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Developmental biology of zebrafish myeloid cells

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ABSTRACT The zebrafish *(Danio rerio)* has emerged as an informative vertebrate model for developmental studies, particularly those employing genetic approaches such as mutagenesis and screening. Zebrafish myelopoiesis has recently been characterized, paving the way for the experimental strengths of this model organism to contribute to an improved understanding of the genetic regulation of myeloid development. Zebrafish have a multi-lineage myeloid compartment with two types of granulocyte (heterophil/neutrophil and eosinophil granulocytes), and monocyte/macrophages, each with characteristic morphological features and histochemical staining properties. Molecular markers have been characterised for various myeloid cell types and their precursor cells, for example: stem cells *(scl, hhex, lmo2),* myeloid lineage precursors *(spi1/pu.1, c/ebp1),* heterophil granulocytes *(mpx/mpo),* macrophages *(L-plastin, fms).* In zebrafish, the sites of early myeloid and erythroid commitment are anatomically separated, being located in the rostral and caudal lateral plate mesoderm respectively. Functional macrophages appear before cells displaying granulocytic markers. By the second day of life, cells expressing granulocyte- and macrophage-specific genes are scattered throughout the embryo, but tend to aggregate in the ventral venous plexus, which may be a site of their production or a preferred site for their residence. Even in early embryos, macrophages are phagocytically active, and granulocytes participate in acute inflammation. Equipped with an understanding of the developmental biology of these various myeloid cells and a set of tools for their identification and functional study, we will now be able to exploit the experimental strengths of this model organism to better understand the genetic regulation of myelopoiesis.

KEY WORDS: *Danio rerio, haematopoiesis, myelopoiesis, leukocyte, granulocyte, macrophage*

Introduction

Danio rerio (zebrafish), a small cyprinid teleost, has emerged as a useful vertebrate model for the study of human diseases, particularly developmental and genetic diseases. Their advantanges for developmental studies include: small size; high fecundity (one female can produce up to 100-200 eggs per week); non-placental embryogenesis external to mother, during which development is independent of maternal care; and rapid development - most tissues are formed within 24 hours post- fertilisation (hpf) and fish reach sexual maturity within 3 months. To these aspects of zebrafish reproductive biology is added the considerable advantage of the optical transparency of the zebrafish chorion and embryo. Futhermore, as a vertebrate, the zebrafish possesses whole developmental systems not present in other genetically tractable model invertebrates such as *C. elegans* and *Drosophila* (Lieschke, 2001).

These attributes have been exploited in several large scale forward genetic studies employing mutagenesis and screening techniques for the unbiased identification of genes involved in specific developmental processes. The first screens exploited the optical transparency of embryos to screen for phenotypes recognizable by direct visual inspection of the developing embryos, identifying mutants affecting a wide range of developmental processes (Driever *et al.*, 1996; Haffter *et al.*, 1996). Recently more focused genetic screens have been based on phenotypes identified by particular molecular markers (exploiting zebrafish optical transparency in whole mount *in situ* hybridization) (Trede *et al.*, 2001), or later developing organ systems (Drummond *et al.*, 1998). Although most screens have been based on phenotypes resulting from chemical germline mutagenesis, other approaches have been successful.

Abbreviations used in this paper: AcP, acid phosphatase; dpf, days post fertilisation; hpf, hours post fertilisation; ICM, intermediate cell mass; LPM, lateral plate mesoderm; MHC, major histocompatibility complex; mpo, myeloperoxidase; mpx, myeloid-specific peroxidase; NSE, non-specific esterase; PAS, periodic acid-schiff; rag, recombinase activating gene.

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Retroviral insertional mutagenesis, incorporating the advantage of flagged mutagenesis sites, has been undertaken by one group (Amsterdam *et al.*, 1999). Direct screening for developmental perturbations induced by environmental chemicals is also practical (Peterson *et al.*, 2000).

Our goal is to exploit the strengths of zebrafish development and genetic versatility to better understand the development of white blood cells (leukocytes), particularly the phagocytes. Although invertebrates like *Drosophila* have phagocytic cells and an effective innate immune system, *Drosophila* lacks the multi-lineage complexity of vertebrate myelopoiesis, and also lacks the context of an adaptive immune system (reviewed in Lieschke, 2001). As we hope our observations will be directly informative for human disease, a vertebrate model organism that retains this degree of cellular and physiological complexity is very appealing.

Already zebrafish have contributed significantly to the study of vertebrate haematopoiesis. At least 2-dozen complementation groups of zebrafish mutants have defects in erythrocyte development and these have comprised the group most systematically pursued to date for the identification of the underlying mutated genes resulting in characterized zebrafish models for nine human diseases (Table 1 and reviewed in Barut *et al.*, 2000; Paw *et al.*, 2000). Although in the majority of cases the fish mutants have represented lesions in genes already known, in one case (*weissherbst* mutant and the *ferroportin1* gene), the fish mutant led to the identification of a new iron transport gene, and then to a new understanding of a human iron storage disease (Long *et al.*, 1997; Richardson, 2000).

The bias towards mutants affecting erythropoiesis primarily reflects the ease with which pink haemoglobinized red cells are visible in zebrafish embryos, and hence the ease with which anaemia could be recognized. Some anaemic mutants have been shown to affect myeloid cell development (e.g. *cloche*) (Lyons *et al.*, 2001), although these are not nearly as numerous.

In this review, we summarise descriptive and molecular data concerning zebrafish myelopoiesis and myeloid cells. Screening for myeloid mutants, our ultimate goal, will be greatly aided by an understanding of adult and developmental zebrafish myelopoiesis at morphological, functional, and molecular levels. Since there are several myeloid cell types, and since myeloid cells are only in low abundance compared with the ubiquitious circulating erythrocyte, this has required a systematic and focused approach. Our particular interest concerns the phagocytic leukocytes, and hence we focus on phagocytes in this review.

Myelopoiesis

Myelopoiesis has been most thoroughly studied in humans and mice. Mammalian myelopoiesis is characterized by four myeloid cell lineages. There are three types of granulocyte: neutrophils, which phagocytose foreign material, presenting a first line of defence against invading bacterial micro-organisms; eosinophils, which are involved in the destruction and removal of parasites; and basophils (and their tissue counterpart, mast cells), which are rich in metachromatic granules and involved in the mucosal immune response (Bainton, 2001). A macrophage lineage (macrophages being the tissue counterpart of circulating monocytes) is capable of engulfing endogenous cellular debris, foreign inanimate particles and invading micro-organisms, killing them where necessary (Douglas *et al.*, 2001; Lehrer *et al.*, 2001). In definitive myelopoiesis, cells of these four lineages arise from a common myeloid stem cell, from which precursors for each of the myeloid cell lineages arise. Precursor cells proliferate, and their progeny undergo a series of differentiation and maturation steps that lead to the various mature myeloid cells. These processes of stem cell replenishment, precursor proliferation, and lineage-specific maturation and differentiation are regulated by a complex interplay of early transcriptional control (reviewed in Orkin, 1995 and Shivdasani *et al.*, 1996) and later, by humoral growth factors interacting with a complex variety of membrane-bound receptors (Lichanska *et al.*, 1999; Yun *et al.*, 2001).

Leukocytes in Fish - A General Overview

Approximately 450 million years of evolutionary divergence separate mammals and bony fish (teleosts, including zebrafish), providing considerable scope for divergence in the molecular and cellular features of myeloid lineages, not only between different species of teleosts themselves, but also between teleosts and mammals (Rowley *et al.*, 1988). Like mammals, teleosts possess several types of granulocyte and a separate macrophage lineage. Descriptive studies are available for a wide range of fish species (reviewed in Rowley *et al.*, 1988 and Secombes, 1996), and indicate that even between major teleost groups (e.g. salmonids and cyprinids, which include zebrafish), significant morphological differences abound (Bielek, 1981). Caution must be exercised in inferring the function or identity of a cell merely from its appearance. Comparison of cells between different species must be based on functional and molecular studies as well as morphology. The evidence reviewed here indicates that at morphological, functional and molecular levels, zebrafish retain the essential features of a multi-lineage myeloid system that will underpin their usefulness as a model for the study of human disease.

Adult Zebrafish Myeloid Cells

In adult zebrafish, the main haematopoietic organ is the kidney (which is hence equivalent to the haematopoietic bone marrow of mammals) and leukocytes circulate in the peripheral blood. Several groups have recognized two types of granulocyte, a heterophil (or

TABLE 1

ZEBRAFISH MUTANTS WITH PRIMARILY HAEMATOPOIETIC PHENOTYPES FOR WHICH THE UNDERLYING MUTATED GENE HAS BEEN IDENTIFIED

Zebrafish mutant	Mutated gene in zebrafish mutant	Human disease resulting from loss-of-function mutation in orthologous gene	Reference
sauternes	*δ-aminolevulinate synthetase*	Congenital sideroblastic anemia	1
yquem	*uroporphyrinogen decarboxylase*	Porphyria cutanea tarda & Hepatoerythropoietic porphyria	2
dracula	*ferrochelatase*	Erythropoietic protoporphyria	3
weissherbst	*ferroportin1*	Haemochromatosis	4
riesling	*β-spectrin*	Hereditary spherocytosis	5
vlad tepes	*gata1*	Familial dyserythropoietic anaemia and thrombocytopenia	6
retsina	*band3*	Congenital dyserythropoietic anaemia type II (HEMPAS)	7
zinfandel	*globin* locus	Resembles thalassaemia	8

References: 1, Brownlie *et al.*, 1998; 2, Wang *et al.*, 1998; 3, Childs *et al.*, 2000; 4, Donovan *et al.*, 2000; 5, Liao *et al.*, 2000b; 6, Lyons *et al.*, 2000; 7, Paw, 2001; 8, Amatruda *et al.*, 1999.

neutrophil) and an eosinophil, readily identified by their appearance and staining characteristics (Fig. 1) (Jagadeeswaran *et al.*, 1999; Bennett *et al.*, 2001; Lieschke *et al.*, 2001).

Zebrafish Heterophil (Neutrophil) Granulocyte

The most abundant zebrafish granulocyte, the heterophil, is characterised by a pale cytoplasm and a multi-lobed (2-3 lobes) segmented nucleus. Heterophils have two distinct populations of granules, one azurophilic by Wright-Giemsa staining and the other larger and not stained by Wright-Giemsa (Bennett *et al.*, 2001; Lieschke *et al.*, 2001). This cell is similar to the human neutrophil, which also has a multi-lobed nucleus and heterophilic cytoplasm filled with azurophilic and non-azurophilic granules (Bainton, 2001).

In mammals, neutrophils pass through several developmental stages (myeloblast, promyelocyte, myelocyte, metamyelocyte, and mature neutrophil) during which the cells become smaller, and the nuclei condense, indent, and become multi-segmented. As the neutrophil matures, so do its granules, expressing and accumulating various enzymes necessary for its function (Bainton, 2001). Myeloperoxidase is an enzyme present in the primary granules of mammalian neutrophils and forms part of the primary defence system of the cell. In the presence of hydrogen peroxide, myeloperoxidase reacts with benzidine dihydrochloride to produce a diaminobenzidine brown-black precipitate (Kaplow, 1965). This histochemical stain is routinely used in clinical diagnostic laboratories. Zebrafish heterophils stain strongly for myeloperoxidase histochemical activity, indicating the presence of a peroxidase enzyme within the cell. Zebrafish myeloblast, promyelocyte, myelocyte, and metamyelocyte are all evident in kidney haematopoietic tissue. On myeloperoxidase staining, they mimic the pattern seen in developing mammalian neutrophils in which myeloperoxidase expression increases up to the myelocyte stage, then becomes more diffuse as expression ceases and the myelocyte distributes its granules (with their myeloperoxidase content) amongst its daughter metamyelocytes (Bennett *et al.*, 2001; Lieschke *et al.*, 2001; Valtieri *et al.*, 1987).

Heterophil granulocytes have a characteristic ultrastructural appearance (Bennett *et al.*, 2001; Lieschke *et al.*, 2001). Highly characteristic elongated electron-dense granules with lamellated axes pack the cytoplasm of mature heterophils, and are present from the promyelocyte stage. A second less abundant granule is

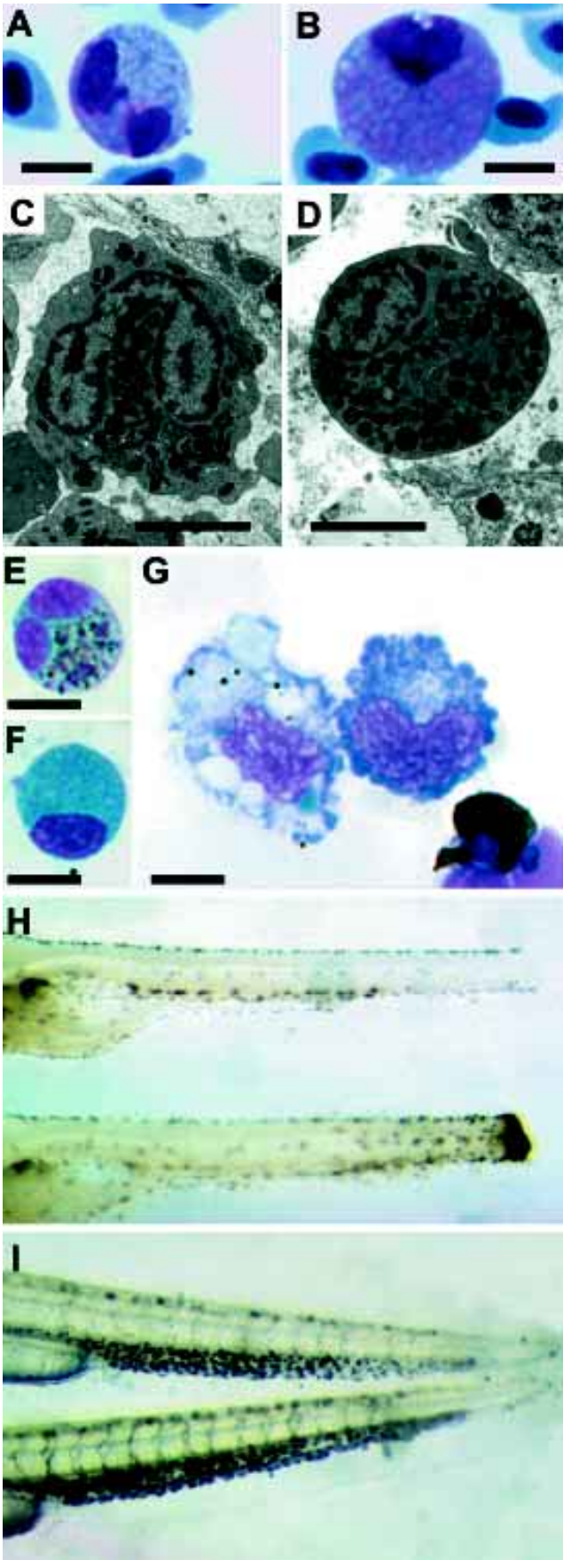

Fig. 1. Histology of zebrafish myeloid cells. (A) *Heterophil granulocyte in adult peripheral blood (May-Grunwald/Giemsa stain).* **(B)** *Eosinophil granulocyte in adult peripheral blood (May-Grunwald/Giemsa stain). (C,D) Heterophil granulocyte* **(C)** *and eosinophil granulocyte* **(D)** *in adult kidney (transmission electron micrograph). (E,F,G) Adult zebrafish leukocytes in cytospin preparations, stained histochemically for myeloperoxidase.* **(E)** *Heterophil granulocyte, showing black cytoplasmic granules indicating myeloperoxidase activity;* **(F)** *Eosinophil granulocyte, negative for myeloperoxidase;* **(G)** *Monocyte and macrophage, both negative for myeloperoxidase.* **(H)** *Whole mount zebrafish embryos (6 dpf) histochemically stained for myeloperoxidase, immediately after tail transection (upper embryo), and 8 hours later (lower embryo). Note the myeloperoxidase-reactive cells in the ventral venous region (upper embryo), and the accumulation over time of myeloperoxidase activity at the trauma site.* **(I)** *Zebrafish embryos photographed* in vivo *several hours after injection with a suspension of carbon particles (India ink), showing the accumulation of black carbon particles within phagocytic macrophages of the ventral vein region. Scale bars: A,B,E,F,G 5 μm; C,D 3 μm.*

round with a homogenous interior. More immature cells have abundant rough endoplasmic reticulum, indicative of high protein synthetic activity. The peroxidase activity of human neutrophils resides in the primary azurophilic granules, but the neutrophil also contains peroxidase-negative spherical or rod-shaped specific (secondary) granules (Bainton, 2001). It remains to be proven which zebrafish heterophil granule contains the zebrafish peroxidase activity.

Zebrafish Eosinophil Granulocyte

Zebrafish eosinophils, characterised by an eosinophilic cytoplasm and a small, non-segmented, peripherally located nucleus, reside in the kidney and circulation (Bennett *et al.*, 2001; Lieschke *et al.*, 2001). Their appearance differs considerably from that of mammalian eosinophils (Bainton, 2001). Zebrafish eosinophil granules are large and contain non crystalline material of broadly variable electron density creating a marbled appearance on electron microscopy. Eosinophils of all developmental stages are found in adult kidney, suggesting this is also the site of their production (Bennett *et al.*, 2001; Lieschke *et al.*, 2001). Given the considerable morphological differences between the zebrafish and mammalian eosinophil, it will take further analysis of granule contents to determine if they are equivalent and whether or not the zebrafish eosinophil in fact has a combined eosinophil-basophil type function. Although it has been postulated that this cell may represent a combined eosinophil/mast cell (Bennett *et al.*, 2001), the physiological role of this cell type is uncertain and will require precise analysis of its gene expression pattern and functional studies.

Other Zebrafish Granulocytes

Although mast cells are identified in many species of fish including teleost species closely related to zebrafish (Rowley *et al.*, 1988; Silphaduang *et al.*, 2001), inconsistencies in previous descriptions of piscine mast cells complicate a morphological recognition of zebrafish mast cells. Strongly eosinophilic cells of the striped bass (*Morone saxatilis* X *M. chrysops*) are called “mast cells” and “eosinophil granule cells” and have recently been shown to contain antimicrobial peptides called piscidins (Silphaduang *et al.*, 2001). Whether zebrafish eosinophils are the functional orthologues of these cells remains to be determined. Surveys with toluidine blue, a stain for the metachromatic granules of mammalian mast cells, failed to identify any positively staining leukocytes (Lieschke *et al.*, 2001), and molecular markers for the zebrafish orthologues of important effector proteins of mammalian mast cells are yet to be isolated. Although the *c-kit* receptor, important in mammalian mast cell development (Broudy, 1997), has been characterized in zebrafish and a mutant *sparse* identified, a basic haematopoietic evaluation of *sparse* did not identify a deficiency of either “eosinophils” or “basophils” (the circulating precursor of mast cells) (Parichy *et al.*, 1999).

Zebrafish Monocytes and Macrophages

Macrophages are obvious in sections of adult kidney and spleen (Bennett *et al.*, 2001; Lieschke *et al.*, 2001). They are large cells with large phagosomes, a high cytoplasm to nuclear ratio, diffuse nuclear chromatin, and an agranular but vacuolated cytoplasm. The vacuoles contain phagocytosed material, including pigment and red cell carcasses. These cells are negative for peroxidase activity. A mononuclear peroxidase-negative phagocytic cell is evident in kidney cytospins and may represent a zebrafish macrophage precursor. These cells are large and round, and are characterised by weak cytoplasmic staining, an elongated curved peripherally-located nucleus, a ruffled cell border, and no cytoplasmic granules.

Myelopoiesis in Zebrafish Embryos

Myelopoiesis refers to the process of formation of all types of leukocytes. Granulopoiesis, monocytopoiesis and lymphopoiesis refer to the lineage-specific processes forming granulocytes, monocyte/macrophages and lymphocytes respectively. At least in the early zebrafish embryo, there is a suggestion that not only are myelopoiesis and erythropoiesis anatomically separated, but that there is some dissociation between the development of the specific myeloid lineages as well (summarised in Table 2).

Embryonic Granulopoiesis

Morphologic studies at the light and ultrastructural level identified early myeloid cells by the second day post-fertilization. By 42-48 hpf, myeloblasts (confidently identified by their typical paracrystalline inclusions on electron microscopy) are evident both in axial tissues and in the circulation (Willett *et al.*, 1999; Lieschke *et al.*, 2001). The site of origin of these granulocytic cells is not precisely determined; a morphologic survey did not identify myeloblasts in the Intermediate Cell Mass (ICM) at 24 hpf, although they were seen over the yolk at this time. Expression of granulocyte-specific marker genes like *mpx/mpo* is first seen in the ICM at 18 hpf, and is strong in some cells over the yolk and in the ICM, including in its most posterior region by 22 hpf. Most of these early granulocytes are in the tissues rather than in circulation, although occasional circulating myeloblasts are undoubtedly seen (Bennett *et al.*, 2001; Lieschke *et al.*, 2001). Whether they arise as a dispersed population, or whether they form in a discrete location and then disperse, is not yet convincingly determined. Following this, particularly in the second and third day of life, the ventral vein region is a prominent site of aggregated *mpo/mpx* expression, suggesting this may be a site of generation of these cells; if not, it is a site of their accumulation. Although the first kidney tubules are evident by 72 hpf, haematopoietic cells are only evident morphologically in the kidney by 96 hpf, at which time myeloblasts and immature heterophil granulocytes are seen amongst erythroid precursors. Over the next 2 weeks renal granulopoiesis expands considerably (Willett *et al.*, 1999).

Even in the first days of development, heterophil granulocytes are functionally competent for participation in host defences. Myeloperoxidase-containing heterophil granulocytes can readily be displayed by whole-mount myeloperoxidase histochemistry (Lieschke *et al.*, 2001). This technique was used to display the migration of these cells to a site of trauma (tail clipping), indicating their involvement in acute inflammation. As the trauma site and associated clot did not contain large numbers of cells with peroxidase activity immediately at the time of wounding, but these cells accumulated over subsequent hours, the processes of cell migration and chemotaxis were presumably involved. The accumulated peroxidase activity extended beyond cellular borders, indicating the release of highly reactive and destructive peroxidase from the granules into the trauma site. Furthermore, in transverse electron microscopic sections, immature heterophil granulocytes were found at unusual sites (*e.g.* between muscle fibres) close to the trauma site, suggesting their rapid recruitment to the trauma site. Cells expressing the *mpx/mpo* gene also accumulated at the trauma site, although fewer in number,

suggesting the cells participating in the inflammation were the more mature cells that had already synthesized stores of peroxidase granules (Lieschke *et al.*, 2001).

Embryonic Macrophage Production

The first population of zebrafish macrophages originates from the rostral lateral plate mesoderm (LPM), anterior to the heart, from whence they migrate over the yolk (Herbomel *et al.*, 1999). At the 11-somite stage, the anterior lateral mesoderm converges to lie beneath the paraxial mesoderm, and by 13 somites, a population of macrophages emigrates from this site underneath the hatching gland onto the anterior surface of the yolk, but by 17 somites, moving onto the posterior half of the yolk sphere as well. These macrophages are highly mobile, phagocytically active, and their appearance on the yolk precedes the arrival of proerythroblasts on the yolk at 25 hpf (30-somite stage). As the circulation establishes, some of these macrophages enter it, distributing throughout the embryo. The expression patterns of molecular markers of macrophages (*L-plastin, fms*) indicate that these cells are widely dispersed throughout embryos of older ages. However, the site of the later wave of macrophage production is not delineated. Some "promonocytes" are described in the ventral vein region at 42 hpf, at a time when this is also a prominent site for the aggregation of cells expressing macrophage markers like *L-plastin* (Herbomel *et al.*, 1999; Willett *et al.*, 1999), suggesting that this may be a site of monocytopoiesis as well as granulopoiesis. Phagocytically-active macrophages are seen in the kidney at 2 weeks post-fertilization (Willett *et al.*, 1997a; Willett *et al.*, 1999), but whether these arise there or not is unclear.

Herbomel *et al.* (1999) used videomicroscopy to visualise macrophage activity in 30 hpf zebrafish embryos challenged with *E. coli* and *B. subtilis* infection. Fifteen minutes after *E. coli* infection, macrophages were covered in adhered bacteria, and actively phagocytosing cell corpses, and within a few hours, the circulation was cleared of bacteria. The embryos were also able to clear lower does of *B. subtilis*, but at higher doses macrophages became highly vacuolated, undergoing a form of frustrated phagocytosis. Erythrophagocytosis was a common activity of infection-activated zebrafish macrophages. The phagocytic activity of macrophages can be exploited to mark them in embryos *in vivo;* following injection of 48 hpf embryos with a carbon particle suspension, carbon is cleared from the circulation and accumulates within the cytoplasm of the phagocytically active macrophages of the ventral venous plexus (Lieschke *et al.*, 2001).

Embryonic and Definitive Haematopoiesis in Zebrafish

Numerous observations in mammals indicate the existence of two types of haematopoiesis - primitive haematopoiesis, occurring in the early embryo, and definitive haematopoiesis, which takes over from primitive haematopoiesis at some stage during embryogenesis and continues into adulthood. The transition from primitive embryonic to definitive haematopoiesis is characterized by changes in gene expression (*e.g.* different globin genes expressed in primitive and definitive erythrocytes) and changes in the site of haematopoiesis. In the mouse, primitive haematopoiesis begins externally in the yolk sac blood islands, then later seeds the aorta-gonad-mesonephros region and fetal liver, with definitive haematopoiesis initiating in the liver but finally residing in the bone marrow (reviewed in Zon, 1995 and Shivdasani *et al.*, 1996).

There is strong evidence in zebrafish also that the processes of primitive and definitive haematopoiesis are separate and different. There are several zebrafish mutants that are anaemic during embryogenesis but viable as adults (*e.g. thunderbird, chardonnay, and chianti*) (Ransom *et al.*, 1996). A large family of zebrafish globin genes exists with progressive switching during development (Chan *et al.*, 1997). While similar observations are yet to be made for myeloid lineages, there is evidence that separate fetal and definitive macrophage populations exist in zebrafish as in other vertebrates. Mature embryonic macrophages arising from the anterior mesoderm in zebrafish maintain an ability to divide, similar to murine and avian fetal macrophages which derive from primitive macrophages and retain their proliferative potential, but unlike the definitive post-mitotic, monocyte-derived mammalian macrophage (reviewed in Herbomel *et al.*, 1999 and Shepard *et al.*, 2000). The waning of expression of all early markers of zebrafish myeloid commitment

TABLE 2

ZEBRAFISH BLOOD CELLS: SITES OF PRODUCTION, HISTOCHEMICAL AND MOLECULAR PHENOTYPES

Leukocyte	Sites of development		Histochemical phenotype	Marker genes	Function
	adult	embryo			
Heterophil granulocyte	kidney (>96 hpf)[1,4,6] spleen[6]	18-24 hpf yolk sac mesoderm[4,6] 24-30 hpf intermediate cell mass >30 hpf - ventral venous plexus?[1]	MPO +[4,6] AcP +[4] PAS -[4] NSE weak	myeloid-specific peroxidase (*mpx/mpo*)[4,6]	acute inflammation[6]
Eosinophil granulocyte	kidney[6] spleen?	unknown	MPO –[4,6] AcP –[4] PAS +[4]	nil	unknown
Monocyte/Macrophage	kidney[1,6] spleen?	12-20 hpf rostral lateral plate mesoderm >20 hpf unknown - ventral venous plexus[1]	MPO –[4] NSE weak	*L-plastin*[2] *fms*[7]	phagocytosis of debris[2]
Lymphocyte	thymus (>65 hpf)[1] kidney (>2 weeks)[1] ovary (?)[9]	earlier site not identified	AcP -[4] Toluidine blue +[5]	*rag1*[5,9] *rag2*	acquired immunity?
Erythrocyte	kidney (>96 hpf)[1]	12-20 hpf lateral plate mesoderm and converging intermediate cell mass[1] 20-30 hpf intermediate cell mass 42-96 dpf ventral vein region[1] 48 hpf dorsal aorta?[1,8]	benzidine o-dianisidine[3]	*gata1*[3] *globin* isoforms[10] *alas-e*[11]	oxygen transport[10]

References: 1, Willett *et al.*, 1999; 2, Herbomel *et al.*, 1999; 3, Detrich, III *et al.*, 1995; 4, Bennett *et al.*, 2001; 5, Willett *et al.*, 1997a; 6, Lieschke *et al.*, 2001; 7, Parichy *et al.*, 1999; 8, Thompson *et al.*, 1998; 9, Willett *et al.*, 1997b; 10, Chan *et al.*, 1997; 11, Brownlie *et al.*, 1998; 12, Yamamoto *et al.*, 2000.

studied to date, if not a technical artifact of the increased difficulty of probe penetration of older zebrafish embryos, would also suggest another wave and site of myeloid cell production is to follow. Morphologic studies suggest this site would be the kidney – it will be interesting to assess by more sensitive methods if the late embryonic and adult kidney remains a site of expression of genes characteristic of haematopoietic stem cells.

There is certainly fluidity in the early locations of zebrafish haematopoiesis. The LPM surrounding the embryo contributes to both early myeloid and erythroid cell production. Myeloid cells emerge first from the rostral LPM at 12-13 hpf and immediately assume their function (Herbomel *et al.*, 1999). At about the same time, erythroid commitment occurs in the caudal LPM, which through a series of morphogenetic movements forms the intraembryonic axial intermediate cell mass (ICM) by progressive convergence in a rostral to caudal direction. Erythroid development only results in circulating haemoglobinized cells at 25-26 hpf, when the circulating erythroblasts still have an immature morphology (Al-Adhami *et al.*, 1977; Detrich, III *et al.*, 1995). Even at this stage, oxygen transport does not appear to be mandatory, as totally anaemic mutants are viable well beyond this age (Stainier *et al.*, 1995a). From 22-30 hpf, many stem cell marker genes are expressed in the posterior ICM or posterior blood island, a structure immediately caudal of the anogenital opening, suggesting this is a further site of haematopoiesis (Blake *et al.*, 2000; Liao *et al.*, 1998; Liao *et al.*, 2000a; Thompson *et al.*, 1998). Whether or not the *myb* expression in the dorsal aorta over this time is a genuine site of haematopoiesis or not also remains unclear (Thompson *et al.*, 1998). During the 3rd and 4th days of development, aggregated populations of cells expressing nearly every haematopoietic marker studied to date suggests the ventral vein region is a site of haematopoiesis (perhaps reflecting seeding from the posterior blood island, which was in its immediate proximity).

TABLE 3

CHARACTERIZED MOLECULAR MARKER GENES FOR ZEBRAFISH MYELOID CELLS AND THEIR PRECURSOR CELLS, WITH THEIR EXPRESSION PATTERN IN VARIOUS MUTANTS AFFECTING HAEMATOPOIESIS

Haematopoietic cell type	Gene	Haematopoietic mutant			
		cloche	*spadetail*	*m683*	*vampire*
Haemangioblast	*scl*[1]	R*	N	N	R (ICM)
	hhex[2]	R*			
	lmo2[3]	0	N		
Haematopoietic stem cell (definitive haematopoiesis)	*myb*[3]	R-0 (ICM, 24 hpf)	R-0 (ICM, 24 hpf)		
	cbfβ[1]	R*		N	R*
Myeloid, not lineage specific	*spi1/pu.1*[4]	R*	N-low		
	c/ebp1[5]	0 (24 hpf)	N	N	
Heterophil granulocyte-specific	*mpx/mpo*[6,7]	R*	N-low		
Macrophage-specific	*draculin*[8]				
	L-plastin[8]	0 (24 hpf)		N	
	fms				
Erythroid-specific	*gata1*[1,9,10]	R*	0	0	R*
Vascular -specific	*fli1*[2,3]	R*	N, disorganised		
	flk1 (vegf-r2)[2,3]	R*	N, disorganised		
	flt4[3]	R*	N, disorganised		

Abbreviations: N, normal expression in lateral plate mesoderm, and intermediate cell mass; 0, absent; R*, reduced expression; absent in the anterior intermediate cell mass (ICM), but with a few expressing cells remaining in the posterior of the ICM of embryos older than 20 hpf

References: 1, Blake *et al.*, 2000; 2, Liao *et al.*, 2000a; 3, Thompson *et al.*, 1998; 4, Lieschke *et al.*, 1999; 5, Lyons *et al.*, 2001; 6, Bennett *et al.*, 2001; 7, Lieschke *et al.*, 2001; 8, Herbomel *et al.*, 1999; 9, Detrich, III *et al.*, 1995; 10, Stainier *et al.*, 1995b; 11, Liao *et al.*, 1997b.

Alternatively, these venous sinuses may simply be a favoured site of residence for the more immature myeloid and erythroid cells. At least by morphologic criteria, definitive haematopoiesis occurs in the kidney from 96 hpf (Al-Adhami *et al.*, 1977; Willett *et al.*, 1999).

Molecular Markers of Early Zebrafish Myelopoiesis

There is extensive conservation of genetic pathways in haematopoiesis and related processes between zebrafish and mammals. In adaptive immunity, the cloning of zebrafish major histocompatibility complex (MHC) genes (Sultmann *et al.*, 1993; Sultmann *et al.*, 1994) and recombinase activating genes, *rag1* and *2*, necessary for V(D)J recombination of Ig and T cell receptors in lymphocytes (Willett *et al.*, 1997b; Willett *et al.*, 1997a), has been reported. Blood clotting in zebrafish involves similar coagulation cascades (Jagadeeswaran *et al.*, 1999; Sheehan *et al.*, 2001) although the cellular participant, the thrombocyte, is somewhat different to an enucleate platelet. A steadily increasing number of haematopoietic-specific genes including transcription factors, receptors, and effector genes has been cloned in zebrafish, providing molecular reagents and markers for specific stages of haematopoietic differentiation and specific cell types (reviewed in Paw *et al.*, 2000). Given the considerable morphologic and functional parallels between zebrafish and mammalian myeloid cells, it is not surprising that zebrafish show conservation of the molecular regulation of myelopoiesis, and of the molecular tools for effector myeloid cell function. Table 3 summarises some of the molecular markers of zebrafish myeloid cells and their precursors that have been characterised to date, and Fig. 2 presents examples of the expression patterns of these genes in early zebrafish development.

Genes marking Zebrafish Haematopoietic Stem Cells: scl, hhex, lmo2

SCL, LMO2 and HHEX are transcription factors implicated in early commitment to haematopoiesis in mammals (Shivdasani *et al.*, 1996). Zebrafish orthologues of these genes have been cloned (Liao *et al.*, 2000a; Thompson *et al.*, 1998) and analysed in expression and functional studies. All three genes are expressed in the LPM at the edge of the embryo from approximately the 5 somite stage: strong expression caudally is separated from rostral domains by a domain of much weaker or absent expression in the LPM abutting the midportion of the embryo. Over-expression of either *scl* or *hhex* augments expression of both vascular markers (e.g. *flk1*, *tie1*) and erythroid markers (o-dianisidine staining, *gata1*) indicating that both transcription factors direct commitment to both fates, suggesting an action at the level of the haemangioblast (Liao *et al.*, 1998). The ability of forced *scl* or *hhex* expression to partially rescue the haematopoietic and vascular defects of the *cloche* mutant place the action of these genes downstream of the unknown gene mutated in *cloche* (Hsu *et al.*, 2001; Liao *et al.*, 1997a; Liao *et al.*, 2000a). The collective data confirm the functional involvement of these genes in haemovascular fate commitment at this early stage and support the use of expression of these genes as markers for early, non-lineage specific, commitment to haemovascular fates.

Genes Orthologous to those marking Definitive Haematopoiesis in Mammals: c-myb, cbfβ

In mice, disruption of the *c-MYB* or *CBFβ* gene results in failure of the switch from embryonic to definitive haematopoiesis, with embryonic lethality (Shivdasani *et al.*, 1996).

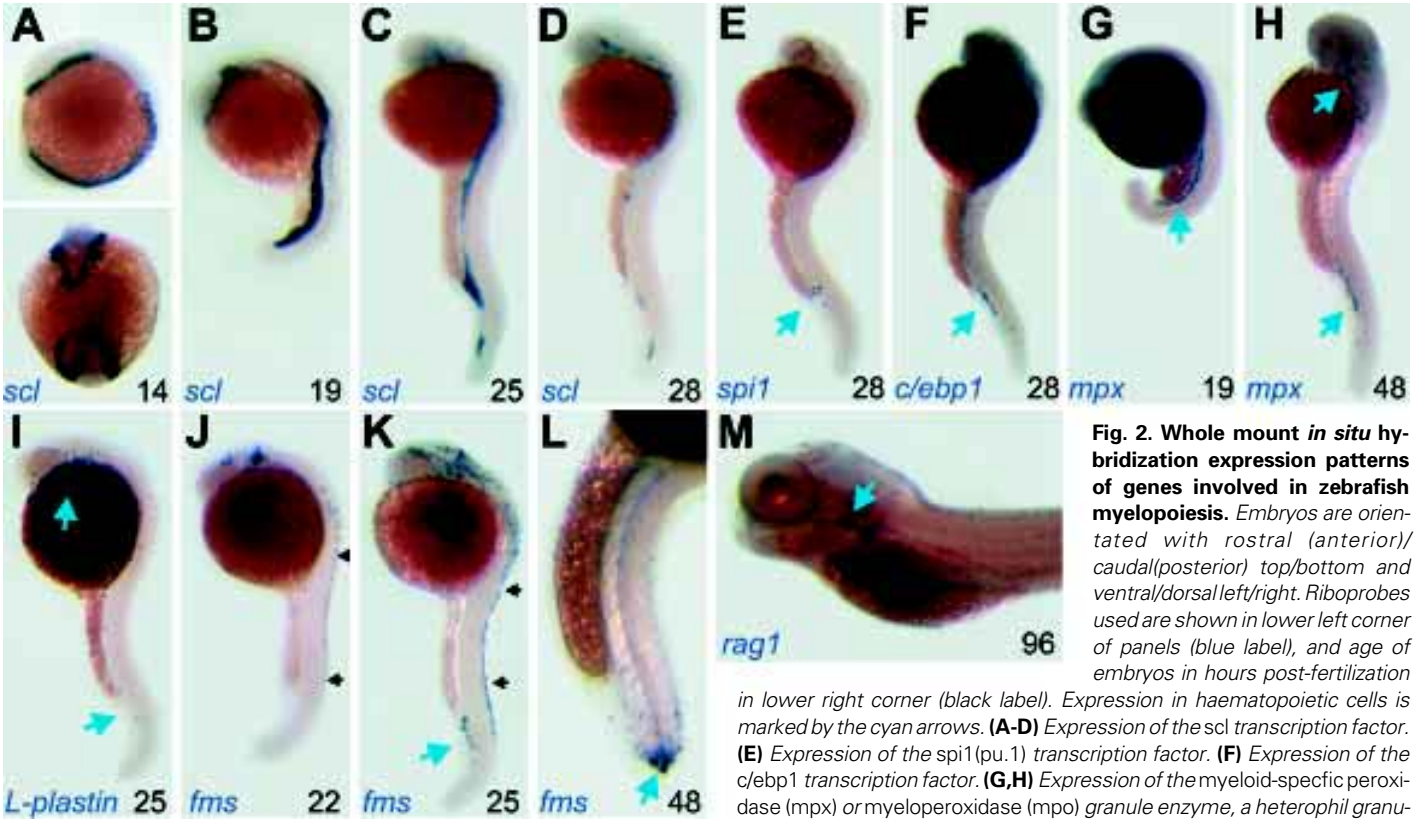

Fig. 2. Whole mount *in situ* hybridization expression patterns of genes involved in zebrafish myelopoiesis. *Embryos are orientated with rostral (anterior)/caudal(posterior) top/bottom and ventral/dorsal left/right. Riboprobes used are shown in lower left corner of panels (blue label), and age of embryos in hours post-fertilization in lower right corner (black label). Expression in haematopoietic cells is marked by the cyan arrows.* **(A-D)** *Expression of the* scl *transcription factor.* **(E)** *Expression of the* spi1(pu.1) *transcription factor.* **(F)** *Expression of the* c/ebp1 *transcription factor.* **(G,H)** *Expression of the* myeloid-specfic peroxidase (mpx) *or* myeloperoxidase (mpo) *granule enzyme, a heterophil granulocyte-specfic marker.* **(I)** *Expression of the* L-plastin *adhesion molecule, a macrophage-specific marker.* **(J-L)** *Expression of* fms*, the receptor for macrophage colony-stimulating factor, in zebrafish expressed in both neural crest derived-cells (black arrows) and in macrophages, most obviously recognized as a new population appearing in the ventral vein region after 24 hpf (K, cyan arrow). Cells expressing* fms *accumulate at a site of trauma (L, 12 hours after transection of the tail of a 2 dpf embryo), a behaviour expected of leukocytes.* **(M)** *Expression of the recombinase gene* rag1 *in the thymus (arrowed).*

Zebrafish *cbfβ* is expressed in anterior and posterior LPM in a pattern resembling that of *scl.* Retention of *cbfβ* expression in a mutant that retains *scl* expression but lacks *gata1* expression (*m683*) suggests that its effect is earlier than the commitment decision to an erythropoietic fate (Blake *et al.*, 2000).

At 18 hpf, zebrafish *myb* expression in the ICM resembles the pattern of *gata1*, with later expression observed in two dispersed populations of cells: one flat and regularly spaced over the yolk surface, and another scattered throughout the embryo (Thompson *et al.*, 1998). Although it was posulated that the yolk sac population may be an early phagocytic compartment, several observations call this into question: this population is retained in *cloche* (although the concurrently expressing ICM population is lost); and this population of cells does not resemble the distribution or shape of other myeloid markers at this age, which are themselves lost in *cloche* (see below). Despite the early expression of *myb* in the ICM, this wave of early zebrafish erythropoiesis is not dependent on *myb*, since the γ-deletion mutant *b316* which lacks *myb* still expresses *gata1* in this region.

Genes marking Myeloid Cells, but not Lineage Restricted: spi1(pu.1), c/ebp1

In mice, deletion of the *PU.1* gene disturbs myelopoiesis, with quantitative and functional defects in neutrophil granulocytes, macrophages, and some subgroups of lymphocytes (Anderson *et al.*, 1998). We have extensively studied the zebrafish *spi1(pu.1)* gene (the orthologue of PU.1; Lieschke *et al.*, in press). *spi1* marks an early site of myeloid commitment in the rostral LPM that corresponds to the site of macrophage development identified by videomicroscopy (Herbomel *et al.*, 1999) and marked by *draculin* (see below).

Although a family of mammalian CCAAT/enhancer-binding proteins (C/EBPs) is known (Hanson, 1998; Lekstrom-Himes *et al.*, 1998), the first characterized zebrafish C/EBP, *c/ebp1*, appeared to be a novel family member with a unique N-terminus (Lyons *et al.*, 2001). The first expression of this gene is in cells on the surface of the yolk at 17-18 hpf, after 24 hpf expressing cells appear in the ventral vein region, and at 48 hpf, positive cells are distributed throughout the embryo. The expression pattern is reminiscent of *L-plastin*, and indeed, most cells are positive for both with double staining. *c/ebp1* expression is preserved in *spadetail*, a mutant retaining myeloid but lacking erythroid cells, but lost in *cloche*, indicating myeloid lineage specificity.

Genes Marking Commitment to the Myeloid Lineage: mpx/mpo

We (Lieschke *et al.*, 2001) and others (Bennett *et al.*, 2001; Hsu *et al.*, 2001) recently reported the isolation of a zebrafish peroxidase gene expressed in zebrafish heterophil granulocytes, but not in eosinophil granulocytes or mature macrophages. Although highly homologous to mammalian myeloperoxidase, it is equally as homologous to several closely related mammalian peroxidases, and phylogenetic analysis indicates that it lies basal to the mammalian peroxidase family, suggesting it may represent an ancestral peroxi-

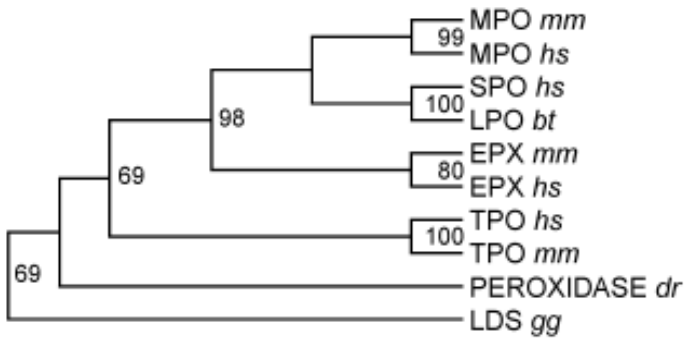

Fig. 3. Phylogenetic analysis of a zebrafish myeloid-specific peroxidase *(mpx)* gene. *Analysis was confined to the region 1 domains (involved in protein-protein interactions) of each protein. The dendrogram was constructed using Clustal X and Treeview, building on a previous comprehensive analysis of the entire peroxidase family (Daiyasu* et al.*, 2000), using linoleate diol synthase (LDS) from* Gaeumannomyces graminis *(gg) as an outgroup. Bootstrap values (n=1000) are indicated at nodes as percentages. The dendrogram complements that given previously for the more conserved catalytic domain (Lieschke* et al.*, 2001),and again places the* Danio rerio *peroxidase basal to the mammalian leukocyte peroxidases MPO and EPX. Abbreviations: MPO, myeloperoxidase; EPX, eosinophil peroxidase; SPO, salivary peroxidase; LPO, lactoperoxidase; TPO, thyroid peroxidase.*

dase gene (Fig. 3). Hence we called the gene *myeloid-specific peroxidase* (*mpx*), rather than *myeloperoxidase* (*mpo*). *Mpx* is expressed first in the ICM at 18 hpf. From 24-72 hpf, *mpx*-expressing cells are scattered throughout the embryo, over the yolk surface, dispersed through the entire body, and with a tendency for a subpopulation of *mpx*-expressing cells to be aggregated in the ventral vein region. Later expression was difficult to discern by whole mount *in situ* hybridization. Correlating with the myeloperoxidase activity, *mpx*-expressing cells localize to a site of acute inflammation. A subset of *spi1* positive cells also co-expressed *mpx* at the time of waning *spi1* expression; this may represent the commitment of a portion of common *spi1*-positive myeloid progenitors to the granulocytic lineage, with the beginning of expression of granule-specific genes (Bennett *et al.*, 2001).

Genes marking Commitment to the Monocyte/Macrophage Lineage: draculin, L-plastin, fms

Although *draculin* was first reported in the context of studies of the early zebrafish macrophage population, its expression pattern is more extensive than just this lineage. *draculin* is expressed from the late blastula at the blastoderm margin, and at gastrulation, becomes confined to the area forming ventral mesoderm, dorsomarginal cells of the organizer, and the presumptive prechordal plate (Herbomel *et al.*, 1999). During gastrulation, *draculin* expression becomes stronger in the LPM, especially in rostral and caudal domains, mimicking other early haematopoietic markers like *scl* and *hhex*, and overlapping the area of *gata1* expression posteriorly. The rostral domain of LPM *draculin* expression lies anterior to *nkx2.5* expression, but between the two areas of *flk* expression believed to represent aortic arch primordia. From the 11-15 somite stage, *draculin*-positive cells converge towards the midline, but progressively *draculin*-expressing cells disperse from the lateral edges of this conglomerate of *draculin*-positive cells. As the cells move over the yolk sac, they lose their draculin expression. Posterior *draculin* expression becomes focused in the posterior ICM, possibly in proerythroblasts as originally described, although as other myeloid markers are also expressed in this site, the identity of the *draculin*-expressing cell at this site warrants further scrutiny.

L-plastin is the zebrafish orthologue of a late marker of macrophage development in mammals, an actin bundling protein involved in adhesion and activation (Jones *et al.*, 1998). *L-plastin* expression initiates as macrophages disperse over the yolk sac, and by 20 hpf, the *L-plastin* expressing cells form a dispersed axial population on the anterior yolk, mostly under the hatching gland and along the pericardium (Herbomel *et al.*, 1999). By 28 hpf, *L-plastin* expression is evident in cells in the posterior ICM and dispersed along the body of the embryo and in the cranial mesenchyme. By 5 days post-fertilization (dpf), expression is drastically reduced, although a few positive cells are observed in the areas of the gill arches and the thymus (Bennett *et al.*, 2001). *L-plastin* expression marks a population of cells distinct from those expressing *mpx/mpo*, although occasional co-expressing cells are observed, and hence this marker seems to distinguish an early zebrafish macrophage population from an emerging granulocytic population (Bennett *et al.*, 2001).

Zebrafish *fms*, a receptor tyrosine kinase gene related to *c-kit*, is believed to be the orthogue of the mammalian *FMS*, which encodes the M-CSF receptor (Parichy *et al.*, 2000). Zebrafish *fms* is expressed in a dispersed population of cells likely to be macrophages, but in addition, there is prominent expression in the neural crest derivatives. Hence the zebrafish *fms* mutant *panther*, has disturbed pigment patterning due to the absence of the yellow-coloured xanthopores and disorganized melanophores. *fms* is expressed in dispersed macrophages over the yolk (confirmed by co-expression with *spi1/pu.1*), and in osteoclasts in the regenerating fin (identified by expression of the protease cathepsin-K) (Parichy *et al.*, 2000). A neural crest role for *fms* has not been suspected from the phenotype of *fms*-defective op/op mice, which have osteosclerosis and impaired macrophage development. Hence the pivotal role for *fms* in zebrafish pigmentation patterning, demonstrated by the *panther* mutant, poses interesting evolutionary issues regarding conservation and divergence of gene function.

Experimental Approaches

Now that the descriptive biology of zebrafish myeloid development enables these processes to be recognized, and now that a group of molecular and histochemical markers is sufficiently characterized for them to be used to identify myeloid cells and their precursors, the genetic strengths of the model organism can be exploited to further our understanding of this developmental process. We are adopting several approaches in our group.

Basic Biology of Myeloid Development

Our major biological interest is in the early commitment of stem cells to a myeloid fate. Several projects in our group seek to exploit the basic biological tools now available in zebrafish to better understand this embryological process.

A Screen for Zebrafish Myeloid Mutants

Our group is committed to undertaking a screen for myeloid developmental mutants in zebrafish, based on a focused screening strategy that will enable recognition of mutants with failure of myeloid commitment but intact erythroid commitment. A pilot screen has demonstrated the feasibility of our approach (J.E. Layton and G.J. Lieschke, unpublished data) and the scale of the

screen is being increased to enable collection of a group of mutants representing lesions in the majority of genes on which this process depends.

Marking the Zebrafish Myeloid Compartment

Early erythroid precursors have been marked in the zebrafish using the zebrafish *gata1* promoter to drive green fluorescent protein (GFP) expression in early erythroid cells. The *gata1*-GFP transgenic fish have also provided the basis for isolating new genes important in early erythroid development (Long *et al.*, 1997). In our laboratory several projects are underway (A.C. Ward and G.J. Lieschke, unpublished data) aiming to mark the myeloid compartment of zebrafish in an analogous fashion. We anticipate that such fish will be valuable tools for facilitating cellular biological studies of early myeloid commitment in zebrafish, and for dissecting the defects in myeloid mutants identified from the screens underway.

Conclusion

A considerable initial momentum in zebrafish haematology focused on the red cell and mutants resulting in anaemia. Recent studies have now characterized myeloid development in this model organism to the point where myeloid lineage-specfic lesions can be identified and studied. Hence it can now reasonably be hoped that the powerful genetic approaches applicable in this model, the genomic resources being collected by the international zebrafish and genomic communities, and the ability to study myeloid development in this model organism, will combine to provide new insights into the myeloid arm of developmental haematology.

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