* expression detected in the activated B cells that populate murine Peyer's patch germinal centers (Han *et al*., 1996). Interestingly, Danilova and Steiner report that patches co-expressing *Igμ* and *Rag-1* in the zebrafish gut resembled Peyer's patches, previously not reported in lower vertebrates (Danilova and Steiner, 2002).

IV. Phenotypic Characterization of Zebrafish Hematolymphoid Cells

Mutagenesis screens in zebrafish have led to the discovery of a wide array of mutants that fail to correctly develop embryonic blood cells. Due to the early time points analyzed, the vast majority of these mutants show defects in the maturation of primitive erythrocytes. As discussed above, definitive, multilineage hematopoiesis does not occur until several days post fertilization in larval development, making simple visualization of non-erythroid mutants difficult. Current screens aimed to uncover deficiencies in definitive hematopoiesis have thus relied upon *in situ*-based approaches for both the lymphoid (Trede and Zon, 1998; Schorpp *et al*., 2000; Trede *et al*., 2001) and myeloid lineages (Bennett *et al*., 2001; Lyons *et al*., 2001a,b; Lieschke *et al*., 2001, 2002). These screens have yielded mutants that fail to specify early larval lymphoid and myeloid populations. It remains to be determined whether these mutants also show defects in adult blood cell production in the kidney, the teleost equivalent of mammalian bone marrow. Additionally, since primitive erythrocytes are thought to derive from different populations of hematopoietic stem cells (HSCs) than their adult, definitive counterparts, it remains to be determined whether the embryonic blood mutants also show defects in producing adult red blood cells. To this end, we have undertaken a thorough characterization of the adult zebrafish hematopoietic system.

A. Lineage Separation by Light Scatter Characteristics

Blood production in adult zebrafish, like other teleosts, occurs in the kidney, which supports both renal functions and multilineage hematopoiesis (zebrafish blood cell types are depicted in Fig. 6). It appears that all blood cell lineages develop here from HSCs, with the exception of mature T lymphocytes, which

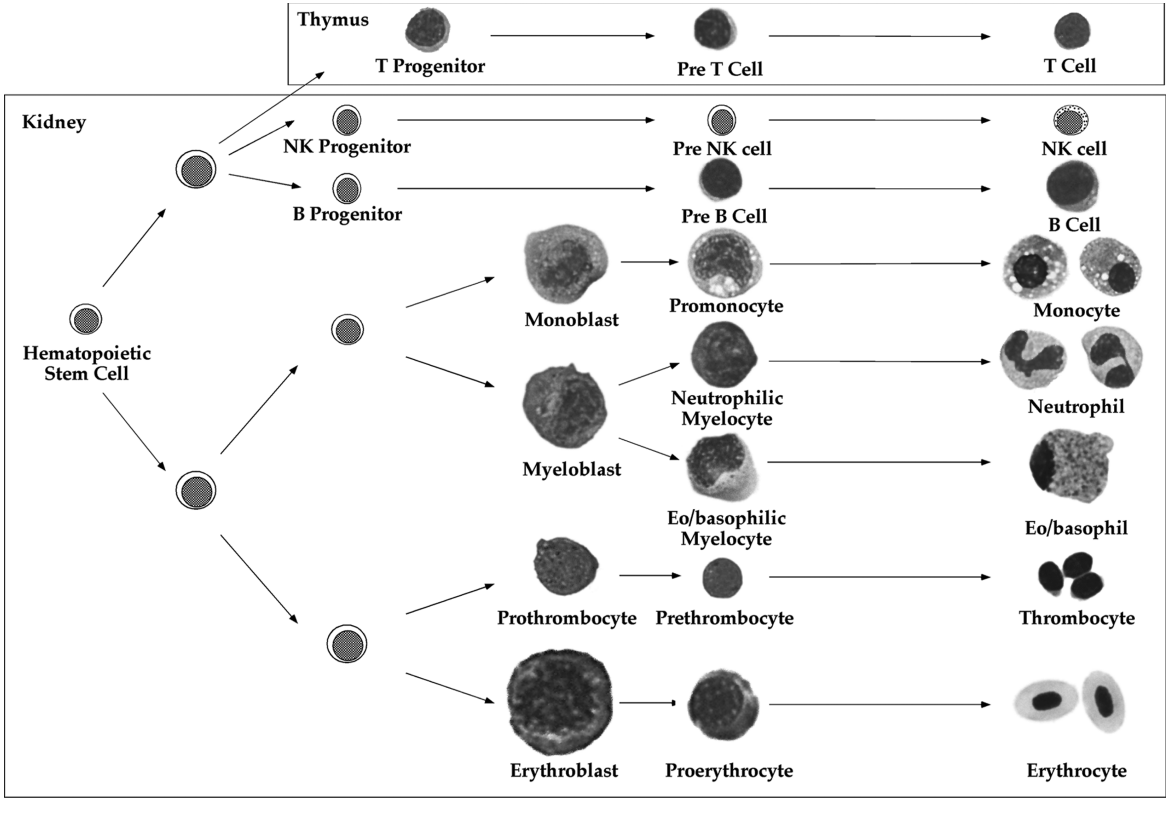


FIG. 6. Model of definitive hematopoiesis in adult zebrafish. Cell types shown are the actual cells found in the zebrafish kidney. May-Grunwald/Giemsa stain. (See Color Insert.)

are educated in the thymus. To assess the parameters of steady state definitive hematopoiesis, we have recently performed differential cells counts in adult hematolymphoid tissues, including the kidney, spleen, and blood (D. T. and L. I. Z., unpublished). Additionally, we have analyzed these tissues by flow cytometry. Examination of kidney marrow, spleen, and blood by light scatter characteristics reveals distinct profiles for each (Fig. 7A). Surprisingly, when compared to mammalian bone marrow scatter characteristics, there exist several discrete "scatter" populations in the zebrafish kidney. From these profiles, the major blood lineages can be isolated to purity from each tissue by cell sorting (Fig. 7B). Mature erythroid cells are found exclusively within a forward scatter $(FSC)^{low}$ fraction, myelomonocytic cells within only a FSC^{high}, side scatter $(SSC)^{high}$ population, lymphoid cells within a FSC^{int} SSC^{low} subset, and immature precursors within a FSC^{high} SSC^{int} subset. Percentages of cells within each scatter population match those obtained by morphological cell counts, demonstrating that this flow cytometric assay is accurate in measuring the relative percentages of each of the major blood lineages. This new technique can thus be used to precisely identify and quantitate defects in definitive blood mutants. In this way, we have analyzed the zebrafish "embryonic" blood mutants. Since many of the primitive mutants are embryonic lethal when homozygous, we have analyzed heterozygous carriers of one mutant allele in adult fish. We find that several mutants show haploinsufficiency as evidenced by aberrant kidney erythropoiesis (D. T. and L. I. Z., unpublished). In general, mutant phenotypes of adult heterozygotes appear intermediate to those of their wild-type siblings and embryonic homozygous mutants. This suggests that many of the gene functions required to make embryonic erythrocytes are similarly required in their adult counterparts. It will be interesting to similarly assess additional lymphoid- and myeloid-specific blood mutants arising from ongoing mutagenesis screens.

B. Use of GFP Transgenes to Define Zebrafish Blood Lineages

To complement the mutant analysis described above and to test autonomy of mutant gene function, we have developed zebrafish hematopoietic cell transplantation (HCT). As a donor cell marker for transplants, we have made use of adult zebrafish expressing GFP under control of the erythroid-specific *GATA-1* promoter. Since erythroid cells are short-lived, they are produced continuously from kidney precursor populations. Continued production of GFP^{+} erythrocytes from transplanted kidney cells thus serves as a surrogate marker of donor-derived hematopoietic stem and progenitor cell activity. One potential complication of transplantation experiments in zebrafish is immune rejection by the host, since it has not been possible to create inbred strains of genetically identical individuals. To lessen this risk, we have chosen 2-day-old

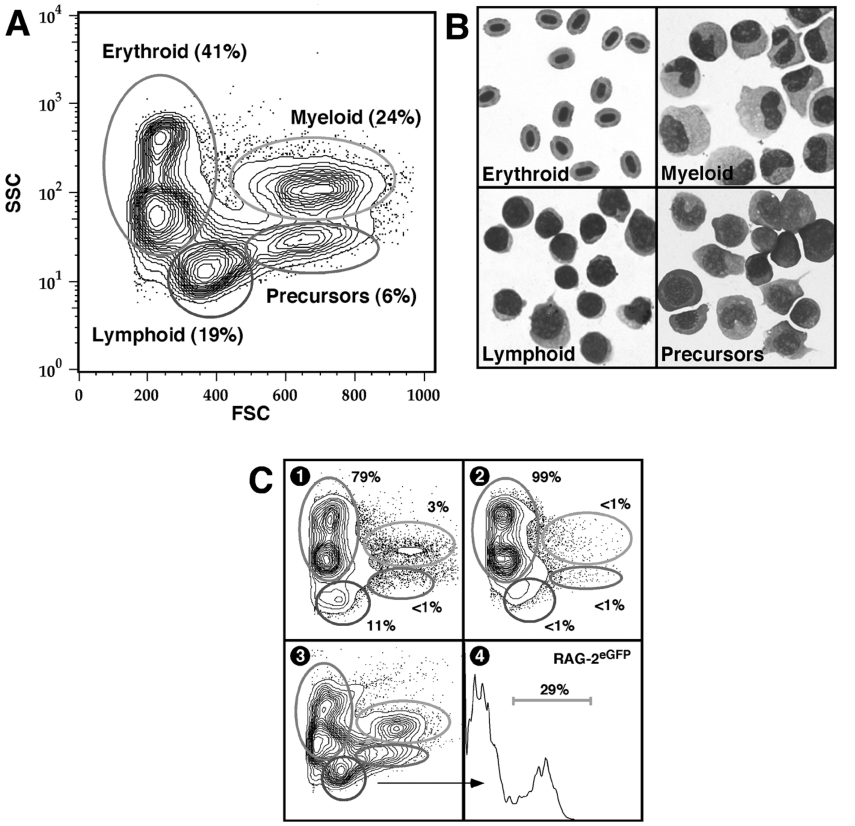


FIG. 7. Separation of definitive blood lineages by flow cytometry. A. Single-cell suspensions of adult kidney cells form distinct populations when analyzed by size (forward scatter; FSC) and granularity (side scatter; SSC). B. Sorting of each population reveals that cells within the red gate are comprised of only mature erythrocytes (upper left panel), that the blue gate contains only lymphocytes (lower left panel), that the purple gate contains immature precursors of all mature blood lineages (lower right panel), and that the green gate contains only myelomonocytic cells (upper right panel). C. Scatter profiles can also be used to identify and quantitate each lineage in the adult spleen (panel 1), and in the peripheral blood (panel 2). Panel 3 shows the kidney profile of a transgenic zebrafish expressing GFP by the lymphoid-specific *RAG-2* promoter. Approximately 30% of the cells within the lymphoid scatter population are GFP-positive (panel 4), whereas all other populations are uniformly negative (not shown). All numbers shown are relative percentages of each respective tissue. (See Color Insert.)

embryos as transplant recipients. Lymphocyte development, as judged by *in situ* expression patterns of *Rag1/2* genes as well as T and B antigen receptor genes, does not begin until 4 dpf (Willett *et al*., 1997; Trede and Zon, 1998; Willett *et al*., 1999; Trede *et al*., 2001). Transplantation before this stage may thus tolerize the host to the graft. Forty-eight hour embryos

are also transparent, allowing transplant success to be easily visualized by circulating GFP^+ cells. Transplantation of whole kidney cells from *GATA-1*^{eGFP} adults into mutant *vlad tepes* embryos, for example, rescues multilineage hematopoiesis for at least six months in these animals that normally die by two weeks of age from erythropoietic failure (D. T. and L. I. Z., unpublished). This demonstrates that *vlad tepes* is required in a cell autonomous manner and strongly suggests that long-term survival of reconstituted individuals is mediated by stable hematopoietic stem cell engraftment.

The ability to isolate the cells of each of the major hematopoietic lineages has facilitated the development of novel reagents to better understand blood development in zebrafish. We have generated cDNA libraries from highly purified myeloid, lymphoid, and precursor cell populations. We are currently sequencing at least 10,000 clones from each library to identify both novel genes and zebrafish orthologues of known mammalian genes. In an approach pioneered by the Thisse lab in zebrafish (Donovan *et al*., 2002), interesting clones will be used to generate *in situ* probes in order to perform high-throughput expression screens over early embryonic and larval development to identify genes expressed in lymphoid, myeloid, or erythroid cell subsets. Genes with interesting expression patterns can then be tested functionally in gain- or loss-of-function experiments by RNA or morpholino injections, respectively. The relative ease of testing functionality in zebrafish is an important advantage over similar approaches in mammals.

Transgenic technology is now commonly used in zebrafish, and many investigators have made a variety of fluorescent reporter lines to mark cell lineages of interest. Lin and colleagues (Long *et al*., 1997) reported the first blood-specific transgenic line in which GFP was driven by the erythroid-specific *GATA-1* promoter. Another GFP line marking early lymphocytes has been created using the zebrafish *Rag-2* promoter (Jessen *et al*., 2001; and D. Langenau and T. Look, unpublished). Analysis of adult kidney marrow shows that the *Rag-2* promoter is active only within the lymphoid scatter population (Fig. 7C, panels 3, 4), and is expressed in approximately 29% of the cells within this fraction. This expression is presumably in early B lymphocytes in the process of rearranging their antigen receptor genes since the adult kidney has been shown to be the site of B cell production in other teleosts (Daggfeldt *et al*., 1993; Hansen and Zapata, 1998; Zapata and Amemiya, 2000). The *Rag-2* promoter is also active in the zebrafish thymus in developing T lymphocytes, but expression is absent in the adult spleen. We have also developed a transgenic line in which GFP is targeted to thrombocytes, zebrafish platelet equivalents, by the CD41 promoter (H.-F. Lin, D. T., C. Abraham, L. I. Z., and R. I. Handin, unpublished). These

transgenic animals are useful not only in tracking *in vivo* a population of interest but can also be used to create lineage-specific cDNA libraries in order to identify lineage-specific genes, expression patterns, and rapid functional testing as discussed above. These lines also afford the possibility of identifying populations enriched for hematopoietic stem and progenitor cells by flow cytometry and transplantation assays.

C. Imaging Techniques

The use of labeled cells also facilitates following specific cell populations in optically transparent zebrafish *in vivo*. Thus, recent developments in the field of intravital microscopy (IVM) have led to important insights into the function and kinetics of the immune system. Using fluorescent dyes or antibodies, cells can be readily tracked *in vivo*. While the applicability of this approach is limited in mammals, it can be a powerful tool to follow circulation and homing of cells in translucent animals, such as the zebrafish. Pigment-deficient strains as well as PTU-treated embryos are convenient subjects for studies. Even in wild-type fish, labeled cells can be easily analyzed in superficial vessels or in the fins (data not shown).

Given the paucity of reagents such as specific antibodies, we utilized the dye CFSE to label harvested cells from spleen and kidney in combination with Fluorescence Microscopy. We decided to use CFSE because of its low toxicity, its uncomplicated use and the excellent staining properties for leukocytes. Particularly in murine lymphocytes, this marker has proven to be an excellent tool for subsequent FACS analysis or IVM and cell tracking for up to six months, as well as for cell division studies (Lyons, 1999; Parish, 1999). Toxicity or specific alterations of cell functions have not been described at recommended concentrations.

Prior to injection into recipient fish, labeled cells can be FACS-sorted and divided into different fractions (Fig. 8A). Thus, labeled populations of lymphocytes or myeloid cells can be injected separately into recipient fish. Migration patterns as well as homing to superficial wounds can then be observed in the living animal by UV fluoroscopy (Fig. 8B, C). In necropsies, the final destination of homing cells can be analyzed. The jelly-like consistency of most internal organs and their translucence also facilitate optical studies (Fig. 8D, E).

In our preliminary studies, we used CFSE to label harvested spleen and kidney cells of zebrafish. On average, a combined 1–2 million cells of hematopoietic origin can be obtained per sacrificed animal. Due to lack of appropriate culture conditions for zebrafish cells, we immediately inject the labeled cells into recipient fish either intraperitoneally or intracardially. These approaches now enable us to address questions of homing of leukocytes to

wounds or sites of infection (see Fig. 8B, C and Section II.B.1.d). Allogeneic recipients can be used to study graft rejection as well as GvHD after irradiation of the host.

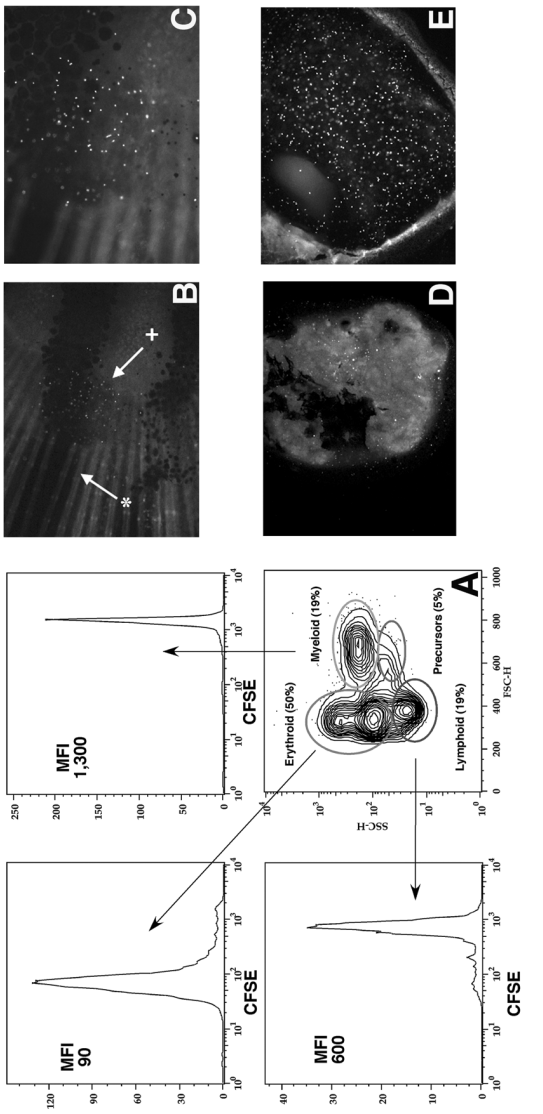
We tested if a GFP signal emanating from internal organs could be detected in live zebrafish. For this purpose we chose a transgenic line, which expresses GFP under the control of the insulin promoter. Consistently, a signal could be observed close to the midline anterior to the anal fins even at low magnification (Fig. 8F). To ascertain the origin of the signal, the fish in Fig. 8F was sacrificed and internal organs were visualized after opening the abdominal cavity. The signal originated from discrete foci in an organ that by macroscopic inspection and position relative to adjacent structures (gut) was identified as the pancreas (Fig. 8G). The ability to visualize GFP signals from internal organs in living adult zebrafish will be a great asset for future immunology screens in adult zebrafish.

V. The Zebrafish as a Vertebrate Model System for Forward Genetic Screens

A. Genetic Screens are a Powerful Method for Identifying Multiple Mutations Within a Genetic Pathway

Forward genetic screening methods are used to identify mutations in novel and known genes within a specific genetic pathway. Large-scale genetic screens in non-vertebrate organisms, such as yeast, worms, plants, and flies have identified hundreds of genes and loci involved in a plethora of biological pathways including the cell division cycle, axon guidance, and embryology, to name a mere few. Selected mouse mutants, dominant screens in mice, and mutations associated with human genetic traits and disease have provided a wealth of knowledge about specific genes. More recently, genetic screens in the zebrafish have revealed hundreds of loci essential for early vertebrate development (Driever *et al*., 1996; Haffter *et al*., 1996; Amsterdam *et al*., 1999; Golling *et al*., 2002). Many mutations can be grouped in specific phenotypic classes, suggesting that mutant gene products may function within the same genetic pathway. Importantly, a number of mutations reside in genes that are highly conserved between fish and humans, and often zebrafish mutant phenotypes resemble human disease conditions (Driever and Fishman, 1996; Dooley and Zon, 2000). With both the zebrafish and human genome sequencing projects completed, determining the function of the predicted 40–60,000 genes in the human genome can be approached by analyzing the effects of mutations in model organisms, such as zebrafish.

Zebrafish characteristics and life cycle facilitate genetic screening (Streisinger *et al*., 1981; Patton and Zon, 2001; Grunwald and Eisen, 2002).


A
B
C
D
E
Myeloid (19%)
Erythroid (50%)
Precursors (5%)
Lymphoid (19%)
SSC-H
FSC-H
CFSE
MFI 1,300
MFI 90
MFI 600

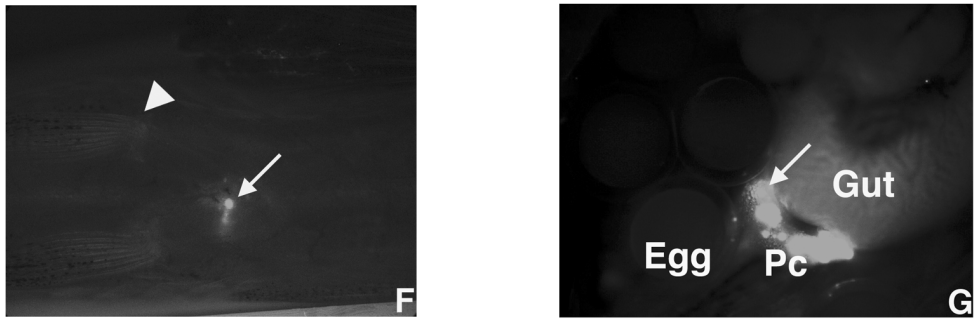


FIG. 8. Intravital microscopy. A. Light scatter characteristics of kidney and spleen cells. After labeling of whole kidney and spleen cells with CFSE, fractions were separately analyzed according to their light scatter characteristics. While red blood cells—although nucleated—show only little staining, myelomonocytic and lymphocytic cells are highly fluorescent. The background fluorescence of unstained cells is <2.0 (MFI = mean fluorescence intensity, based on the geometric mean). B., C. Accumulation of labeled mixed leukocytes at the site of inflammation. Two hours after superficial injection of complete Freund's adjuvant, cells cluster specifically at the site of injection. (Magnification 50× in C, 100× in D; * indicates background at the tail fin, + indicates the site of injection; pictures were taken with an inverted Epifluorescence Microscope, Leica DMIRE2.) D., E. Homing of leukocytes to kidney and spleen. Kidney (D) and spleen (E) taken from fish, two hours after injection of CFSE labeled mixed leukocytes. While the spleen shows homing of a homogeneous cell population, cells of different sizes, including several large cells, can be seen in the kidney (Magnification 50× for kidney and 100× for spleen). F., G. Imaging insulin-promoter-GFP transgenic female zebrafish. A live female zebrafish transgenic for insulin-promoter-GFP was anesthetized with tricaine and placed in a plastic container with abdomen pointing down. Pictures were taken with an inverted Epifluorescence Microscope, Leica DMIRE2. Arrowhead indicates anal fin, arrow indicates fluorescent signal (F, magnification 25 ×). Female was then sacrificed, abdominal cavity opened and placed on plastic dish (G, magnification 100 ×). Arrow indicates green fluorescent signal emanating from discrete foci in pancreas (Pc).

From the mid-1960s through to the 1980s, in an effort to develop a vertebrate genetic system, George Streisinger established that zebrafish genetic mutations could be generated and inherited in Mendelian fashion (Grunwald and Eisen, 2002). As small fishes (3–4 cm long as adults), zebrafish can breed efficiently and thrive within a laboratory setting, and the large numbers required for genetic screening can be housed in the laboratory. Sexually mature at three months of age, females typically lay 50–200 embryos, called a "clutch," and the fertilized embryos are collected and grown to adulthood. Zebrafish embryos are transparent, and because they are extruded prior to fertilization, the fundamental stages of early vertebrate development can be visualized and studied in live embryos under a dissecting microscope. By 24 hpf, zebrafish have a visible beating heart and blood flow, and behavioral responses to touch and light are readily observed.

Genetic screening techniques in zebrafish have expanded to encompass the initial visual assessment of developmental abnormalities and the more recent screening for mutants with defects leading to aberrant movement, metabolism, vision, behavior, learning, or drug addiction. Once isolated, zebrafish mutants are a powerful starting point for dissecting a genetic pathway when coupled with other mutants, small molecules, gene knockdown and overexpression technologies (see Section VII). Mutants also provide a starting point for the next genetic screen for new mutations that enhance or suppress the original phenotype (Trede *et al*., 2001; Patton and Zon, 2001; Kramer *et al*., 2002). Detection of the mutant phenotype, size of the fish facility, the type and number of mutations, and the method of cloning the mutant gene are important considerations in the design of a forward genetic screen. Here, we briefly review the genetic screening methods and techniques commonly used in zebrafish.

B. Generating Genetic Mutations in Zebrafish

The number and type of mutations generated depends on the choice of mutagen used in the genetic screen. Large numbers of mutations in founder fish reduce the number of individuals in subsequent generations that require screening before finding a phenotype of interest. The chemical mutagen most commonly used in zebrafish, ethylnitrosourea (ENU), generates point mutations throughout the genome, albeit with varying mutation rates among loci (Mullins *et al*., 1994; Solnica-Krezel *et al*., 1994; Riley and Grunwald, 1995; Knapik, 2000). Gamma and X-rays can generate a wide range of mutations, including point mutations, large genomic deletions and translocation events (Walker, 1999). The chemical mutagen trimethylpsoralen can generate smaller deletions in the range of 100 bp to 15 kb (Ando and Mishina, 1998). Point mutations can generate alleles of varying phenotypic strength,

while deletions can be an effective method to generate null alleles of known and unknown genes. Large deletions and translocations can complicate genetic screening, however, by affecting more than one gene, and possibly altering phenotypes within the clutch. Mutations generated by the above mutagens can be isolated by positional cloning (Talbot and Schier, 1999), and many mutations responsible for zebrafish phenotypes have been cloned. While labor-intensive, positional cloning has been greatly facilitated by the complete sequence and assembly of the zebrafish genome by the Sanger Institute (www.sanger.ac.uk). Furthermore, sequence homologies and overall close syntenic relationship to humans and the pufferfish, two organisms with fully sequenced and isolated genomes, greatly facilitates positioning and eventual cloning of mutated genes.

An alternative and complementary approach to chemical and radiation mutagens is insertional mutagenesis. Developed in zebrafish by Nancy Hopkins' laboratory (Amsterdam *et al*., 1999; Golling *et al*., 2002), insertional mutagenesis provides an extremely efficient means of cloning mutant genes. Rather than soaking fish in an ENU solution, parental fish are generated by injecting high titers of retroviruses into the 1000–2000-cell stage embryo. The virus infects a multitude of cells, including primordial germ cells, and insertions into germ cells are transmitted to offspring, some causing mutant phenotypes. Mutagenic frequency of insertional approaches is seven to nine-fold lower than ENU mutagenesis. However, this may be balanced by the ease of cloning mutant genes (Golling *et al*., 2002) and improved methods of generating high-titer virus are being developed (Chen *et al*., 2002). The Cambridge screen has isolated over 500 insertional mutants, and over 75 of the disrupted genes have been cloned (Amsterdam *et al*., 1999; Golling *et al*., 2002). Importantly, all of the genes identified have human orthologs or related human genes (Golling *et al*., 2002).

C. F2 Genetic Screens: Large-Scale Genetic Screens for Early Development Mutants

Successful small-scale genetic screens (Kimmel, 1989) and the ability to recover mutations in the germline (Mullins *et al*., 1994; Solnica-Krezel *et al*., 1994; Riley and Grunwald, 1995), coupled with the optical clarity and external development of the zebrafish embryo, laid the foundation for two groups of scientists in Boston, USA (Driever *et al*., 1996) and Tübingen, Germany (Haffter *et al*., 1996) to undertake the first large-scale genetic screens in a vertebrate. Both groups designed an F2 genetic screen and analyzed the F3 generation embryos for early development abnormalities (Fig. 9A). In F2 screens, the founder male fish (P) are treated with a mutagen to generate a multitude of germ-line mutations, which are inherited by their offspring, the

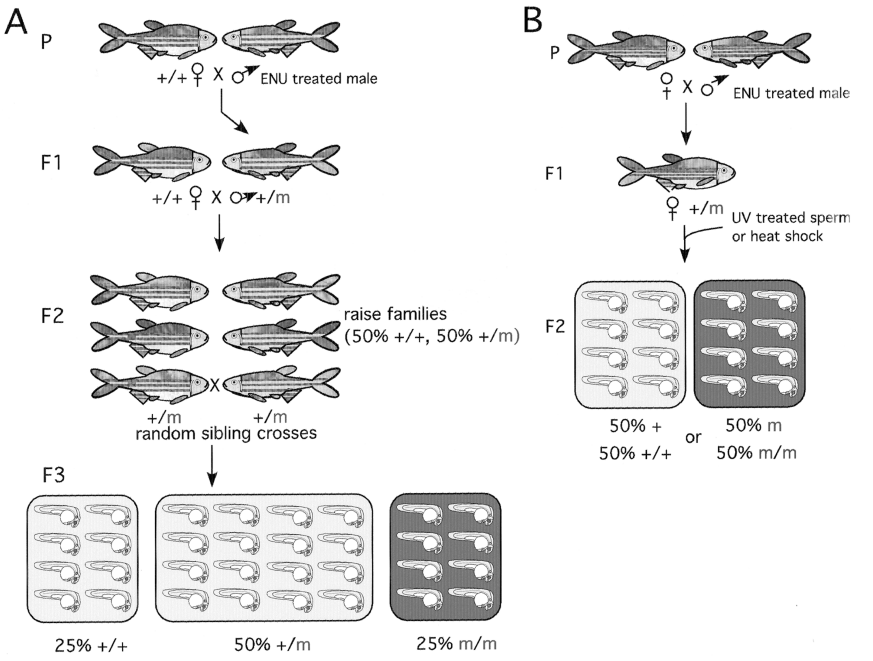


FIG. 9. Zebrafish F2 genetic screen design. A. Founder fish (P) are mutagenized (with a mutagen such as ENU) and mated to wild type fish to create the F1 generation. F1 siblings are crossed to each other to generate the maximum number of mutations in the F2 generation. A mutation of interest (m) that is heterozygous in a F1 fish is inherited by 50% of the F2 generation. Random F2 sibling crosses reveal a recessive mutation in 25% of the population (m/m), and 50% will be heterozygous (+/m) for the mutation, while 25% will be wild type for the mutation (+/+). In the Cambridge screen, founder fish are mutagenized by insertional mutagenesis at the 1000 cell stage embryo, and crossed to each other as adults to maximize the frequency of mutation in the F1 generation. Seven pair-wise crosses in the F2 generation gives a 95% probability of identifying a recessive mutation. B. Zebrafish F1 genetic screen design. Creating F2 haploids and homozygous gynogenetic diploids eliminates the need for F2 families in a genetic screen. As in F2 screens, the parent (P) is mutagenized, and F1 families created. F1 females are squeezed gently to release their eggs, which are fertilized with UV-treated sperm (genetically impotent) to create haploid embryos, or treated with heat shock to generate homozygous gynogenetic embryos. Clutches of haploids and homozygous diploids are 50% mutant (m, or m/m) and 50% wild type (+, or +/+) for a specific mutation.

F1 generation. To increase the frequency of mutations screened, F1 siblings are typically crossed to one another to raise F2 generation families, in which half of the fish are heterozygous for the mutation of interest. For saturation screens, approximately 5000 F2 families were raised, derived from over 300 ENU treated founder males. For a recessive mutation with Mendelian inheritance patterns, 25% of the F3 embryos are wild type (+/+), 50% are

heterozygous (−/+), and 25% of the clutch are homozygous (−/−) for a specific mutation (Fig. 9B).

The Tübingen and Boston screens identified over 2000 developmental mutants within the first five days of fertilization (Driever *et al*., 1996; Haffter *et al*., 1996). The breadth of mutant phenotypes discovered is unparalleled in other animal systems, and include mutants in the cell cycle, gastrulation, epiboly, brain development, axonal growth, somite formation, touch, and eye movements. Embryonic mutants were also isolated with defects in specific embryonic organs, such as the heart and blood system (Thisse and Zon, 2002). Similarity between fish and human disease phenotypes and disease genes, suggests that some mutants may be used as models for the human disease condition. For example, the *pickwick* mutant has a thin-walled heart which contracts poorly (Xu *et al*., 2002). The *pickwick* gene encodes a mutation in *titin*, a homolog to the human gene that is mutated in families with dilated cardiomyopathy. Zebrafish embryos have also been identified with defects in diverse and different aspects of hematopoiesis (Ransom *et al*., 1996; Weinstein *et al*., 1996). Zebrafish embryos with erythropoietic porphyria syndromes, *dracula* and *yquem*, encode *Ferrochelatase* (Childs *et al*., 2000) and *Uroporphyrinogen Decarboxylase,* respectively (Wang *et al*., 1998), and closely resemble the rapid, light dependent lysis of red blood cells seen in the human condition. Anemic mutant phenotypes, such as in *merlot/chablis*, *sauternes* and *chardonnay* mutants, resemble human anemias and encode the erythrocyte structural *Protein 4.1* (Shafizadeh *et al*., 2002), *delta-aminolevulinate synthase* (*ALAS2*) (Brownlie *et al*., 1998), and *Divalent Metal Transporter 1* (Donovan *et al*., 2002), respectively.

The hypochromic anemic mutant *weissherbst* has a mutation in the highly conserved iron exporter gene *Ferroportin 1* (Donovan *et al.*, 2000). When first identified in zebrafish, Ferroportin 1 was a novel protein, found to be conserved in humans and was postulated to be the predicted iron transporter that exported iron into circulation from the intestine (Donovan *et al*., 2000). In humans, classic hereditary hemochromatosis is a condition inherited as an autosomal recessive trait, and is caused by mutations in the *HFE* or *TFR2* genes. However, the mutation responsible for the prevalence of a genetically different subtype of hemochromatosis that occurs particularly in Southern Europe, and is inherited as an autosomal-dominant disorder, had been elusive. Ferroportin 1 mutations have since been identified in patients with this atypical hemochromatosis, thereby solving an important clinical puzzle (Montosi *et al*., 2001; Njajou *et al*., 2001). This sampling of zebrafish mutants illustrates the power of zebrafish genetic screens to identify novel and known genes important for organ-specific development, many of which have direct implications for our understanding of human disease.

D. Haploid and Gynogenetic Diploid Screens

The length of time and amount of space required for an F2 genetic screen can be cumbersome and extensive. To streamline a large-scale F2 screen and to circumvent the generation of a large number of F2 families, oocytes can be manipulated to undergo embryogenesis as haploid or gynogenetic animals (Streisinger *et al*., 1981; Beattie *et al*., 1999; Walker, 1999) (Fig. 10). During ovulation, eggs undergo the first stages of meiosis, including recombination between aligned, duplicated sister chromatids (Fig. 10). Extracted eggs have completed meiosis I and, upon fertilization, will undergo meiosis II, during which the separation of sister chromatids occurs. Normally, one set of maternal DNA combines with paternal DNA to generate a diploid animal, while the remaining maternal DNA is extruded as a polar body. Haploid embryos are generated by gently squeezing the F1 female, collecting her eggs, and fertilizing them with UV-inactivated sperm. Upon fertilization with genetically inactivated sperm, one set of maternal DNA is extruded as the polar body. As the paternal DNA is unable to make a genetic contribution to the embryo, haploid embryos are generated. In this way, recessive mutations are revealed in F2 generation embryos (rather than in F3 embryos, as in an F2 screen), and mutant phenotypes are seen in 50% of the clutch (see Fig. 10).

Haploid animals can live up to 3 days. Initially they are grossly similar to diploid embryos, and screening for the mutation of interest in early development in F2 embryos saves time and space. However, specific, abnormal phenotypes of haploid embryos after day 2 of development (e.g., degeneration of neural tissue, abnormal blood circulation) and their limited life span, makes them unsuitable for particular genetic screens. For example, lymphocytes are first detected on day 3 post fertilization (see Section III.B.2), at a point when haploid embryos are too abnormal to be scored. As an alternative, gynogenetic diploid embryos can be generated by treating eggs, fertilized with UV-inactivated sperm, with early pressure (EP) or heat shock (HS) (Beattie *et al*., 1999). Hydrostatic pressure applied to oocytes within the first few minutes post fertilization prevents anaphase by breaking down the meiotic II spindle. If fertilized with UV-inactivated sperm, EP treated eggs develop normally into diploid animals, where all the genetic information is derived from the mother. Recombination events that occur on average once per chromosome arm prior to metaphase I alter the percentage of mutants that will be expected within an EP derived clutch (Fig. 9). EP treated embryos will be homozygous for mutations close to the centromere (50% of the clutch will have the mutant phenotype) and heterozygous for mutations distal to the centromere (less than 50% of the clutch will have the mutant phenotype). The number of homozygous mutant embryos in a given clutch of EP embryos

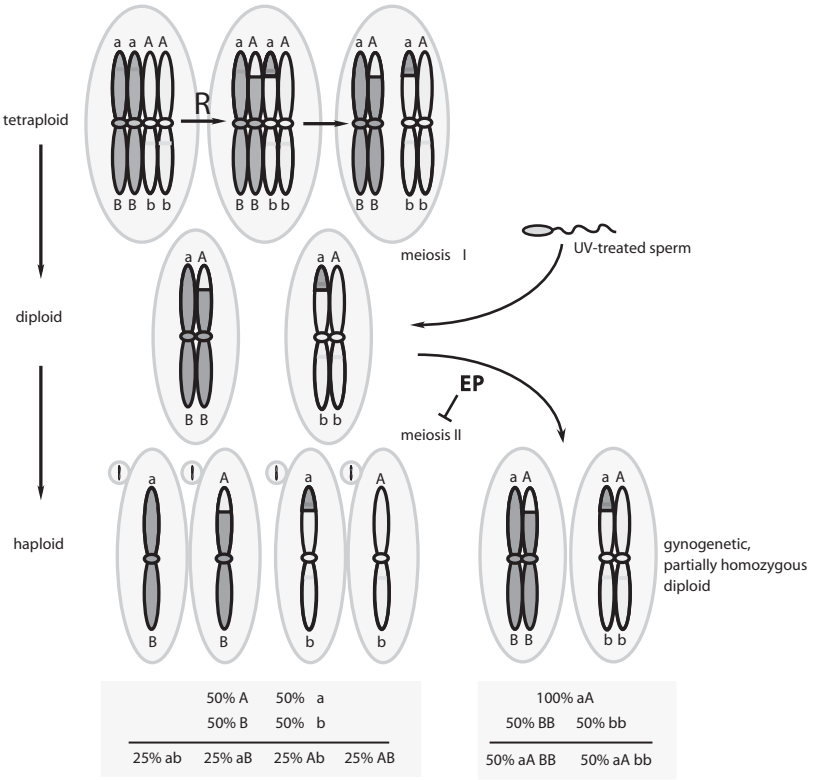


FIG. 10. Meiosis I and II, and the generation of haploid and partially homozygous diploid embryos in zebrafish. Oocytes squeezed from an F1 female have completed meiosis I, and initiate meiosis II upon fertilization. Sister chromatids separate in meiosis II, and one genome is extruded as a polar body. When fertilized with UV-treated sperm, the remaining genome from meiosis II becomes the genome for the haploid embryo. Early Pressure (EP) inhibits the meiotic II spindle, and both genome copies are retained in the embryo. Recombination events (R) occur on average once per chromosome arm during metaphase of meiosis I, and alleles distal to the centromere (A, a) are exchanged. Alleles proximal to the centromere are exchanged with low frequency (B, b). In haploid (and HS diploids) animals, each mutation is in 50% of the embryos (50% A, 50% a; 50% B, 50% b), regardless of recombination events. For EP embryos, recombination events result in a heterozygous diploid genome for mutations distal to the centromere and homozygous for mutations proximal to the centromere. Shown here, the clutch is 100% heterozygous for the A alleles (Aa), and 50% of the clutch is homozygous for the B alleles, (bb, BB).

thus decreases as a function of the distance of the mutation from the centromere [distance in centi Morgan = 50 (1−(2 × mutant number/total number of embryos))]. Homozygous diploid animals can also be generated from a heterozygous mother by heat-shocking activated oocytes. HS inhibits

the first mitotic division, and thus, HS derived embryos are homozygous at all loci and mutant phenotypes are detected in 50% of the clutch. Despite the genetic advantages of HS in genetic screens, HS embryos have notoriously poor viability (10–20%), and EP thus seems to be the method of choice in gynogenetic diploid screens.

EP screens have been used successfully to pioneer small-scale screens in a range of biological processes. For example, primary motor neurons have distinct axonal trajectories, which can be visualized using a set of antibodies to mark the cell bodies and axons (Beattie *et al*., 1999, 2000; Gray *et al*., 2001). Because motor neuron morphology is abnormal in haploid embryos, an EP screen was selected to identify mutants with aberrant primary motor neuron axonal trajectories (Beattie *et al*., 1999, 2000; Gray *et al*., 2001). Also, an adult EP screen has been designed to isolate mutants with blood clotting defects by measuring the conversion rate of human fibrinogen to fibrin in zebrafish blood (Jagadeeswaran *et al*., 2000). Similarities between human and fish blood coagulation pathways (Sheehan *et al*., 2001) suggest that this type of screen should yield insight into the genetic regulation of zebrafish and human coagulation pathways (Jagadeeswaran *et al*., 2000).

E. Combinations and Variations of Genetic Screens

The number of mutants desired, the type of mutation, and the size and time available are variables of screen design that can be tailored to fit an individual facility. In the future, the availability of high-titer virus for insertional mutagenesis should make this approach more attractive for smaller laboratories designing both F2 and F1 genetic screens. Screening tools, such as antibody staining, RNA expression patterns, and fluorescence can reveal cells, organs, and processes invisible to the eye (Patton and Zon, 2001). For example, a fluorescent lipid reporter to visualize lipid metabolism in live embryos was used to identify the morphologically normal mutant *fat-free* in a small-scale F2 genetic screen (Farber *et al*., 2001). Isolation of temperature-sensitive mutants provides an approach to create alleles that act in a controlled environment, such as in the identification of temperature-sensitive fin regeneration mutants (Johnson and Weston, 1995; Poss *et al*., 2002).

With a mutant in hand, genetic screens can be designed to identify mutations that suppress or enhance a phenotype. For example, the mutant *squint* was initially identified as a strain-specific background enhancer of *cyclops* (Feldman *et al*., 1998), and novel *smad5* alleles have been isolated in a dominant enhancer screen of the zebrafish *smad5* mutant, *somitabun* (Kramer *et al*., 2002). Furthermore, novel genes with aberrant gene expression patterns in a mutant can be identified, as shown by screening for genes downregulated in the midline mutant *one-eyed pinhead* (Hoshijima *et al*., 2002). Small

molecules can be used to dissect vertebrate ontogeny, and used as a tool to induce temporal control over gene product function (Peterson *et al*., 2000, 2001; Chan *et al*., 2002). Of note, the small molecule concentrimide can mimic a heart-specific mutant phenotype, and a vascular endothelial growth factor receptor inhibitor prevents blood vessel formation in zebrafish embryos (Chan *et al*., 2002). Overexpression of the gene *AKT* can partially rescue the angiogenic inhibitor effects (Chan *et al*., 2002), suggesting that genetic screens for mutations altering the small molecule induced phenotypes may provide insight into the genetic mechanism of drug action and open avenues for treatment of disease.

VI. Zebrafish Screens for Lymphoid Mutants

In planning a screen for zebrafish lymphoid mutants, the first issue to be considered is the relatively late development of lymphocytes during zebrafish development. Most screens in zebrafish have been designed to uncover early developmental defects, such as dorsal-ventral axis patterning (Hammerschmidt *et al*., 1996; Mullins *et al*., 1996). Even mutations affecting formation of organs such as the cardiovascular system (Chen *et al*., 1996; Stainier *et al*., 1996) are discernible at 2 or 3 dpf. The optimal time-point for a lymphoid screen is 5 dpf, when wild-type embryos have strong thymic staining with most of the T cell probes. This precludes the option to carry out haploid screens, where embryos degenerate after 48 h. This leaves classic F2 screens or EP screens as alternatives. Space requirements of F2 screens, where 5000 F2 families have to be raised to approach saturation (see Section V.C), appear prohibitive when screening for a restricted number of genes that affect only lymphopoiesis. However, F2 screens for a specific organ system or developmental process can be done successfully in conjunction with other screens. This strategy has recently been accomplished by the group of Tom Boehm (see below) as part of the Tübingen 2000 screen consortium (Habeck *et al*., 2002). The most feasible focused screen for lymphoid mutants therefore appears to be an EP screen. We (see below) and others (Schorpp *et al*., 2000) have initiated EP screens for lymphoid mutants and have recovered a number of interesting mutants.

A screen for lymphoid mutants was initiated after having assembled the necessary components of the lymphoid program in zebrafish. *Rag-1* was selected as a lymphoid probe due to its robust thymic expression pattern. F1 females derived from ENU mutagenized males were screened by WISH for gynogenetic diploid offspring with defects for *Rag-1* expression. To date, eight mutants lacking *Rag-1* expression have been identified and are undergoing phenotypic and genetic characterization (N. S. T. *et al.*, unpublished). All *Rag-1*$^{-}$ mutants identified in this screen have a marked reduction or complete

absence of lymphoblasts in the thymus by ultrastructural analysis and WISH. Further characterization revealed defects in thymic development, and some mutants have abnormal pharyngeal arch development. As pharyngeal arch development relies on an intact neural crest cell and endodermal compartment (Manley and Capecchi, 1995; Conway *et al*., 1997; Peters *et al*., 1998; Miller *et al*., 2000), the defect in this latter group of mutants might lie in the initial formation of the thymic rudiment. As a possibility, the Hoxa-3-Pax-1 pathway could be perturbed in this group of mutants (Manley, 2000). In mutants where pharyngeal arch architecture appears grossly normal, and endodermal pouches three and four are unaffected, the defects appear to affect differentiation of the thymic anlage. Seven of the eight mutants have been mapped to zebrafish chromosomes, and close genetic markers have been identified. Cloning of the mutated genes promises to yield new insights into thymic organogenesis.

VII. Reverse Genetic Approaches

Reverse genetics, the ability to inactivate a given gene in an entire animal, is just beginning to be addressed in the zebrafish. Established techniques and materials required for generating both germline and somatic gene inactivation in mammals and lower organisms have only recently become available to the zebrafish community. Two approaches have been attempted in order to develop somatic gene inactivation in zebrafish embryos. As discussed below, the use of morpholino oligonucleotides has been successful (Ekker and Larson, 2001; Lele *et al*., 2001) whereas RNAi has not (Oates *et al*., 2000). Among the multiple approaches being explored to generate targeted-gene disruption, there is currently only one reverse genetic method capable of producing germline gene inactivation. This method uses target-selected mutagenesis to screen for mutants in specific genes.

A. Targeting Induced Local Lesions in Genomes (TILLING)

An alternative to standard mammalian gene targeting has recently yielded the first mutant fish by a reverse genetics approach (Wienholds *et al*., 2002) (Fig. 11). This approach, which is also known as TILLING (Targeting Induced Local Lesions In Genomes), has been used in other organisms such as *Arabidopsis* (McCallum *et al*., 2000). A library of approximately 2500 cryopreserved sperm samples from the F1 progeny of ethylnitrosourea (ENU) mutagenized zebrafish was generated. Genomic DNA from the sperm donors was arrayed for high-throughput mutation screening by PCR and sequencing. Exons of *Rag1* were amplified and analyzed to identify heterozygous mutations, which were not polymorphic in other fish. Lines of fish carrying the

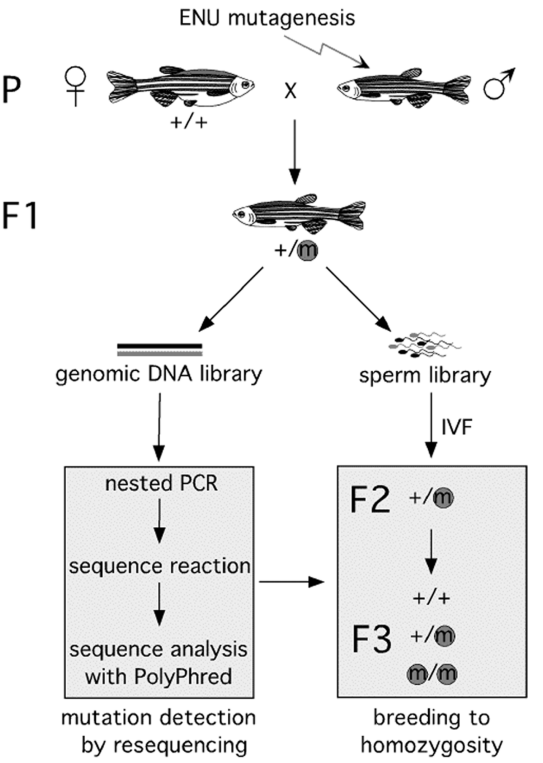


FIG. 11. Overview of target-selected mutagenesis in zebrafish. Adult male zebrafish are mutagenized with ENU as per standard procedure (Solnica-Krezel *et al.*, 1994; Haffter *et al.*, 1996). Mutagenized males are crossed with wild-type females to produce nonmosaic F1 generation of fish. Sperm is isolated and cryopreserved from fertile F1 males. Genomic DNA is isolated, arrayed in PCS plates, and screened for mutations by nested PCR amplification of the target gene and subsequent DNA sequence analysis. After a particular mutation is identified, *in vitro* fertilization (IVF) is performed to recover the F2 line carrying the mutation. Finally, mutations can be bred to homozygosity and analysed for phenotypes. (Reproduced from Wienholds *et al.* (2002) with permission.)

mutations of interest were then established by thawing the corresponding sperm samples and performing *in vitro* fertilization.

Using this target-selected mutagenesis approach, a series of 15 *Rag1* mutations were found including a premature stop codon in the catalytic domain. In the homozygous mutant context the expected phenotype—failure of antigen receptor rearrangement—was observed (Wienholds *et al.*, 2002). Only complete loss of function has been evaluated thus far. Other missense mutations in the *Rag-1* gene may be hypomorphic. The null mutant fish live to adulthood in a non-sterile environment without signs of infectious disease, but

upon tail clipping quickly die of unknown causes where wild type fish would survive (E. Wienholds, personal communication). These fish are now available for study of the teleost immune system.

There are multiple mutation detection strategies that might be applied to target-selected mutagenesis. Sequencing was the method used initially by Plasterk's group, but a much cheaper alternative is already in use. PCR amplification of exons of the gene of interest is followed by denaturation/renaturation of the samples, creating hetero-duplexes where point mutations and SNPs occur in heterozygous individuals. The celery mismatch repair enzyme CEL I is an endonuclease that cuts at the site of single base pair mismatches. Incubation of the DNA with CEL I followed by electrophoresis makes a very sensitive mutation detection system, where altered migration pattern of bands indicates the presence of a change at the nucleotide level (Oleykowski *et al*., 1998). These mutations are then analyzed by sequencing and evaluated for their potential effects on protein function.

TILLING has its advantages and disadvantages when compared to classic gene disruption in mice. A major advantage is that it is much cheaper to screen for mutants by this method than to generate mutants by gene disruption. Another advantage of the target-selected method is the generation of an allelic series of mutations of varying severity. For example, a viable, hypomorphic allele of a gene that confers lethality when completely disrupted can be useful for genetic studies such as suppressor/enhancer screens. A disadvantage is that the probability of finding a mutation is directly related to the size of the exons. Thus, the identification of a mutation in a gene with a small coding region would require screening a very large number of F1 progeny DNA. Also, this approach cannot replace specialized gene-targeting constructs such as the conditional alleles typically generated in mice with cre/loxP systems. Therefore, for a number of genes and purposes, targeted gene disruption will still be the method of choice when it becomes available in the zebrafish.

B. Other Gene-Targeting Strategies

Gene targeting in zebrafish has not previously been possible because embryonic stem cell (ES) culture, the key to the technique pioneered in mice, is still being developed in the fish. ES-like cells have been cultured from dissociated zebrafish blastulae and used to create chimeras (Ma *et al*., 2001). The ES-like cells are cultured with a trout cell feeder layer to prevent differentiation so that they can be transplanted back into the host blastula. Homologous recombination of a targeting construct has not yet been attempted with these ES-like cells. Transient transgenic experiments suggest

that homologous recombination does occur in zebrafish embryos (Hagmann *et al*., 1998), providing hope for this strategy.

Targeted-gene disruption has been accomplished in other mammalian organisms by avoiding the ES cell culture problem using nuclear transfer of cultured cells (McCreath *et al*., 2000; Lai *et al*., 2002; Rideout *et al*., 2002). Nuclear transfer has recently been achieved in the zebrafish using long-term cultured donor cells. Embryonic fibroblast cells from disaggregated (5–15 somite) embryos were cultured for at least 12 weeks before transplanting their nuclei into enuclueated, unfertilized eggs (Lee *et al*., 2002a). Now long-term cultured cells may potentially be used to select for gene disruption by homologous recombination or gene traps and can subsequently be used for nuclear transfer.

Another targeting strategy has been used in *Drosophila* to produce gene disruption by homologous recombination. FLP recombinase and a site-specific restriction enzyme (I-SceI) in combination with transgenic animals containing DNA surrounded by FRT sites, can generate excised recombinagenic DNA fragments that undergo a high frequency of homologous recombination (Rong and Golic, 2001). When homologous recombination occurs in the germline of these animals, the mutation can be passed on to the next generation. This method is currently being examined in zebrafish.

C. Transient Gene Knockdown Using Morpholinos

While germline gene inactivation generates very important data that cannot always be obtained through somatic gene inactivation, the latter approach is often much cheaper and faster. Morpholino oligos, first developed for clinical applications, have been successful in inducing antisense effects in zebrafish embryos (Summerton and Weller, 1997; Nasevicius and Ekker, 2000). These 25 bp DNA analogs operate by blocking mRNA translation. They only operate when complementary to a sequence between the 5′ UTR through the first 25 bases 3′ of the AUG start site or to splice junctions. Morpholinos are typically injected into zebrafish embryos at the 1–8 cell stage at a final concentration range of 0.1–1.0 nM. The DNA analogs are immune to DNAse degradation and are thus stable in the embryo for extended periods (Summerton and Weller, 1997; Nasevicius and Ekker, 2000). Furthermore their small size allows for even distribution to all cells in the developing embryo at concentrations sufficient for inhibition.

There is variability in the severity of the phenotypes for a given antisense of a gene. This has been studied by comparison of morphants to genetic mutants. Sometimes there is only a weak phenotype, or only a portion of the embryo shows any phenotype. It is also possible for morpholinos to cause more severe phenotypes than null mutants, where only the zygotic gene products are affected. In these cases inactivation of translation of maternal

transcripts is the basis for the observed effect. Morpholinos can cause side effects, often at high concentrations, such as cell death, defects in epiboly, and neural degeneration. The appearance of side effects is very oligo-dependent. It is unclear if the observed toxicity is a result of unexpected complementarity to other genes or other nonspecific effects of the oligonucleotides (Heasman, 2002).

The duration of the antisense effect is variable but, in isolated cases, can last up to 10 days post injection (Nasevicius and Ekker, 2000). The loss of antisense effect is thought to be due to dilution of the morpholino as the embryo grows in size. A number of studies have suggested that 100% of genes can be affected by morpholinos when a number of different sequences are targeted (Nasevicius and Ekker, 2000; Lele *et al*., 2001).

RNAi, which has had recent success in mammalian systems, appears to have a nonspecific interference effect in zebrafish (Oates *et al*., 2000). Initially a number of reports suggested that RNAi was causing reduced levels of specific endogenous mRNAs (Wargelius *et al*., 1999), but now it is generally accepted that RNAi induces a nonspecific degradation of mRNAs transcribed from zygotic genes (Zhao *et al*., 2001). It is unclear if short double stranded RNA would have a more specific effect in zebrafish embryos as it does in mammalian cells.

With the ability to do reverse genetics, the zebrafish has become a useful vertebrate model system to examine the function of genes found in mammalian studies or in zebrafish gene expression screens (see Section VIII). Given the long-lasting effects of morpholinos (up to 10 days) this method should also be applicable to immunology, as T cells appear in the zebrafish thymus at 72 hpf (see Section III.B.2). Known or novel genes expressed in lymphocytes can now be interrogated regarding their role in lymphopoiesis using morpholinos. A variety of read-outs can be devised, ranging from screening for a reduction in lymphocyte number by WISH, or for a decrease in the fluorescent signal of *Rag-1* in transgenic fish lines. The speed with which these experiments can be carried out makes this model a very attractive "first-pass" method to assess gene function, prior to launching germline gene disruption in mice or zebrafish. Many genes are currently being examined by morpholino knockdown, and many more will soon be examined by target-selected mutagenesis. As a number of potential methods are being perfected, targeted gene disruption will most likely soon be streamlined in the zebrafish.

VIII. Gene Expression Screens

The advantages of the zebrafish over other vertebrates, i.e., optical transparency and rapid development as well as the option of performing

WISH during embryonic and larval stages provide the opportunity to uncover genes that are expressed in specific organ systems. Based on this rationale, a WISH-based screen was recently implemented using organ- or stage-specific cDNA libraries (Donovan *et al*., 2002). cDNAs matching interesting expression profiles are then directly sequenced and the corresponding genes identified by gene homology searches. 5′ rapid amplification of cDNA ends (RACE) can be instrumental in identifying incomplete cDNAs (often only the 3′UTR is represented). In addition, mapping of the gene in question facilitates establishment of syntenic relationships with chromosomes of vertebrates with fully sequenced and assembled genomes, for e.g., FUGU and humans, is an alternative strategy. Coupled with the expression profile of the gene in question, its identity can often be established. Map positions can then also be compared with chromosomal locations of ENU-generated mutants with defects corresponding to the expression profile of the identified gene. This approach has been successfully implemented in the cloning of the zebrafish mutant *chardonnay*, which has a defect in the hematopoietic iron transporter divalent metal transporter-1 (DMT-1) (Donovan *et al*., 2002).

We adapted this strategy to probe the embryonic cDNA library, which was used to identify DMT-1, for genes involved in the immune system (N. S. T., J. Galloway, L. I. Z., C. Thisse, B. Thisse, unpublished). Inclusion in the WISH procedure of wild-type embryos 5 dpf permitted screening for genes expressed in the thymus at that time-point. Given that the starting material was derived from embryonic tissues, we expected to obtain genes that were expressed not only in the thymus but also in other tissues. To date, we have identified 28 genes, which can be classified into several groups. Some are known genes with established roles in lymphoid development, for example TdT, or thymus development (e.g., keratin). Another group consists of genes, which have so far been unsuspected to play a role in thymus or lymphoid development. Still another group contains genes with interesting expression patterns outside the thymus, such as the dorsal aorta. An example of this is CORO-1A, a gene thus far shown to bind actin with a role in chemotaxis, cell motility, cytokinesis and phagocytosis (Suzuki *et al*., 1995; de Hostos, 1999). Finally there is a group of novel genes. These genes can now be tested in a variety of hematopoietic and lymphoid mutants. Furthermore, the function of these genes can be transiently inactivated by using morpholinos (see Section VII.C). This approach promises to yield interesting and potentially novel genes involved in hematopoiesis and the development of the immune system.

An alternative approach was implemented by FACS sorting lymphocytes by light scatter characteristics (see Section IV.A) to generate a lymphoid library. We are in the process of sequencing the genes contained in this library (D. T., N. S. T., A. C., L. I. Z, unpublished).

IX. Use of Genomics: Getting Started with the Zebrafish

Zebrafish laboratories are often contacted by scientists from various fields of biology who wish to use the zebrafish system for the study of a particular topic. The first issue usually centers around the isolation of zebrafish orthologs of genes that they study. There are many reagents and tools that can be instrumental in this endeavor. The ZFIN database (http://www.zfin.org) lists general information about zebrafish, and gives the contact information for most zebrafish investigators. Contacting a zebrafish investigator in the field of study may obviate the need for cloning, as the gene of interest may already have been isolated, but not yet published. Fruitful and mutually beneficial collaborations may thus be forged. It can also be helpful to inspect the MGH Website (http://zebrafish.mgh.harvard.edu/), the Tübingen Website of R. Geisler (http://www.eb.tuebingen.mpg.de/dept3/research_interests/geisler_lab/gen_mapping.html), our laboratory web site (http://zfrhmaps.tch.harvard.edu/ZonRHmapper/) or the Sanger Center Web site (http://www.sanger.ac.uk/Projects/D_rerio/), which offer links to BLAST sites to examine zebrafish orthologs. Orthologous sequences can then be used to search for expressed sequence tags (ESTs) in the NCBI site (http://www.ncbi.nlm.nih.gov/BLAST/), which has the Genbank entries for the ESTs. An alternative is the Washington University Website (http://zfish.wustl.edu/) which gives a list of cloned zebrafish ESTs. Once satisfactory homology is established, the zebrafish EST of interest can be ordered from RZPD in Berlin, Germany (http://www.rzpd.de/). In order to obtain full-length clones, the EST can be used to hybridize into zebrafish cDNA libraries, available from many investigators, and RZPD, which sells cDNA libraries assembled on gridded filters. In particular, we have made available our zebrafish kidney marrow cDNA library at RZPD, a library that was used to clone a variety of hematopoietic and immune genes, including *lck* (N. S. T. *et al*., in preparation). This procedure has a good likelihood of success and should lead to the isolation of the cDNA clone of interest.

Once the cDNA of interest is obtained, it can be used in a variety of assays to explore its function. First, the cDNA can be used for gene expression studies by *in situ* hybridization. This provides an ontogenetic analysis and can yield important new insights into a developmental process. For example, close scrutiny of *Rag-1* and $c\mu$ expression by *in situ* hybridization provided the first clue that B cell development is initiated in the zebrafish pancreas (see Section III.C.2, and Danilova and Steiner, 2002). Furthermore the expression of both *Rag-1* and *Rag-2* in the olfactory placodes of the developing zebrafish is a surprising finding, which may reveal a hitherto undefined role of the recombination activating genes.

Second, the availability of a full-length cDNA clone allows analysis of gene activity and function by employing two approaches, which can proceed fairly rapidly. This next step should involve a collaboration with a zebrafish laboratory as detailed knowledge of normal zebrafish development is required to interpret the results. First, it is possible to overexpress the gene and see if this perturbs normal development. For instance, the injection of the T cell oncoprotein SCL leads to the conversion of mesodermal tissues such as muscle or pronephros to become blood (Gering *et al*., 1998). Second, it is possible to use an antisense technology such as morpholinos to transiently knockdown the function of the gene. This technology has been employed successfully in a number of organ systems (*Genesis*, Volume 30, issue 3, 2001 and Section VII.B.1). However, it is important to realize that the inactivating effect of the morpholino is time-limited, and in some cases restricted to the first four to five days of development (Huang *et al*., 2001), although it can persist for up to 10 days (Nasevicius and Ekker, 2000). It is therefore pivotal to acquire complete understanding of the biologic process of study prior to launching gene knockdown experiments. Furthermore, determining an appropriate read-out for the expected effect is essential. If these criteria are met, these two studies can be helpful in determining if a gene is necessary and/or sufficient for the biologic process of interest.

It is possible to set up the technology in one's own lab (after training in a zebrafish lab for about 6 weeks, the time usually required to acquire proficiency in the injection technique). The lab set-up needed for morpholino and overexpression studies is about 200 square feet, an $8000 tank system, and a microinjection apparatus ($10,000).

Third, knowing the map position of the gene of interest can potentially lead to the identification of a pre-existing zebrafish mutant that was generated by the Tübingen large-scale screens. Many of the ESTs have already been mapped as indicated by the Washington University Website with links to our Website. Map positions can be determined using zebrafish-hamster radiation hybrid panels. This can be done as a service by our laboratory (http://zfrhmaps.tch.harvard.edu/ZonRHmapper/) or by R. Geisler's laboratory (http://www.eb.tuebingen.mpg.de/dept3/research_interests/geisler_lab/gen_mapping.html). The map position can then be correlated to the map position of mutants that were previously identified in large-scale screens by R. Geisler's laboratory in Tübingen. The stock center in Tübingen will send out the respective mutant for analysis.

Other approaches are feasible, but are more involved and require a full collaboration with a dedicated zebrafish laboratory, or a major upstart investment. These include establishment of stable transgenics, which constitutively express the gene of interest or drive GFP under the control of a particular promoter. This takes approximately 4–6 months and allows direct

observation of organ development or cell trafficking under a variety of experimental conditions. If a mutant zebrafish for the gene of interest is available through the Tübingen stock center and the investigator wants to proceed further with genetic screens (based on enhancer or suppressor genetics), this is a full time project for about 1.5 years. For instance, a large-scale screen for mutants that have a decrease in SCL expression was accomplished in that timeframe. The zebrafish system is a versatile model for the investigation of many biologic pathways. If a signal transduction pathway is clearly conserved in yeast, worms, or flies, it may be preferable to study it in those organisms rather than the zebrafish. In many scenarios the biology or pathophysiology of the pathway of interest requires the use of a vertebrate, and in those cases the zebrafish may elucidate pathway components that could not be achieved in other species that are by far more cost- and labor-intensive.

X. Concluding Remarks: Impact of Zebrafish on Immunology

Early events in the development of the primitive and definitive blood forming system are still poorly understood. Additionally, the specification of both B and T cells occurs during embryogenesis and, given the completion of this process before birth, are difficult to study in mammals by forward genetics. Historically, the major strength of the zebrafish has been the opportunity it offered to carry out forward genetic screens in a vertebrate organism in a relatively restricted space. Establishing the zebrafish as a model system for the study of the immune system will provide an alternative and complementary tool to the use of forward genetic screens in mice. Small- and large-scale screens of the adaptive immune system in zebrafish based on defective *Rag-1* expression have been carried out in Boston, Tübingen and Freiburg, Germany. Cloning of the mutated genes leading to the *Rag-1*$^{-}$ phenotype is greatly facilitated by recent advances in zebrafish genomics, including the recent completion of the zebrafish genome sequencing project to three-fold coverage by the Sanger Center. The mutants resulting from these screens will probably reveal novel genes or known genes with novel functions affecting T cell development and thymic organogenesis and will be instrumental in elucidating physiology and pathology of the immune system.

In recent years, rapid advances in a variety of fields have allowed the zebrafish to become a more versatile tool for immunology. For example, progress in FACS analysis permitted sorting for all major hematopoietic lineages, including lymphoid cells, and the subsequent generation of lymphoid libraries. Screening the genes contained within them for expression in lymphoid organs coupled with a gene knockdown technique using morpholinos promises to be a rapid and specific method to assess the

function of novel genes in the immune system. Genes identified in those screens will then be available for more in-depth studies in mouse (where the arsenal of molecular tools available to the immunologist is leagues ahead of the zebrafish) e.g., through gene targeting approaches. Another success story is the recent progress in generating transgenic lines of zebrafish expressing fluorochromes under the control of specific gene promoters. Tied with advances in *in vivo* imaging, immune-specific transgenic lines will not only allow the development of novel screens for immune deficiencies and autoimmunity, but will likely impact on our understanding of lymphocyte trafficking in health and disease. In this setting, probing innate immunity appears an attractive option. Finally, new methods, such as ES cell gene inactivation and TILLING will permit the generation of specific mutants (e.g., *Rag-1* mutant), which will be of great value and will add to the resourcefulness of the zebrafish as an instrument for immunological research.

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