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Chapter 3

Regulation of Teleost Macrophage and Neutrophil Cell Development by Growth Factors and Transcription Factors

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53589

1. Introduction

Macrophages and neutrophils are the sentinel cells of the innate immune response of vertebrates, such as bony fish (teleosts). As phagocytic myeloid cells, they are involved in homeostatic mechanisms, wound healing, and the detection, elimination and clearance of foreign entities including tumors, virus-infected cells and invading pathogens. Furthermore, macrophages and neutrophils are responsible for producing hundreds of bioactive molecules that are important in pathogen recognition and destruction, cellular communication and activation, initiation of an adaptive immune response and later, resolution of an inflammatory response and tissue repair. Neutrophils and macrophages, while essential to survival, have a finite lifespan. Therefore, a manufacturing centre, the hematopoietic niche, is needed for the production of myeloid cells. The hematopoietic niche must maintain basal myeloid cell production levels during homeostasis, yet retain the flexibility to ramp-up cell production in response to physiological demands, such as pathogenic insult. The development of macrophages (monopoiesis) and neutrophils (granulopoiesis) is collectively known as myelopoiesis, and is regulated by the complex interaction of colony-stimulating factors (CSFs), their receptors, and intracellular transcription factor machinery that control lineage fate decisions and terminal differentiation events.

Over the past 50 years, research using the mouse model system has culminated in the identification of the site(s) of myelopoiesis, the progenitor cell types that give rise to mature myeloid cells, the extracellular and intracellular cues required, and a detailed understanding of the complex intracellular and extracellular milieu of factors that drive this tightly controlled

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process. From these studies we understand hematopoiesis as an exquisitely fine-tuned, highly regulated, process whereby all blood cells develop from a small number of hematopoietic stem cells (HSCs). HSCs are characterized as long-term repopulating, pluripotent, quiescent cells that undergo symmetrical self-renewal to sustain the population of HSCs within the hematopoietic niche, or asymmetrical division to give rise to hematopoietic progenitor cells (HPCs) [1]. HPCs can develop along the lymphoid lineage, termed lymphopoiesis, to give rise to B-cells, T-cells, natural killer (NK) cells and dendritic cells (DCs). Alternatively, HPCs can develop along an erythroid lineage, termed erythropoiesis, to give rise to erythrocytes and megakaryocytes, or develop along a myeloid lineage to give rise to granulocytes (neutrophils, basophils, eosinophils, mast cells), mononuclear phagocytes (monocytes and macrophages), and DCs. The lymphoid lineage represents the adaptive arm of the immune response, while the myeloid lineage represents the innate arm of the immune response. Regardless of lineage, the decisions made to commit and develop along a given lineage are controlled by extracellular growth factors and intracellular transcription factors that act in concert to regulate gene and protein expression to achieve the desired outcome.

When compared to the mechanisms of myelopoiesis in the mouse, studies using lower vertebrates, such as teleosts, have identified both evolutionary conservation as well as divergence in the mechanisms of myelopoiesis. With over 30,000 identified species, teleosts are the most expansive class of vertebrates, and represent an excellent model system to study the evolution of vertebrate myelopoiesis as they are one of the ancient classes of vertebrates to retain the production of myeloid cells. Within the teleost system, much research surrounds the characterization of teleost cytokines and receptors involved in inflammation and their cellular targets (primarily macrophages). In comparison, little is known about the mechanisms that govern myeloid cell production. Research on teleost myelopoiesis is hampered by the lack of reagents, the difficulty in isolating appreciable numbers of relatively pure populations of HSCs/HPCs, and in identifying key growth factors important for myeloid cell development due to evolutionary selection pressures. As such, the focus of this review is to provide an overview of the current knowledge of the fish model systems used and the growth factors, receptors and transcription factors involved in teleost myelopoiesis, using information from the mammalian model systems as a scaffold to put the advances into context.

2. Teleost model systems of myelopoiesis

2.1. Zebrafish model system

The zebrafish model has been instrumental in advancing our knowledge of the sites of hematopoiesis/myelopoiesis in teleosts, the development, differentiation and migration of HSCs, and through genetic manipulation, the characterization of the early acting growth factors, receptors and transcription factors involved in hematopoiesis. By far, the major advantage provided by zebrafish is the ease of generating transgenic zebrafish, morphant zebrafish (morpholinos) and knockout zebrafish (zinc finger nucleases), as well as many others, due to

http://dx.doi.org/10.5772/53589

genetic manipulation. In conjunction, the rapid generation of embryos, embryonic transparency, and small embryo size allows for mass screening strategies. The advantages of zebrafish as a model system, and their contributions to hematopoiesis have been extensively reviewed elsewhere [2-5], and thus will not be covered in this review. While the zebrafish is an excellent *in vivo* model, it does not lend itself to *ex-vivo* studies due to the small size of the fish and the difficulty of isolating sufficient number of cells for *in vitro* studies.

2.2. Ginbuna crucian carp model system

In vivo transplantation to test the repopulation activity of donor cells has been the gold standard for the characterization of HSCs and HPCs [6-9]. In cyprinid fish, there is a unique transplantation model system for detecting HSCs and HPCs using clonal ginbuna crucian carp (*Carassius auratus langsdorfii*, S3n strain) and ginbuna-goldfish (*Carassius auratus*) hybrids (S4n strain). Ginbuna crucian carp have advantages for transplantation experiments because they are easily maintained, tolerate handling and are large enough to allow for the collection of sufficient hematopoietic cells. Clonal ginbuna are unisexual triploid fish (all female, 3n = 156) that principally reproduce gynogenetically. A unique clone (S3n) can reproduce by not only gynogenesis but also bisexual reproduction. When eggs from the S3n clone are inseminated with UV-irradiated goldfish sperm, triploid clones result. In contrast, when the eggs are inseminated with normal goldfish sperm, tetraploid hybrids (S4n) are obtained [10]. These S4n fish possessed four sets of chromosomes, three from the S3n clone and one from the goldfish. Therefore, when the cells from S3n clones are transferred into S4n recipients, transplants are accepted, whereas the reverse transplants are rejected [11, 12]. Moreover, the donor cells in the recipient tissues are easily distinguished by their difference in DNA content by flow cytometric analysis (ploidy analysis) [13].

The ginbuna crucian carp model system has been instrumental in serving as a close parallel to the mouse model system in terms of hematopoietic reconstitution experiments to demonstrate the existence of HSCs in teleosts. Identification of donor and recipient HSCs/HPCs and characterization of their progeny by ploidy analysis is useful for assessing the multipotency of different progenitor cell populations. Furthermore, the use of this model system has allowed for the determination of the location of HSCs within the hematopoietic organs of cyprinids. Use of the ginbuan crucian carp system will be particularly important for future work as antibodies are developed against markers on the surface of fish HSCs and HPCs to allow for the analysis of the potency of progenitor cell subpopulations.

2.3. Goldfish model system

The goldfish model system represents a unique opportunity to study myelopoiesis *in vitro*. Firstly, teleost monopoiesis can be examined using the previously developed primary kidney macrophage (PKM) culture system [14, 15] and has provided information on the growth factors, receptors, and transcription factors involved. Secondly, large numbers of relatively pure neutrophils can be isolated from the goldfish kidney [16] and represents a starting point for studying granulopoiesis in goldfish and will be discussed in the following sections.

Together, these two model systems will prove instrumental in understanding the factors that regulate teleost myelopoiesis.

In the *in vitro* PKM system, small mononuclear cells isolated from the goldfish kidney proliferated and differentiated over 8-10 days giving rise to three cell sub-populations, R1-, R2- and R3-gated cells [14, 15]. The cytochemical, molecular and functional characterization of these cell sub-populations demonstrated the presence of putative progenitor cells (R1 gate), monocytes (R3 gate), and mature macrophages (R2 gate). These three cell sub-populations in PKM cultures represent distinct junctures of macrophage development simultaneously occurring *in vitro* [14, 15].

The spontaneous proliferation and differentiation of PKMs suggested the production of endogenous growth factors and prompted the examination of the target cell sub-population(s) upon which they acted and their effects on cell proliferation and differentiation. The putative progenitors (R1 cells) and macrophages (R2 cells), but not monocytes, were determined to be responsible for the production of endogenous growth factors that act in an autocrine and paracrine fashion [15]. Addition of cell-conditioned medium (CCM) to sorted cell populations demonstrated the capacity of putative progenitors and monocytes to proliferate and differentiate in response to endogenous growth factors. However, treatment of macrophages (R2 cells) with CCM demonstrated their apparent terminal differentiation, while their capacity to proliferate suggested they were capable of self-renewal [15, 17]. Clearly, different endogenous growth factors present in CCM exert distinct actions on macrophage cell sub-populations.

Two pathways of macrophage development were proposed to occur in the PKM cultures. The predominant pathway was classical macrophage development in which progenitor cells differentiated into monocytes and then macrophages [17]. The second was an alternative pathway of macrophage development in which progenitor cells differentiated into macrophages without a prominent monocytic stage [17]. The possible retention of the alternative pathway of macrophage production in addition to the classical pathway may provide a mechanism for rapid generation of macrophages during injury or infection *in vivo*.

The observed kinetics of the PKM cultures suggested three phases of growth. Initially, there is a lag phase (days 1-4) where many cells die, followed by a proliferative phase (days 5-9) where cell numbers rapidly increase [14], and finally, a senescence phase (days 10-14) characterized by cell clumping and cell apoptosis [17, 18]. Differential cross screening of proliferative versus senescence phase PKMs identified a number of differentially expressed genes including those involved in hematopoiesis, signal transduction, transcription, translation and protein processing [19]. The involvement of the identified transcripts in the regulation of cell development [20-22] will be discussed in the following sections.

These seminal observations from PKM cultures established three important ideas regarding goldfish monopoiesis: (1) kidney leukocytes produce their own endogenous growth factors important for driving proliferation and differentiation [14, 15]. (2) Within the population of small leukocyte R1 cells, a population of macrophage progenitor cells must exist. (3) Unlike mammalian systems, the progenitor cell population gives rise to fully differentiated macro-

http://dx.doi.org/10.5772/53589

phages *in vitro* in the absence of exogenous growth factors. Thus, the goldfish PKM model system allows for comprehensive analysis of the interactions between developing macrophage subpopulations in vitro.

3. Site of hematopoiesis/myelopoiesis

3.1. Two waves of hematopoiesis in vertebrates

There are two waves of hematopoiesis in vertebrates. The first wave is primitive hematopoiesis and occurs during embryonic development. Definitive hematopoiesis follows primitive hematopoiesis and occurs in the post-natal or adult animal. Primitive and definitive hematopoiesis are different on a temporal scale, a spatial scale, and in the types of cellular progeny generated. With the exception of T-cells, that undergo maturation in the thymus, lymphopoiesis and myelopoiesis occur in the major hematopoietic organs. The major hematopoietic organ of teleosts is the kidney, akin to that of mammalian bone marrow.

3.2. Primitive myelopoiesis in teleosts

The development of myelopoiesis in fish has primarily been studied using the zebrafish model system. Primitive myelopoiesis is predominated by HPCs with primarily erythroid and myeloid development potential. Initially, primitive hematopoiesis is initiated in the anterior lateral mesoderm (ALM), that gives rise to the rostral blood island (RBI), and in the posterior lateral mesoderm (PLM), that gives rise to the intermediate cell mass (ICM). The RBI is the site of primitive myeloid cell development, generating primarily primitive macrophages that undergo rapid differentiation, lacking or having a very short monocytic stage [23] and a few neutrophils [24], while the ICM is the site of primitive erythroid cell development [25]. This stage of primitive hematopoiesis occurs early during development of zebrafish, approximately 11 hours post fertilization (hpf). Following the onset of circulation, at around 24 hpf, the site of hematopoiesis then switches to the posterior blood island (PBI) [26] and produces multi-lineage progenitor cells capable of producing both primitive erythroid and myeloid cells [27]. Primitive macrophages act as phagocytes during tissue remodeling throughout embryonic development and in clearance of bacterial pathogens [23]. While primitive neutrophils also migrate to a site of infection, they were not observed to phagocytose bacteria [24]. The temporal, spatial and transcriptional control of zebrafish primitive hematopoiesis has been reviewed by [28-30]. Differences in the initial site of hematopoiesis occur between fish species, however, the production of erythrocytes and macrophages during primitive hematopoiesis is consistent [31, 32].

3.3. Definitive myelopoiesis in teleosts

The onset of definitive myelopoiesis occurs around 36 hpf in the zebrafish. Here, HSCs seed the aorta-gonad-mesonephros (AGM) and the caudal hematopoietic tissue (CHT) [33, 34]. By 48 hpf, the HSCs seed the kidney [33], the final hematopoietic site equivalent to mammalian bone marrow [35-37].

The existence of teleost kidney HSCs and HPCs capable of generating all hematopoietic lineages was demonstrated using transplantation studies in zebrafish and ginbuna crucian carp. Transplantation of whole kidney marrow from *gata1*^{eGFP} zebrafish into pre-thymic *vlad tepes* (*gata1*$^{-/-}$) zebrafish [37] or whole kidney marrow from *β-actin*^{eGFP} zebrafish into lethally irradiated zebrafish [38], resulted in rescue of the phenotype and produced lymphoid and myeloid cell types suggestive of the presence of HSCs capable of long-term reconstitution. However, these studies were complicated by the use of whole kidney marrow during transplantation. Using ginbuna crucian carp, HSCs, found to be associated with the trunk kidney renal tubules, were identifiable by their ability to efflux Hoechst 33342 using the ATP-binding cassette (ABC) transporter, ABCG2a, and HPCs were identified by their ability to efflux rhodamine 123 by another ABC transporter, P-glycoprotein [39-42]. HSCs, consisting of 0.33% ± 0.15 of the total body kidney cells, were capable of engraftment and long-term production (>9 months) of all hemopoietic progeny, including erythrocytes, granulocytes, monocytes, thrombocytes and lymphocytes [40, 41, 43]. HPCs, while they could also give rise to all hemopoietic progeny, were only capable of short-term reconstitution [42]. However, engraftment of donor HSCs and HPCs only occurred in anemia-induced or gamma irradiated recipients [40, 43, 44] suggesting that space within the hematopoietic niche is required for successful engraftment of HSCs to occur [40, 43]. Experiments using zebrafish and ginbuna crucian carp provide strong evidence that the teleost trunk kidney contains HSCs and HPCs capable of multi-lineage differentiation, including myelopoiesis [45].

4. Commitment to the myeloid lineage

4.1. Progression of cell development

From the mouse model we know that the commitment of a pluripotent, self-renewing HSC to a common myeloid progenitor (CMP) is a progression of lineage fate decisions controlled by extracellular cues, such as growth factors, within the hematopoietic niche [46-48], as well as the modulation of intracellular transcription factors [49-52]. The process of committing to a CMP begins with long-term HSCs (LT-HSCs), capable of self-renewal and multi-lineage differentiation. LT-HSCs give rise to short-term HSCs (ST-HSCs) with limited capacity for self-renewal, which then differentiate into multipotent progenitors (MPPs) with no ability to self-renew, reviewed by [53]. The MPPs can give rise to the CMP or the lymphoid-myeloid primed multipotent progenitors (LMPPs) [54-57]. The CMP can differentiate into megakaryocyte/erythroid progenitor (MEP) or to a granulocyte/macrophage progenitor (GMP) [58] (Figure 1). The LMPPs can differentiate into a common lymphoid precursor (CLP) that gives rise to T- and B-lymphocytes, or can also give rise to GMPs [54-57, 59, 60], and reviewed in [61].

On the other hand, the "myeloid-based model" of hematopoiesis, in which myeloid potential is retained in erythroid, T, and B cell branches even after these lineages have segregated from each other, has been proposed [62]. Notably, there is no CLP in this model [63-65]. According to this model, hematopoiesis can be understood as follows: specification toward er-

http://dx.doi.org/10.5772/53589

ythroid, T, and B cell lineages proceeds on a basis of a prototypical developmental program to construct myeloid cells [66, 67]. Indeed, several findings in teleosts are supportive of the myeloid-based model [68, 69]. In the future, the myeloid-based model may bring a paradigm shift in the concept of blood cell lineage development. In the following sections the key growth factors and transcription factors studied in the teleost system will be discussed.

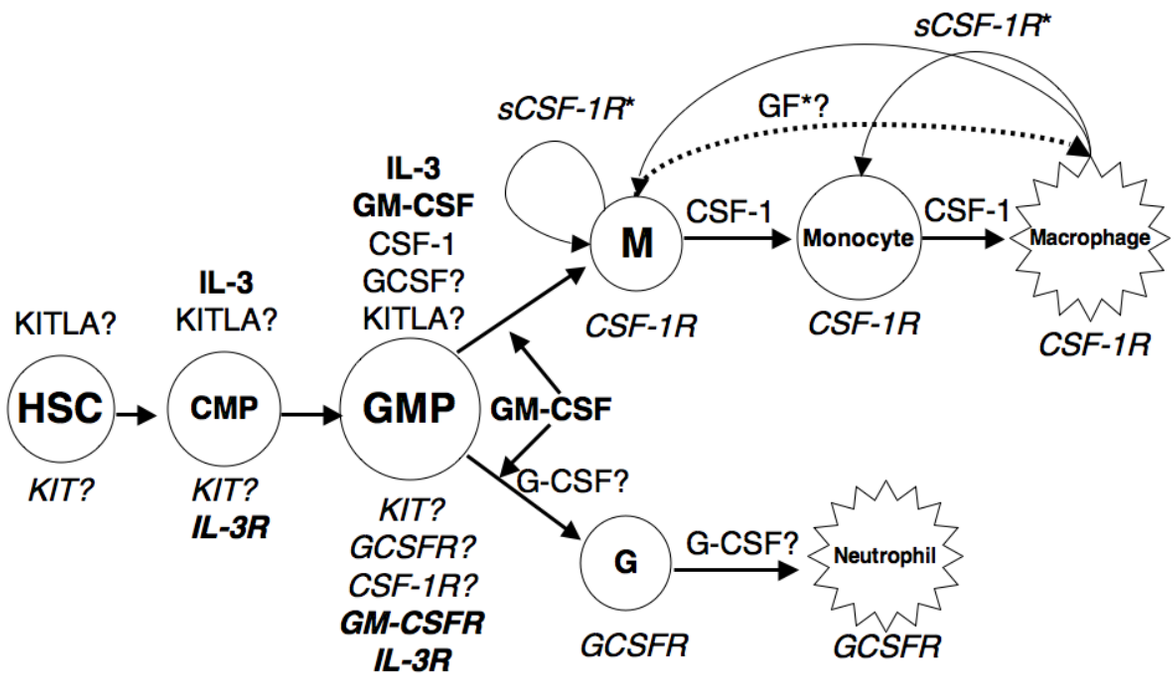


Figure 1. Growth factors and their receptors involved in goldfish myelopoiesis. Goldfish growth factors are shown in uppercase lettering, goldfish growth factor receptors/surface receptors are shown in uppercase italics lettering, and growth factors and their receptors important in mammalian myelopoiesis, but have yet to be identified in teleosts are shown in uppercase ***italics***. The dashed arrow denotes the alternative pathway of macrophage development in goldfish, the solid curved arrows denote negative regulation of macrophage development by sCSF-1R. Question marks denote the hypothesized role of growth factors or receptors and further studies are required to test the hypothesis. Asterisks mark differences between teleosts and mammals. Abbreviations used: (1) **Cellular stages**: HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; M, monocytic precursor; G, granulocytic precursor. (2) **Growth factors**: KITLA, kit ligand a; IL-3, interleukin 3; GM-CSF, granulocyte-macrophage colony-stimulating factor; CSF-1, colony-stimulating factor 1 (macrophage colony-stimulating factor); GCSF, granulocyte colony-stimulating factor; GF, growth factor. **Receptors**: IL-3R, interleukin 3 receptor; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; CSF-1R, colony-stimulating factor-1 receptor (macrophage colony-stimulating factor receptor); sCSF-1R, soluble colony-stimulating factor-1 receptor; GCSFR, granulocyte colony-stimulating factor receptor.

4.2. Receptors and growth factors

4.2.1. Mammalian stem cell factor and Kit receptor

Stem cell factor (SCF) was identified [70-72] as short-chain four-helix bundle [73] encoded by the *Steel* locus in the mouse [74]. Mutations in the *Steel* locus were associated with defects in stromal cells, and resulted in reduced numbers of HSCs and HPCs [75]. The *SCF* gene

produces two alternatively spliced mRNAs that differ in the presence or absence of exon 6 [71]. Although the two *SCF* splice variants can be expressed in the same tissues, they have tissue specific regulation of expression [71, 76]. Both SCF isoforms are produced as extensively glycosylated [77, 78] membrane bound forms (mSCF) that can undergo proteolytic cleavage to produce a soluble form of SCF (sSCF) [79, 80]. In human blood, sSCF is at a concentration of 3.0 ± 1.1 ng/mL [77]. Alternatively, mSCF may provide a means for cell-to-cell contact with the stromal cells in the hematopoietic niche [71], and may act to increase the signal strength provided to the HSC/HPCs, reviewed in [81]. Both mSCF and sSCF are capable of forming dimers [78, 82] and signal through their receptor, c-KIT.

The SCF receptor, c-KIT (CD117), was first identified as the cellular oncogene (*c-onc*) equivalent of the viral oncogene (*v-onc*), *v-Kit*, isolated from the Hardy-Zuckerman 4 feline sarcoma virus [83]. Based on structural analysis, the c-KIT protein was grouped within the Type III tyrosine kinase receptor family that includes colony-stimulating factor-1 receptor (CSF-1R), platelet derived growth factor receptor (PDGFR), and FLT3/FLK2 receptor [84-87]. Studies mapped *c-KIT* to the *White* locus (*W*) in the mouse [74, 83], and demonstrated that mice with mutations in the *White* or *Steel* loci exhibit hypopigmentation, mast cell deficiency, macrocytic anemia, and sterility, while the complete loss of either of these genes was lethal [74, 88].

The c-KIT protein is primarily found on hematopoietic cells and is a marker of long-term reconstituting HSCs in humans [89] and mice [90-92]. c-KIT is expressed on pluripotent and multipotent HSCs and myeloerythroid precursors, but not on differentiating or mature cell types [90-92], with the exception of mast cells [93]. Approximately 2×10^4 c-KIT receptors are found on normal human HPCs [94], and can undergo proteolytic cleavage to release a soluble form of c-KIT [95-97]. The soluble c-KIT receptor is thought to regulate membrane bound c-KIT activity, *in vivo*, by blocking SCF binding [95, 98].

Binding of homodimeric SCF to c-KIT results in receptor homodimerization, conformational changes in the extracellular and intracellular domains and autophosphorylation of the intracellular tyrosines (reviewed extensively in [73, 78, 99-105]) leading to a number of downstream signaling pathways that mediate the action of SCF through c-KIT. These signaling pathways include phosphatidylinositol-3-kinase (PI3K), phospholipase Cγ (PLCγ), members of the Janus family of protein tyrosine kinases (JAK) and signal transducers and activators of transcription (STATs), Src family members, the Ras/Raf/MAP kinase pathway, and others. The signaling pathway initiated depends on the cell type, and the strength and duration of the signal, reviewed in [106-108].

4.2.2. Biological functions of stem cell factor

SCF and its type III tyrosine kinase receptor c-KIT, are involved in hematopoiesis [81, 107, 108], spermatogenesis [109-111], and development of melanocytes [110, 112-114] and mast cells [93, 96, 115-120]. Within the hematopoietic niche, one role of SCF/c-KIT is to mediate HSC and HPC survival, important for the generation of spleen, interleukin-3 (IL-3), granulocyte/macrophage, and macrophage colony-forming units (CFU-S, CFU-IL-3, CFU-GM, and CFU-M) [121]. Further studies have confirmed SCF/c-KIT to mediate the survival of long-

term HSCs by blocking cell cycling or by inhibiting apoptosis [122, 123]. Furthermore, SCF can synergizing with other growth factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) [124], granulocyte colony-stimulating factor (G-CSF), IL-1, IL-3 [98], IL-6, and IL-7, among others, to promote the proliferation and differentiation of HPCs [125, 126] and reviewed in [101]. Often, the progeny of HPC differentiation depends on the particular growth factor and SCF. Lastly, SCF acts as a homing signal to HPCs, such as CFU-GEMM (granulocyte-erythrocyte-macrophage-megakaryocyte), CFU-GM, CFU-Meg (megakaryocyte) and burst forming units-erythrocyte (BFU-E) [127]

4.2.3. Teleost Kit and Kit ligand

Whole genome duplication has resulted in two orthologues of *c-KIT* and *SCF* in teleosts. Teleost orthologues of *c-KIT,* termed kit a (*kita*) and kit b (*kitb*), were first identified in zebrafish and have subsequently been predicted from genomic analysis of *Takifugu rubripes* and *Tetraodon nigroviridis* [128, 129]. The *kita* orthologue has also been identified and characterized in *Carassius auratus* [130]. The two orthologues of mammalian *SCF* are termed kit ligand a (*kitla*) and kit ligand b (*kitlb*) [128, 131]. The *kitla* and *kitlb* have been identified in zebrafish, and predicted in fugu, medaka, and stickleback genomes [131]. The *kitla* orthologue has been identified and characterized in goldfish [130].

Zebrafish *kita*, located on chromosome 20, and *kitb,* located on chromosome 1, are the orthologues of human and mouse *c-KIT* [128, 129]. Both *kita* and *kitb* genes contain 21 exons, however, their respective proteins only retain 55% identity to each other [129]. The partitioning of gene distribution and function was proposed to explain the retention of *kita* and the duplicated gene, *kitb* [128, 129]. From studies on developing zebrafish, *kita* is expressed in hematopoietic progenitors, melanoblasts and melanocytes derived from the neural crest, along the lateral line, the notochord and pineal gland [128, 129]. The expression of *kitb* occurs by 9 hpf and does not overlap that of *kita*. Instead, *kitb* expression is restricted to the Rohon-Beard neurons, trigeminal ganglia, and otic vesicle [129]. Together, the expression of *kita* and *kitb* approximates that of *c-KIT* in the mouse model system, with the notable exception of *c-KIT* expression in primordial germ cells (PGCs).

The *kitla* gene is located on chromosome 25 and the *kitlb* gene is located on chromosome 4 of the zebrafish genome [132]. *Kitla* has 9 exons while *kitlb* has 8 exons [131]. The nine *kitla* exons correspond to the 9 exons of mammalian SCF isoform 1, including exon 6 which allows for cleavage of membrane bound SCF into a soluble form [131]. However, *kitlb* appears to correspond to SCF isoform 2, in which exon 6 has been spliced out. The expression of *kitla* is first observed at 19 hpf in the zebrafish and is found in the developing tail bud, pineal gland, sensory epithelium of the ear, ventral otic vesicles, and in the somites [131]. Similar to the expression of goldfish *kita*, *kitla* showed constitutive mRNA levels in tissues [130] and this expression pattern was similar to what was observed in adult zebrafish tissues [132]. Goldfish *kitla* showed high levels of mRNA in isolated putative progenitor cells and monocytes compared to macrophages [130]. Zebrafish *kitlb* mRNA expression was observed in the brain ventricles, ear and cardinal vein plexus and at lower levels in the skin as zebrafish development progressed [131].

4.2.4. Biological functions of teleost kit ligands and receptors

Based on the non-overlapping expression of *kita* and *kitb*, the functional roles of c-KIT in mammals may be partitioned between teleost KITA and KITB. The zebrafish mutant *sparse*, shown to map to *kita* [128], or kit^{w34} mutants [133] show defects in their pigmentation pattern. Zebrafish KITA was shown to be involved in the dispersion and maintenance of melanocytes [128], and may play a transient role in melanocyte differentiation when melanoblast development is perturbed [134]. Furthermore, knock-down of zebrafish *kitla* or *kitlb* using morpholinos supported the involvement of KITLA in the migration and survival of melanocytes [131]. Teleost *kit* expression in melanocytes has been implicated in the pigment pattern formation in a number of fish species [128, 135-137] and suggests that the functions in myelocyte development have been partitioned to the *kita* orthologue.

The role of teleost *kita/kitla and kitb/kitlb* during hematopoiesis is not clear. Examination of hematopoiesis in zebrafish *sparse* mutants revealed no obvious defects in hematopoiesis during development. Although, slight decreases in promyelocyte and neutrophil cell numbers, and slight increases in band cells and monocytes were observed in the kidney [128]. In addition, zebrafish injected with *kitla* morpholinos or *kitlb* morpholinos also did not show defects in hematopoiesis. However, studies in the goldfish model system demonstrated the expression of *kita* mRNA in isolated kidney progenitor cells, and the functional role of goldfish KITLA in progenitor cell chemotaxis, proliferation, and maintenance [130]. Taken together, these data suggest that KITA and KITLA proteins play a central role in myelopoiesis (Figure 1). However, redundancy between the two ligands and receptors may account for the absence of hematopoietic defects in the zebrafish system, or there may be redundancy with another tyrosine kinase receptor. Additionally, the absence of hematopoietic defects in the zebrafish may represent KIT-independent and KIT-dependent stages of hematopoiesis. The function of KITLB and KITB during hematopoiesis in teleosts remains to be determined.

Lastly, c-KIT plays a role in the development of primordial germ cells (PGCs) in mice. Examination of primordial germ cell development in fish revealed that *kita* and *kitb* expression was not detected in PGCs, and suggests teleost KITs do not play a role in the development of PGCs [128, 129]. However, it appears that *kita*, *kitb*, *kitla* and *kitlb* play a role in ovarian folliculogenesis in zebrafish and provides evidence of neofunctionalization of these genes [132].

4.2.5. Interleukin-3 and Interleukin-3 receptor

Interleukin-3 (IL-3) is a multi-lineage colony-stimulating factor (multi-CSF) that acts through the IL-3 receptor alpha and common beta chain on multipotent erythro/myeloid HPCs to promote their self renewal, proliferation and differentiation [138-140]. IL-3 can also act on committed myeloid progenitors to promote their proliferation and differentiation [138-142]. Interestingly, *IL-3*, *IL-4*, *IL-5* and *GM-CSF* are all found on chromosome 5q in humans. The close proximity of the CSFs on the chromosome, along with their similar structure and function may suggest they arose from a common ancestral gene [143]. However, genes encoding IL-3 and the specific IL-3 receptor alpha (IL-3Rα) have not been identified in any teleosts to date, despite genome sequencing (Figure 1). The lack of IL-3 in teleosts may be due to diffi-

culties in identifying the IL-3 orthologue in teleosts due to the low sequence conservation of IL-3 observed between mammals, or may represent the evolutionary loss of IL-3 in teleosts. As IL-3 and IL-3R have not been identified in teleosts, IL-3 and IL-3R will not be discussed here. The structure, function and regulation of mammalian IL-3 and its receptor have been extensively reviewed elsewhere by [144-146].

4.2.6. Granulocyte-macrophage colony-stimulating factor/Granulocyte-macrophage colony-stimulating factor receptor

GM-CSF shares redundancy with IL-3 in terms of its function. However, GM-CSF acts on a more mature population of HPCs and has been associated with the formation of both granulocyte and macrophage colonies from CFU-GM [147, 148]. GM-CSF is produced by activated T-lymphocytes [147, 149], endothelial cells [150], and lung fibroblasts [151] and suggests the importance of GM-CSF during emergency hematopoiesis. GM-CSF promotes the survival, proliferation and differentiation of GMPs [147, 148, 152]. Furthermore, GM-CSF is chemoattractive to immature and mature neutrophils *in vitro* and *in vivo* [153, 154] and enhances neutrophil anti-microbial functions and neutrophil survival [155]. GM-CSF can also promote monocytes to differentiate into inflammatory dendritic cells [156, 157]. The GM-CSF receptor (GM-CSFR) is composed of heterodimeric alpha and beta chains as described for IL-3. Since the βc chain is common to IL-3, IL-5 and GM-CSF, the βc chain signals through JAK/STAT, MAPK, and PI3K pathways [145, 158].

Similar to that of *IL-3*, *GM-CSF* has not been identified in teleosts (Figure 1). The close proximity of *IL-3* and *GM-CSF* on the same chromosome may suggest that a genomic deletion occurred on this chromosome, subsequent to the divergence of fish and mammals. The hematopoietic CSFs that compensate for the loss of IL-3 and GM-CSF in teleosts are not known.

4.3. Transcription factors

Commitment of LT-HSCs to the myeloid lineage is an intricate regulation of the transcription factors expressed, their relative levels to one another, and their expression on a temporal scale. Transcription factors (TFs) can act antagonistically or co-operatively. Thus, the presence or absence of a TF partner, or the relative levels of a TF to its antagonistic counterpart, determine lineage fate decisions. Furthermore, the expression of a transcription factor in an HSC does not exert the same effect as when it is expressed in a committed progenitor cell. The transcriptional regulation of mammalian hematopoiesis/myelopoiesis has been extensively reviewed elsewhere [159-162], and will only be briefly described here for the purpose of putting advances in the teleost model systems into context. A visual representation of which stages these transcription factors are important is shown in Figure 2.

4.3.1. MafB

MAFB, a bZIP transcription factor family member, is highly expressed in LT-HSCs, but not in MPPs, CMPs, or GMPs and was recently found to be involved in restricting proliferation and myeloid lineage differentiation of LT-HSCs [163]. $MAFB^{-/-}$ LT-HSCs showed increased

proliferative activity and gave rise to large numbers of primarily myeloid progeny in a mouse repopulation assay [163]. The MAFB$^{-/-}$ HSCs had higher proliferative ability and gives rise to greater numbers of myeloid progeny in response to CSF-1 compared to wild type HSCs, *in vitro*. Furthermore, *in vitro* studies demonstrated that treatment of MAFB$^{-/-}$ HSCs with CSF-1 led to the rapid activation of *PU.1* transcription that suggested MAFB must be down-regulated to allow expression of *PU.1* in MPPs [163]. It appears that MAFB plays an important role in antagonizing the expression of PU.1 and the commitment of MPPs to CMPs. Furthermore, MAFB has been shown to bind ETS-1 though its zipper-binding domain and can act to repress erythroid lineage commitment in CMPs [164].

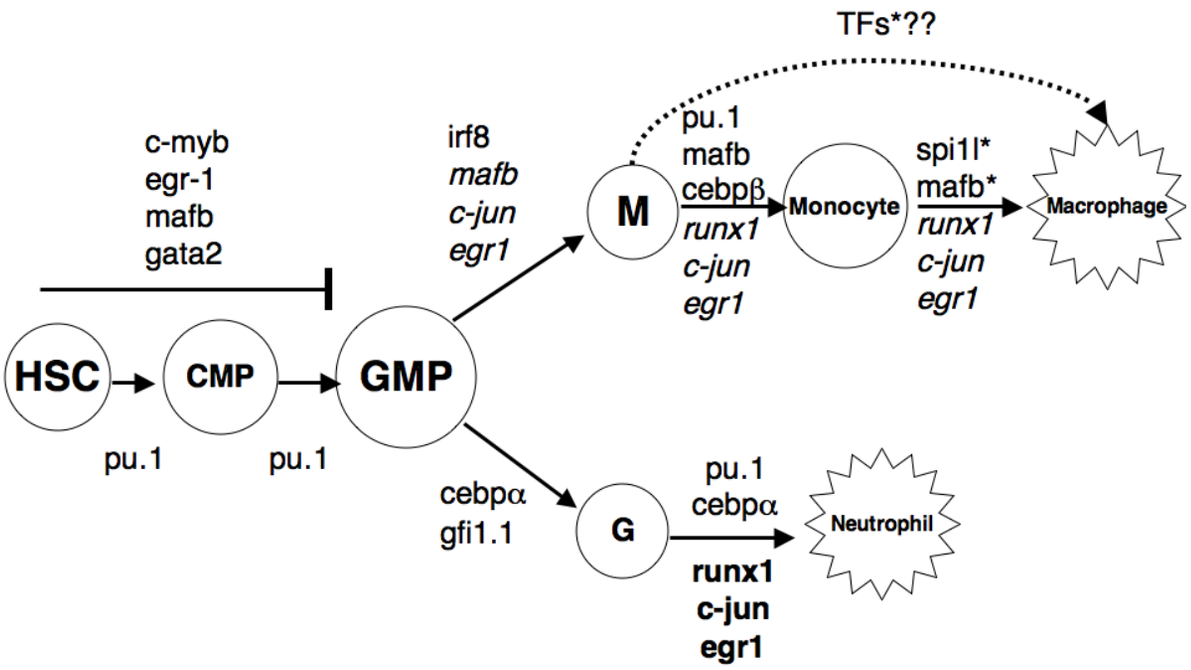


Figure 2. Transcription factors involved in goldfish myelopoiesis. Goldfish transcription factors shown in lower case lettering are up-regulated, goldfish transcription factors shown in bold are down-regulated, transcription factors that are important in cellular differentiation in mammalian systmes but have yet to be studied in the teleost system are shown in italics. The dashed arrow denotes the alternative pathway of macrophage development in teleosts. Question marks denote unknown transcription factors involved in the alternative pathway of macrophage development. Asterisks mark differences between teleosts and mammals. Abbreviations used: (1) Cellular stages: HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; M, monocytic precursor; G, granulocytic precursor. (2) Transcription factors: c-MYB, cellular myelobastosis oncogene; EGR-1, early growth response-1; MAFB, musculoaponeurotic fibrosarcoma oncogene homologue B; GATA2, GATA binding protein 2; IRF8, interferon regulatory factor 8; CEBPα, CCAAT/enhancer-binding protein alpha; GFI1, growth factor independent 1; RUNX1, runt-related transcription factor 1.

In zebrafish, the *mafb* orthologue has been identified and mRNA was found expressed in the blood forming regions of the developing embryo [165]. However, the role of MAFB in zebrafish HSCs has not yet been assessed.

http://dx.doi.org/10.5772/53589

4.3.2. C/EBPs

CCAAT/enhancer binding proteins (C/EBPs) are members of the family of transcription factors that contain a C-terminal basic leucine zipper domain (bZIP) comprised of a basic region involved in DNA binding and a leucine zipper domain involved in protein interactions [166]. Six members of the C/EBP family have been identified in mammals: alpha, beta, gamma, delta, epsilon and zeta [167]. Orthologues of the C/EBP family of transcription factors have been identified in teleosts [168-171], corresponding to C/EBPα, C/EBPβ, C/EBPγ, C/EBPε, and C/EBPδ.

Expressed in HSCs, CMPs and GMPs [172, 173], C/EBPα has been shown to be involved in directing granulocyte cell fate and terminal differentiation of neutrophils, along with C/EBPε. Mice deficient in C/EBPα show diminished numbers of CFU-GM, CFU-M, CFU-G, macrophages and neutrophils [174, 175]. The loss of myeloid cells in C/EBPα deficient mice is reflective of the role that CEBPα plays in determining the fate of a CMP to a GMP lineage versus an MEP lineage [176]. C/EBPα is capable of binding to the *PU.1* promoter [175] and up-regulating *PU.1* expression, to dictate a GMP cell fate [175, 177] (see discussion on PU.1 below). The increase in C/EBPα in GMPs has been shown to inhibit monocyte/macrophage differentiation [178] and initiate differentiation along the granulocyte lineage by regulating *GCSFR*, elastase and myeloperoxidase gene expression [179-181].

The zebrafish CEBPα orthologue showed 66% amino acid identity to human C/EBPα, while the bZIP domains showed 99% amino acid identity [168]. In zebrafish, *cebpa* was expressed in myeloid cells on the surface of the yolk sac during embryogenesis [168]. At 16 hpf, a population of blood cells co-expressed the transcription factors *gata1*, *pu.1* and *cebpa*, and by 22 hpf, the majority of the *cebpa*$^+$ cells co-expressed *pu.1*, however, not all *pu.1*$^+$ cells expressed *cebpa* [182]. Furthermore, *cebpa* was co-expressed with myeloperoxidase (*mpo*), a marker for granulocytes, but *cebpa*$^+$ cells did not always express *mpo* [182]. These three cell sub-populations likely represent distinct junctures in myeloid cell development: erythromyeloid cells, GMPs and committed neutrophils and their precursors, respectively. The expression of *cebpa* with these additional markers mirrors the importance of C/EBPα in the mammalian system in which C/EBPα is important for committment to a myeloid lineage versus an erythroid lineage, to a granulocyte lineage over a macrophage lineage, and in terminal differentiation of neutrophils. An orthologue of *cebpa* was also identified in Japanese flounder and mRNA was observed in the head and posterior kidney, spleen, liver, gill, heart, brain, skin, intraperitoneal cells, and weakly in the intestine, muscle and PBLs [171]. However, expression of *cebpa* in isolated cells populations was not performed.

Two studies have examined the function of CEBPα in zebrafish primitive myelopoiesis. The injection of a deletion mutant of *cebpa* into zebrafish embryos functioned as a dominant-negative mutation and blocked the production of full-length CEBPα. These embryos exhibited an increase in *gata1*$^+$ expression in the posterior lateral plate mesoderm at 22 hpf and in the intermediate cell mass at 26 hpf, reflective of an erythroid progenitor cell expansion. This expression corresponded to a subsequent increase in circulating erythrocytes based on the increase in *α-hemoglobin* expression, indicative of erythrocytes [182]. However, the expressions of the myeloid specific genes, *mpo* and *l-plastin*, were normal [182]. Based on the pat-

tern of expression, it was suggested that PU.1 acts upstream or in parallel with C/EBPα during zebrafish primitive myelopoiesis [182]. Recently, it has been shown that the sumoylation (a post-translational protein modification) of zebrafish CEBPα inhibited CEBPα transcriptional activity and its ability to interact with and repress GATA1, thus driving lineage commitment of a myelo-erythroid progenitor to that of the erythroid lineage [183]. Taken together, these studies demonstrate the conserved role of CEBPα in the commitment of a CMP to a GMP. However, due to the toxicity of *cebpa* morpholinos to zebrafish embryos, knockdown experiments could not be performed.

Cebpb was identified in rainbow trout as a single intron-less gene and the predicted CEBPβ protein showed 30-34% amino acid identity to mammalian C/EBPβ [169]. The *cebpb* mRNA was detected in the head and posterior kidney, spleen, liver, gill, intestine, muscle and peripheral blood leukocytes (PBLs) [169]. Japanese flounder CEBPβ also showed a low (33-38%) amino acid identity to other vertebrate sequences, but retained 95% amino acid identity in the bZIP domain. The *cebpb* mRNA was expressed in the head and posterior kidney, liver, gill, brain, peritoneal cavity fluid and PBLs, with low mRNA levels in the heart, intestine, mucus, eye and spleen [170]. In zebrafish, CEBPβ showed 49% amino acid identity to human C/EBPβ and *cebpb mRNA* was detected in cells on the surface of the yolk sac, corresponding to the myeloid cells that normally spread over the yolk sac early in embryogenesis [168]. A *cebpb* transcript was also identified in a differential cross-screen of goldfish proliferative phase and senescence phase PKMs, and was up-regulated in goldfish monocytes, and expressed in low levels in progenitors and macrophages [19]. However, the functional role of CEBPβ has not been examined in teleost myelopoiesis.

The orthologues of C/EBPδ, C/EBPγ and C/EBPε exist in teleosts. The *cebpd* and *cebpg* transcripts were identified in zebrafish and show a ubiquitous expression pattern in embryos [168]. CEBPδ and CEBPγ showed 57 and 50% identity to their human counterparts on the amino acid level. However, their bZIP domains showed higher conservation to their human counterparts, with 86% and 76% amino acid identity, respectively [168]. The *cebpe* orthologue was identified in Japanese flounder and its corresponding predicted protein had a 27% overall amino acid identity and a 90% amino acid identity in the bZIP domain compared to the mammalian counterparts, but failed to cluster with other *cebpe* sequences in phylogenetic analysis [170]. The *cebpe* mRNA was detected in the head and posterior kidney, spleen, brain, peritoneal cavity fluid and at low levels in the PBLs. However, the functional role of these C/EBPs in teleost myelopoiesis is unknown.

4.3.3. PU.1

The Ets transcription family member PU.1 is well known as the master transcriptional regulator of mammalian myelopoiesis through an antagonistic relationship with GATA1, recently reviewed by [184]. At the N-terminus, PU.1 comprises of an acidic domain and a glutamine rich domain that are involved in activation of transcription, and a PEST domain important for protein interactions [184]. At the C-terminus, PU.1 has an Ets domain important for binding the DNA consensus sequence AAAG(A/C/G)GGAAG [185]. Mice deficient in PU.1 (*PU.1*$^{-/-}$) have reduced CLPs, and GMPs, increased numbers of MEPs, and lack B-

cells, T-cells, monocytes/macrophages as well as severely reduced numbers of granulocytes [186-190]. *PU.1* is expressed in HSCs, CLPs and at varying levels in CMPs, increasing as these progenitors are induced to differentiate into monocytes/macrophages and neutrophils [191]. At the CMP stage, PU.1 antagonizes with GATA1 to determine whether the CMP commits to a GMP or a MEP. PU.1 binds to GATA1 and inhibits GATA1 from binding to and initiating transcriptional activation of a number of erythroid genes that are important for commitment to an erythroid lineage [184, 192, 193]. The reverse is also true; GATA1 can bind to PU.1 and inhibit the binding of PU.1 and transcriptional activation of a number of myeloid genes [184, 192, 193], including to the promoters of *CSF-1R* [194-196] and *GCSFR* genes [181, 196, 197]. Therefore, the lineage fate decision along a GMP or a MEP fate is a balancing act in timing and relative protein levels of PU.1 and GATA1.

PU.1 also plays a role at the GMP stage to regulate commitment to a granulocyte or macrophage lineage. Increased levels of PU.1 at the GMP stage, along with AP-1 association, drives a monocyte cell fate, while lower levels of PU.1 drives granulocyte cell fate [175, 177]. Furthermore, PU.1 induces *EGR-2* and *NAB-2* expression [177]. The EGR-2/NAB-2 transcription factors function to repress neutrophil genes by antagonizing GFI1, an important transcription factor in the initiation of neutrophil differentiation [177], discussed in section 5.3.2.

An orthologue of PU.1 has been identified in teleosts. In the Japanese flounder, *pu.1* mRNA was detected in the head and posterior kidney, spleen, heart, PBLs, intraperitoneal cells, and weakly in the intestine and gill, but was absent from the liver, skin, muscle and brain [171]. In zebrafish, *pu.1* was identified as a single gene copy and analysis of the predicted protein sequence showed the conserved transactivation, PEST, and DNA-binding domains. Although the overall amino acid identity to other PU.1 proteins was 48-53%, the DNA-binding domain of zebrafish PU.1 showed 83% amino acid identity to mammalian PU.1 [198]. Examination of the zebrafish *pu.1* promoter region predicted potential binding sites for PU.1 and CEBPα [199]. The expression of *pu.1* is first detected at 12 hpf in blood cells from the PLM, later in the ICM, and finally in the kidney, and these *pu.1*$^+$ blood cells give rise to myeloid cells [198-200]. The population of *pu.1*$^+$ cells represents myeloid HPCs, myeloid precursors, monocytes/macrophages and neutrophils during both primitive and definitive myelopoiesis in the zebrafish [24, 200].

Knockdown of *pu.1* in zebrafish using morpholinos showed a large reduction in the number of cells positive for *mpo* and *l-plastin* mRNA, markers of granulocytes and monocytes/macrophages [201, 202]. In addition to the loss of myeloid cells, an increase in *gata1* expression was observed, and these *gata1*$^+$ cells gave rise to mature erythrocytes [201]. Conversely, *gata1* morphants failed to develop mature erythrocytes and showed an increase in the number of *pu.1*$^+$, *mpo*$^+$ and *l-plastin*$^+$ cells [201, 202]. Ectopic expression of *pu.1* or *gata1* was observed in *gata1* or *pu.1* morphants, respectively, suggesting the conversion of progenitors to an alternate lineage [201, 202]. Microarray analysis of genes regulated by PU.1 revealed the regulation of ~250 genes, including *cebpa*, *csf-1r* and myeloid-specific peroxidase *(mpx)*, among others [203]. Taken together, PU.1 has a conserved role in dictating a myeloid lineage, opposing GATA1 and the transcriptional activation of erythroid genes.

A pu.1-like gene (spi-1 like, *spi-1l*) was also identified in zebrafish. The predicted amino acid sequence of SPI-1l showed 45% amino acid identity to zebrafish PU.1, and retained all three domains [204]. *In situ* hybridization revealed a population of blood cells positive for *pu.1* and *spi-1l*, in addition to a population of single positive *pu.1* cells [204]. However, only a few single-positive *spi-1l* cells were observed. *Spi-1l* morphants showed a loss of *mpx* and *l-plastin* positive cells, indicative of a loss in granulocytes and monocytes/ macrophages [204]. Unlike *pu.1* morphants, no change in *gata1* expression was observed, suggesting that SPI-1l acts downstream of PU.1, and plays an important role in myeloid cell differentiation [204].

5. Commitment of bi-potent myeloid progenitors to the macrophage or neutrophil lineage

5.1. Macrophage development

5.1.1. Progression of cell development

In mammalian systems, the progression of macrophage development proceeds from a committed macrophage progenitor, monoblast, promonocyte, monocyte and then to a mature tissue macrophage, reviewed by [205-207] (Figure 1). While the presence of a unipotent committed macrophage progenitor has yet to be unequivocally demonstrated in the teleost systems, progenitor/precursor cells that give rise to monocytes and macrophages have been demonstrated. *In vitro*, a spontaneous proliferating trout RTS-11 cell line has two predominant cell types; a round non-adherent cell type that appears to be a pre-monocyte or myeloid precursor and an adherent macrophage-like cell, arising from the non-adherent cell type [208]. The cultivation of trout kidney progenitor-like cells developed a trout primary kidney monocyte culture that contained progenitor cells, promonocyte-like cells, and monocytes [209]. Furthermore, the generation of goldfish primary kidney macrophage cultures demonstrated that small mononuclear cells became monocytes and mature macrophages, *in vitro*. In the zebrafish model system, whole kidney marrow was added to a kidney fibroblast layer and was shown to maintain HPCs and precursor cells that then differentiated into myeloid and lymphoid cells [210]. Recently, the development of a zebrafish methylcellulose colony forming unit assay suggested the presence of a common erythro-myeloid HPC [211]. *In vivo* studies, primarily in the zebrafish, have demonstrated that monocytes/ macrophages arise from the hematopoietic organ [45, 212-215], migrate to various tissues [216], and both primitive and definitive macrophages are motile, migrate to the site of insult, and readily phagocytose particles or pathogens [23, 45, 217-219]. The identification of progenitor cells that are capable of differentiating into monocytes and macrophages suggests a conserved macrophage differentiation pathway in vertebrates.

5.1.2. Receptors and growth factors

5.1.2.1. Colony-stimulating factor-1

The central growth factor that regulates the survival, proliferation, and differentiation of macrophages and their precursors is colony-stimulating factor-1 (CSF-1) [220-223]. Alternative splicing of *CSF-1* transcripts leads to production of a secreted glycoprotein, a secreted proteoglycan, or a membrane-bound glycoprotein that can be proteolytically cleaved from the surface, reviewed by [144, 221]. However, only the first 149-150 aa of the N-terminal portion of the CSF-1 core protein has shown to be important for biological function [224, 225]. CSF-1 homodimers, are covalently linked by an interchain disulphide bond to form a dimer [226] that then binds the CSF-1 receptor. CSF-1 is produced by an array of cell types including fibroblasts, endothelial cells, and bone marrow stromal cells, reviewed by [144]. In addition, activated T-cells [227-229], monocytes, macrophages [230, 231], fibroblasts, and endothelial cells [144] can produce CSF-1. CSF-1 production by activated cell types suggests a role for CSF-1 at the site of inflammation, which may be necessary for the rapid recruitment, differentiation and activation of macrophages and their precursors.

5.1.2.2. Interleukin-34

Recently, IL-34 was identified as another growth factor involved in mediating macrophage development in mammals, in addition to CSF-1 [232-234]. The IL-34 protein does not show homology to any other human protein and or contain any known conserved structural motifs [232]. Homodimeric IL-34 binds to CSF-1R, although with a different affinity than that of CSF-1, and to different sites on the receptor [232, 233] [235]. The hierarchy in binding of the CSF-1R ligands may provide a mechanism for differential signaling depending on the bound ligand. To date, IL-34 has not been identified in teleosts.

5.1.2.3. Colony-stimulating factor-1 receptor

The *CSF-1R* gene, shown to map to the proto-oncogene *c-fms*, is a member of the type III tyrosine kinase family of receptors [236, 237], reviewed by [238]. The binding of homodimeric CSF-1 to CSF-1R, triggers receptor homodimerization and activation [239]. Receptor activation triggers autophosphorylation of the intracellular tyrosine residues and activation of JAK/STAT, PI3K/Akt, and MAPK pathways, as well as pathways for receptor-mediated internalization and destruction, reviewed by [162, 238, 240]. Within the hematopoietic system, CSF-1R protein is primarily found on macrophages and their precursors and has been used as a marker of cells along the macrophage lineage in mammalian systems [222, 237]. CSF-1R progressively increased with macrophage differentiation [144].

5.1.2.4. Biological functions of colony stimulating factor-1

In addition to the regulation of survival, proliferation, and differentiation of macrophages and their precursors [220-223], CSF-1 has been shown to exert pro-inflammatory effects on monocytes and macrophages. These effects include the enhancement of macrophage chemo-

taxis, phagocytosis of pathogens, and the production of antimicrobial agents, reviewed by [162, 238]. CSF-1 is a pleiotropic cytokine and functions in a number of other biological systems such as regulation of macrophage and osteoclast numbers, bone remodeling, tooth production and fertility and breast development [241-245]

5.1.2.5. Teleost colony stimulating factor-1

Teleost *csf-1* (*mcsf*) was first identified in the goldfish as a 600 bp mRNA transcript that was present at high levels in spleen tissue, monocytes, and phorbol ester-activated monocytes [246]. The *csf-1* transcript encoded for a 199 aa precursor protein, with the mature CSF-1 protein predicted to have a molecular weight of 22 kDa. The goldfish CSF-1 has 27% aa identity to human CSF-1 [246]. Alignment of goldfish CSF-1 with mammalian CSF-1s showed conservation of four cysteine residues required for protein folding, similar to that of mammalian CSF-1 [246]. Ligand-receptor binding studies demonstrated that homodimeric CSF-1 could bind to soluble CSF-1R (see teleost CSF-1R section below, Figure 1). Functional characterization of a recombinant goldfish CSF-1 was shown to induce monocyte proliferation and differentiation (Figure 1), which was abrogated in the presence of sCSF-1R or in monocytes transfected with *csf-1r* RNAi oligos [246, 247]. Recombinant goldfish CSF-1 also aided in the long-term survival of mature macrophages *in vitro* [247]. The recombinant CSF-1 protein was chemoattractive to PKMs, and promoted their ability to perform phagocytosis and produce antimicrobial compounds [248], suggesting a pro-inflammatory role for CSF-1 in goldfish.

Two *csf-1* genes were later identified in trout and zebrafish, termed *mcsf-1* and *mcsf-2*, and a second goldfish *mcsf* transcript was identified [249]. The trout and zebrafish *mcsf-1* genes encoded for proteins of 593 and 526 aa, the trout and zebrafish *mcsf-2* genes encoded for proteins of 276 and 284 aa, respectively, while the goldfish *mcsf* gene encoded for a 544 aa protein [249]. All of the identified transcripts possessed a signal peptide, a CSF-1 domain, a transmembrane domain, and a short cytoplasmic domain [249]. However, the N-terminal region of all teleost CSF-1 proteins showed high homology (46-88%), consistent with the important role of the CSF-1 N-terminal portion for biological function.

The genomic structure of the identified *mcsf*s also differed. The zebrafish *mcsf-1*, found on chromosome 11, possessed seven exons and *mcsf-2*, found on chromosome 8, possessed nine exons. Based on syntenic analysis, the two *mcsf* genes appeared to have arose through a chromosomal or genome duplication [249]. Examination of the intron-exon structure of trout *mcsf*s showed *mcsf-1* to possess 10 exons and 9 introns, and *mcsf-2* to have 9 exons and 8 introns [249].

Along with differing genomic organizations, trout *mcsf-1/-2* are differentially expressed in tissues. The *mcsf-1* transcript was predominantly expressed in the spleen, intestine and brain, while *mcsf-2* was predominantly expressed in the head kidney, gills, muscle and liver [249]. While a recombinant trout MCSF-1 protein was produced and demonstrated to induce the proliferation of head kidney macrophages, a recombinant trout MCSF-2 protein was not produced to examine whether there was differential regulation of macrophage function by

the MCSFs [249]. Whether MCSF-1 and MCSF-2 are functionally redundant or functionally partitioned (sub-functionalization), remains to be determined.

5.1.2.6. Teleost colony-stimulating factor-1 receptor

The *csf-1r* sequences have been identified in a number of teleost species including puffer fish [250, 251], zebrafish [252], rainbow trout [253], gilthead seabream [254] and goldfish [20]. CSF-1R protein appears to be a marker of monocytes and macrophages in teleosts [20, 254, 255] (Figure 1). Analysis of the puffer fish *csf-1r* gene shows a 21 exon gene structure in fish, same as in mammals. However, the puffer fish *csf-1r* gene only spans 10.5 kbp versus the mammalian 55 kbp, due to the decrease in the size of the intronic sequences [250]. The *csf-1r* mRNA open reading frame encodes for a 975 aa protein, with a signal peptide, an extracellular domain with 10 conserved cysteine residues characteristic of immunoglobulin domains, transmembrane domain, and an intracellular kinase domain with an interruption of 70 bp [250]. While CSF-1R of puffer fish is only 39% similar to human CSF-1R, the kinase domain is considerably more conserved, particularly in the motifs associated with signaling. The fish *csf-1r* gene was linked with *pdgfrb-1* [250].

A second *csf-1r* gene (*csf-1r-2*) was also identified in puffer fish, and linked with a second *pdgfrb* (*pdgfrb-2*). The *csf-1r-2* gene was comprised of 22 exons and had a different intron-exon organization than *csf-1r-1* [251]. Despite the similar protein structure of the two CSF-1Rs, the amino acid sequences were only 39% identical. The *csf-1r* mRNAs were differentially expressed in tissues. The *csf-1r-1* was expressed in blood, brain, eye, gill, heart, kidney, ovary, skin, and spleen, while *csf-1r-2* was expressed in the blood, brain, eye, gill, heart, kidney, liver, muscle, skin, spleen and testis. [251].

The duplication of *csf-1r* genes was also observed in cichlids, the green-spotted pufferfish, medaka, and *Tetraodon* (found on chromosomes 1 and 7), with the *csf-1r-2* duplicated genes appearing to have undergone evolutionary selection or diversification while the *csf-1r-1* gene appeared to resemble that of the ancestral gene [256]. It was proposed that the fish specific whole genome duplication generated the two paralogues of *csf-1r* in fish, as well as two *pdgfrb* and *kit* genes, and that *kit* and *csf-1r-2* may have been retained to play a role in the survival, migration and differentiation of melanocytes and xanthophores, important pigment cells involved in fish coloration patterns [256].

The *panther* (*fms*) mutant zebrafish have a defect in the *csf-1r* gene, and mutant fish fail to develop their characteristic pigment pattern of black and yellow stripes. The CSF-1R was found to be important in the survival, migration and differentiation of precursors to yellow xanthophores in zebrafish [257, 258]. However, unlike that of the CSF-1R$^{-/-}$ mice, there were no reports of hematopoietic defects in *panther* zebrafish. The lack of hematopoietic defects may be due to the presence of another *csf-1r* gene, a low level of *csf-1r* expression, or a differential requirement for CSF-1R during embryonic macrophage development versus adult macrophage development in teleosts. However, CSF-1R was shown to be important in the migration of primitive macrophages to tissues, such as the brain, retina and epidermis upon comparing primitive macrophage distribution and migration in wild-type and *panther* zebrafish [252]. Furthermore, *csf-1r* mRNA was detected in inflammatory macrophages from 3

dpf zebrafish embryos [219]. Taken together, these results support a role for CSF-1R in teleost macrophage biology.

A full-length *csf-1r* cDNA sequence was identified in trout, with an open reading frame of 2904 bp encoding for a 967 aa protein, predicted to be ~109 kDa. Trout CSF-1R had 40% aa identity to that of human and mouse, and 54% and 52% identity to that of puffer fish and zebrafish CSF-1R [253]. The trout *csf-1r* gene was similar to that of the ancestral gene, and mRNA was found in the head-kidney, spleen, blood, ovary, and showed lower mRNA levels in the liver, brain, heart, muscle, gill, and skin [253]. Southern blotting revealed two bands in each lane, suggestive of a second *csf-1r* gene in trout. However, a second *csf-1r* gene was never identified.

CSF-1R was also identified in goldfish as a 975 aa integral membrane bound protein (mCSF-1R) that possessed the five Ig extracellular domains with multiple N-linked glycosylation sites, a transmembrane domain, and an intracellular tyrosine kinase domain [20]. The mRNA of mCSF-1R could be detected in progenitor, monocyte and macrophage subpopulations, and an antibody produced against the first two Ig domains of CSF-1R was able to recognize monocytes and macrophages [20]. However, unlike mammalian neutrophils, zebrafish and goldfish neutrophils do not appear to express mRNA for *csf-1r* [16, 219]. Additionally, alternative splicing of the *csf-1r* transcript encoded for a soluble form of the CSF-1R (sCSF-1R), possessing only the D1 and D2 Ig domains, important for binding of CSF-1. The s*csf-1r* mRNA was expressed by leukocytes within the progenitor and macrophage populations, but not in the monocyte subpopulation [20]. Furthermore, addition of a recombinant purified sCSF-1R dampened the proliferation of spontaneously growing and differentiating PKMs [20]. The increased production of the sCSF-1R by PKMs during senescence phase suggested that sCSF-1R was involved in the negative regulation of CSF-1 signaling through mCSF-1R [20, 246] (Figure 1).

5.2. Neutrophil development

5.2.1. Progression of cell development

Following the commitment of the CFU-GM to a committed granulocyte progenitor cell, terminal differentiation through a promyelocyte, myelocyte, and metamyelocyte stages occur to give rise to a mature neutrophil, and are regulated through growth factor and transcription factor signaling, reviewed by [259] (Figure 1). Similar to that of mammals, the differentiation of fish neutrophils appears to occur through various stages, based on morphological and cytochemical characteristics, and include the promyelocyte, myelocyte, metamyelocyte and the mature neutrophil, which sometimes had a segmented nucleus [45, 212, 213, 215, 260]. These neutrophils were shown to migrate from the hematopoietic organ to the site of wounding, pathogen injection, or transformed cell injection [24, 45, 261], in response to a hydrogen peroxide attractant produced by cells at the site of damage [217]. However, the responding neutrophils had low phagocytic activity [24], or engulfed small fragments of the pathogen [217]. *In vitro*, treatment of zebrafish kidney marrow cells with G-CSF gave rise to CFU-GM in a methylcellulose assay [211]. However, there is a lack of *in vitro* culture sys-

tems for studying progenitor cell to neutrophil differentiation. The identification of functional neutrophils and their precursors suggests the presence of a committed granulocyte progenitor cell in teleosts.

5.2.2. Receptors and growth factors

5.2.2.1. Granulocyte colony-stimulating factor

Neutrophils contribute to both innate and adaptive immune responses. They are capable of chemotaxis, phagocytosis, antimicrobial molecule production, and formation of extracellular traps [262-267]. Upon activation, neutrophils produce a number of chemokines, pro-inflammatory and anti-inflammatory cytokines, as well as the colony-stimulating factors G-CSF, CSF-1, GM-CSF, IL3 and SCF, reviewed by [268, 269]. However, neutrophils are short lived, 6-90 hrs, and need to be continuously replaced.

GCSF, a member of the class I cytokine family, is the primary CSF that mediates the proliferation, differentiation, survival and activation of neutrophils and their progenitors, and has been reviewed extensively by [144, 270]. The transcription of *GCSF* is controlled by an upstream promoter region with a tumor necrosis factor alpha response region that is bound by NF-kB p65 and NF-IL6, reviewed elsewhere by [144, 271]. As such, GCSF can be produced by activated monocytes/macrophages, neutrophils, fibroblasts and endothelial cells in response to a number of pro-inflammatory stimuli, reviewed elsewhere by [144, 270, 271]. In humans, the normal GCSF concentration in blood ranges from 30-162 pg/mL, and can be massively up-regulated during infection up to 3200 pg/mL [272-274].

5.2.2.2. Granulocyte colony-stimulating factor receptor

The protein structure of GCSFR is comprised of a signal peptide, an immunoglobulin-like domain, a cytokine receptor homology (CRH) domain containing the class I cytokine receptor superfamily motif W-S-X-W-S, three fibronectin domains, a transmembrane domain, and an intracellular cytoplasmic signaling domain containing three motifs termed Box 1, Box 2, and Box 3, important for signal transduction [270, 275]. Based on their protein structure and conserved motifs, the human and mouse integral membrane GCSFR proteins were placed in the type I cytokine receptor family.

While there are reports of GCSFR on other hematopoietic cells such as monocytes [276] and lymphocytes, as well as some non-hematopoietic cells, GCSFR is primarily found on neutrophils and their precursors [270, 277]. Neutrophils up-regulate their levels of GCSFR as they differentiate from progenitor cell to mature neutrophil, with 50-500 GCSF receptors per cell [278]. Structural analysis showed GCSF forms a homodimer, binds two GCSFRs, and leads to receptor homodimerization in a 2:2 complex [279-281]. Binding of a homodimeric GCSF to two GCSF receptors triggers intracellular signaling through the JAK/STAT, Ras/Raf/Erk, or PI3K pathways [275, 277, 282]. These signaling pathways ultimately lead to the migration, survival, proliferation, and differentiation of neutrophils. Control of GCSFR signaling in neutrophils is modulated through (1) transcriptional activation of the *GCSFR* by AP-1, AP-2,

C/EBPα, NF-IL6, GATA-1, and PU.1/SPI1 transcription factors [181, 197], (2) the production of a soluble receptor through alternative splicing [275], and (3) cleavage of surface GCSFR by elastase [283].

5.2.2.3. Biological activity of granulocyte colony stimulating factor

The targeted gene disruption of *GCSF* and *GCSFR* has demonstrated the important functional roles of GCSF *in vivo*. GCSF and GCSFR deficient mice display severe neutropenia (70%-88% reduction in circulating neutrophils), reduction in monocyte and macrophage numbers, and ~50% reduction in the numbers of neutrophil precursors present in the bone marrow [284, 285] [282, 286] and are unable to control *Listeria monocytogenes* infections [284, 285]. GCSF treatment of bone marrow cells, *in vitro*, induced CFU activity to produce mainly neutrophil colonies [287] and promoted the proliferation of neutrophil precursors [270]. The release of mature neutrophils, their terminal differentiation, survival, and activation, is also mediated by GCSF *in vitro* and *in vivo*, reviewed by [270]. Lastly, GCSF has been used in the clinical setting to increase peripheral blood neutrophil numbers for treatment of disease and for stem cell mobilization from the bone marrow into the peripheral blood, reviewed by [288, 289].

5.2.2.4. Teleost granulocyte colony-stimulating factor

The teleost *gcsf* gene was first identified in Japanese flounder, fugu, and the green-spotted pufferfish [290]. Both the fugu and green-spotted pufferfish have two *gcsf* genes, termed *gcsf-1* and *gcsf-2*, while only an orthologue of *gcsf-2* was identified in flounder [290]. Phylogenetic analysis of vertebrate *gcsf*s predicted fish *gcsf-1* to be the ancestral gene, while *gcsf-2* was predicted to be the duplicated gene. Alignment of the fish GCSFs with human and mouse GCSF showed low identity, ranging from no significant identity to 34% amino acid identity [290]. Despite the low amino acid identity of fish to mammalian GCSF, all fish *gcsf* genes retained a 5 exon/ 4 intron structure with a conserved tumor necrosis factor alpha response element in the promoter region. Furthermore, the predicted transcripts have an open reading frame of 561-636 bp, corresponding to a predicted protein of 20-23 kDa, and 4-5 AU rich sequences in their 3′ UTRs shown to be involved in mRNA instability and degradation [290]. Determination of the ratio of synonymous to asynonymous nucleotide substitutions (Ks/Ka) in fish *gcsf* genes ranged from 0.467 to 0.961 with an average of 0.793, demonstrating that positive selection was occurring in GCSFs of fish (and chicken) [290]. Two *gcsf* genes were also identified in the black rockfish (*Sebastes schlegelii*) [291] and in zebrafish [292] (O. Svoboda and P. Bartunek, personal communication), while only one *gcsf* gene has been identified in trout (NM_001195184).

Flounder *gcsf-2* mRNA levels were highest in the spleen, kidney, and gill. However, *gcsf-2* mRNA was still detected in the brain, eyes, heart, peripheral blood leukocytes, ovary, skin, and stomach, but was not detected in intestine, liver, or muscle tissue [290]. As expected, *gcsf-2* mRNA levels were up-regulated in kidney and peripheral blood leukocytes following treatment with lipopolysaccharide (LPS) or a mixture of concanavalin A and phorbol esters (ConA/PMA) [290]. The black rockfish *gcsf-1* showed expression in the peripheral blood leu-

kocytes, spleen, gill, intestine and muscle [291]. However, black rockfish *gcsf-2* was ubiquitously expressed in the peripheral blood leukocytes, head and trunk kidney, spleen, gill, intestine, muscle, liver and brain [291]. Although both *gcsf-1* and *gcsf-2* black rockfish mRNA levels were upregulated in PBLs treated with LPS or ConA/PMA, differential kinetics and levels of expression were observed between the two *gcsfs* [291]. It appears that *gcsf-1* may be rapidly induced with sustained levels following stimulation, whereas *gcsf-2* is only slightly upregulated and showed a drastic increase in mRNA levels after ConA/PMA treatment for 24 hrs [291]. Taken together, these data suggest that GCSF-1 may play an important role during inflammation, although functional studies are required to determine the roles of GCSF-1 and GCSF-2 in teleost granulopoiesis and inflammation.

Functional studies on fish GCSF-1 are limited. Only two manuscripts report on the function of GCSF-1 and both utilize the zebrafish model system. *In vitro*, precursor cells from whole kidney marrow were sorted, plated in a methylcellulose colony forming unit assay and treated with either GCSF or a combination of GCSF and erythropoietin (EPO). While both treatments led to CFUs containing granulocytes and macrophages, the combination of GCSF and EPO also supported the formation of erythroid CFUs [211]. *In vivo*, morpholino mediated knockdown of *gcsfr* in zebrafish showed a decrease in numbers and migration of cells expressing both neutrophil and macrophage specific transcripts, during both primitive and definitive hematopoiesis in the zebrafish embryo. However, a population of myeloid cells remained, despite morpholino mediated knockdown of *gcsfr*, suggesting the presence of a GCSFR-independent pathway of myeloid cell development and migration [292]. Injection of wild-type zebrafish with *gcsf* mRNA increased the number of myeloid and *gcsfr*$^{+}$ cells, while injection of *gcsf* mRNA into *gcsfr* morpholino zebrafish did not result in an increase in myeloid cell numbers [292]. These studies suggested GCSF-1 participates in myeloid cell development, similar to that observed in mammalian systems (Figure 1). No functional studies have been performed using GCSF-2, and the role(s) of GCSF-2 in myelopoiesis remain to be elucidated.

5.2.2.5. Teleost granulocyte colony-stimulating factor receptor

The *gcsfr* has been identified in zebrafish [292], goldfish [293], and trout (AJ616901). Only one gene copy has been identified, although Southern blotting for goldfish *gcsfr* suggested the presence of more than one gene [293]. Analysis of the upstream promoter region of the 16 exon zebrafish *gcsfr* gene showed conserved putative sites for binding of the transcription factors HOXA5, PU.1 and CEBP family members [292], similar to the human *gcsfr* promoter region. These data suggest the conserved regulation of *gcsfr* gene expression in teleosts.

The predicted protein structure of zebrafish and goldfish GCSFRs is conserved across vertebrates. The teleost GCSFR extracellular domain is comprised of a signal peptide, an Ig-like domain, a cytokine homology domain containing the WSXWS motif and four cysteine residues, and three fibronectin domains. Following the transmembrane region, the intracellular region contains predicted Box1, Box2, and Box 3 signaling motifs and 6 tyrosine residues [292, 293], shown to be involved in receptor activation and internalization in higher vertebrates.

In zebrafish, *gcsfr* mRNA is expressed by 14 hpf in the RBI, followed by the yolk sac, the ICM, and finally in the kidney by 96 hpf, consistent with the production of neutrophils during primitive and definitive hematopoiesis. In adult goldfish, *gcsfr* mRNA levels were highest in kidney and spleen, followed by the gill, intestine, heart, brain and blood [293]. The *gcsfr* mRNA was highly expressed in goldfish neutrophils and was up-regulated in response to mitogens or pathogens [293] (Figure 1).

5.3. Transcription factors

In addition to the transcription factors described in section 4.3, there are a number of transcription factors downstream that participate in determining GMP fate decisions and that play a role in macrophage and neutrophil cell development, reviewed by [51, 294]. A visual representation of the stage(s) in which these transcription factors are important are shown in Figure 2.

5.3.1. Early growth response (Egr)

The four Egr proteins, EGR1 [295, 296], EGR2 [297], EGR3 [298] and EGR4 [299], are members of the zinc finger transcription factor family and have an N-terminus activation domain, a repressor domain capable of binding to NAB1/2, and a DNA binding domain comprised of three zinc fingers that bind to the GC rich sequence, 5'-GCGGGGGC'3' [300]. EGR1 promotes commitment to the macrophage lineage at the expense of granulocytic lineage [301, 302] and has been shown to be essential for myeloblast differentiation into monocytes/macrophages [303, 304]. Treatment of mouse bone marrow cells with CSF-1 has been shown to induce *EGR1* mRNA levels by 6-7 fold three hours post treatment, as well as *EGR2* and *EGR3* mRNA levels by 2-4 fold [305]. Although *EGR*$^{-/-}$ mice display normal macrophage development [306], it is thought that there is redundancy amongst the Egr transcription factors. Consistent with this idea, EGR2 is also abundant in monoblasts and monocytes [307], and may be involved in monocyte differentiation. Although a zebrafish orthologue of *EGR1* has been identified [308], the role of *egr1* in teleost macrophage development has not been examined.

5.3.2. Growth factor independence 1 (Gfi1)

Growth factor independence 1 (GFI1) is a zinc finger transcription factor comprised of an N-terminal Snail/Gfi1 (SNAG) domain that is involved in recruiting proteins to modify histones, and a C-terminal domain containing six zinc fingers involved in DNA recognition [309]. *GFI1* is expressed in T-cells, B-cells, mature granulocytes and activated macrophages [310, 311]. GFI1$^{-/-}$ mice showed slight defects in lymphocyte development, increased monocyte and monocyte precursor numbers, an absence of granulocytes and were highly susceptible to infections [310, 311]. Furthermore, myeloid progenitors from GFI1$^{-/-}$ mice did not differentiate into mature granulocytes in the presence of GCSF *in vitro* [310] or *in vivo* [311]. C/EBPα can up-regulate *GFI1* expression, promoting a neutrophil cell fate, and GFI1 also acts as a negative regulator on *PU.1* to decrease its expression [177, 180]. This lower level of *PU.1* drives granulocyte cell fate [175, 177]. GFI1 is important for neutrophil differentiation [177,

310, 312] and acts by activating Ras guanine nucleotide releasing protein 1 (RasGRP1) which is necessary for activating Ras in the Ras/MEK/Erk pathway that is initiated during GCSF signaling [313]. The expression of GFI1 is sustained during differentiation and the transcription factor functions by blocking the expression of *EGR-2*/*NAB-2*, effectively antagonizing the EGR1/2 transcription factor and preventing initiation of a monocytic differentiation pathway, thereby promoting neutrophil differentiation [177, 312]. Like that of PU.1 and GATA1, GFI1 and EGR-1/EGR-2 act as an antagonistic pair to regulate neutrophil versus macrophage lineage fate.

In zebrafish, two *gfi1* genes have been identified, termed *gfi1* and *gfi1.1*. *gfi1* is primarily expressed in neural tissues, and not in the hematopoietic system [314], suggesting that this is not the functional orthologue of mammalian *GFI1*. However, *gfi1.1* was expressed in the different hematopoietic organs of the developing zebrafish embryo, suggesting that *gfi1.1* is expressed in hematopoietic cells [315]. Zebrafish *gfi1.1* morphants displayed a three-fold increase in the number of *pu.1*$^{+}$ cells, along with an increase in *l-plastin* expression and a decrease in *mpo* expression [315]. These data are consistent with the known functional role of mammalian GFI1, suggesting that zebrafish GFI1.1, and not zebrafish GFI1, is the functional orthologue of mammalian GFI.

5.3.3. Interferon response factor-8 (IRF-8)

Interferon response factor-8 (IRF-8, also known as ICSBP) is one out of nine members of the IRF transcription factor family and is characterized by an N-terminal DNA binding domain and a C-terminus IRF association domain that can associate with other IRF or Ets transcription family members [316, 317]. IRF8$^{-/-}$ mice and BXH-2 mice with a mutation in their IRF association domain show a drastic expansion of granulocytes at the expense of macrophages [318, 319]. Enforced expression of *IRF8* in myeloid progenitor cells *in vitro* led to the induced expression of a number of macrophage lineage differentiation transcripts including *CSF-1R* and *EGR1*. Additionally, enforced expression of *IRF8* in myeloid cell lines prevented their differentiation into granulocytes when treated with GCSF [320]. It is clear from the *in vivo* and *in vitro* studies that IRF8 promotes the commitment of myeloid progenitors along the macrophage lineage at the expense of the granulocyte lineage.

The homologue of *irf-8* was identified in rainbow trout [321] and zebrafish [322] with 53-55% amino acid identity to human IRF8 [321, 322]. In trout tissues, *irf8* mRNA was detected in the spleen, head kidney, gill, brain, intestine, skin, muscle, and liver [321] and mRNA levels could be up-regulated in splenocytes upon treatment with Poly I:C, PMA, PHA and recombinant IL-15. However, the role of IRF8 in GMP fate decisions or during macrophage development was not assessed. In zebrafish developing embryos, *irf8* mRNA was first detected in the rostral blood island, the site of primitive myelopoiesis, and was co-expressed with *csf-1r* mRNA, but not in cells positive for *mpx*, suggesting that *irf8* is expressed in cells committed to the macrophage lineage [322]. In zebrafish *irf8* morphants, *csf-1r*$^{+}$ cells were absent, while *mpx*$^{+}$ cells and mature neutrophils were increased by approximately three-fold, suggesting IRF8 is required for macrophage development. This phenotype could be rescued by injecting embryos with *irf8* mRNA.

Conversely, the over-expression of *irf8* mRNA in zebrafish resulted in an increase in macrophages by approximately 50% and a decrease in neutrophil numbers by about 40% [322]. These data are similar to those of the mammalian system and suggest a conserved role for IRF8 in determining macrophage over neutrophil cell lineage during primitive myelopoiesis. However, whether IRF8 plays the same role during definitive myelopoiesis in teleosts remains to be determined.

5.3.4. MafB

In addition to the previously described role of MAFB in HSCs and CMPs (see section 4.3.1), *MAFB* is highly expressed in monocytes and macrophages [164, 323] and has been shown to induce differentiation of myeloblasts into monocytes and macrophages [163, 307, 324, 325]. Furthermore, MAFB and c-MAF double knockout mice displayed differentiated macrophages that were capable of proliferating in response to CSF-1 in semi-solid and liquid culture [325]. Therefore, it appears that *MAFB* expression is sustained in monocytes and macrophages in order to prevent proliferation in these terminally differentiated cell populations.

Studies examining the role of MAFB in teleost myelopoiesis are limited. In the goldfish PKM system, a *mafb* transcript was identified and showed increasing mRNA levels with macrophage development [19]. The increasing mRNA levels of *mafb* during macrophage differentiation are similar to what has been observed in mammalian systems and suggest that MAFB may play a role in teleost macrophage differentiation.

6. Conclusion

Myelopoiesis is an orchestration of a multitude of growth factors and transcription factors that control cell fate decisions and differentiation along a chosen cell lineage. It is evident that there exists some functional redundancy in the action of myelopoietic growth factors, most likely put in place to ensure the production of these critical innate immune cells. Studies have focused on examining the regulation of myelopoiesis in the mouse model system, and have only just begun in the teleost model system.

The divergence of teleosts and mammals occurred approximately 400-450 Mya, thus teleosts represent one of the most basal groups of vertebrates [326]. Comparison of soluble factors and their receptors in teleosts and mammals show retention of many of important hematopoietic growth factors and receptors, including PDGFR [250, 251, 327], c-KIT [128-130], FLT3 (accession number DQ317446), CSF-1R [20, 250-254], GCSFR [292, 293], and their ligands PDGF [328], KIT ligand [128, 130, 131], CSF-1 [246, 249], and GCSF [290-292], although FLK2, the ligand to FLT3, has not yet been reported. However, it appears that teleosts do not possess the key myeloid growth factors IL-3 and GM-CSF, and their cognate receptors. In addition, teleosts possess all of the TF families required for hematopoiesis in higher vertebrates, reviewed in [28, 30]. Based on studies performed to date, the regulation of hematopoiesis is largely similar between mammals and teleosts. However, teleosts often possess a

http://dx.doi.org/10.5772/53589

number of gene duplications for many of the soluble factors, receptors, and to some extent, transcription factors as a result of a teleost-specific whole genome duplication predicted to have occurred approximately 350 Mya, and is believed to be responsible for the radiation of teleosts [329, 330]. Many of these teleost genes are rapidly evolving, often undergoing sub-functionalization or neo-functionalization making the identification of teleost orthologues difficult. By developing an understanding of the soluble mediators, receptors, and the intracellular machinery that govern teleost myelopoiesis, we may be better equipped to develop strategies to promote host defense against pathogens, particularly in aquaculture in which fish are predisposed to infection.

Acknowledgements

This work was supported by a grant from Natural Sciences and Engineering Research Council of Canada (NSERC) to MB. BAK was supported by NSERC and Alberta Ingenuity Fund (AIF) doctoral scholarships, FK was supported by a Japan Society for the Promotion of Science (JSPS) research fellowship.

List of abbreviations:

ABC ATP-binding cassette; **AGM** aorta-gonad-mesonephros; **ALM** anterior lateral mesoderm; **ATP** adenosine triphosphate; **BFU-E** burst-forming unit-erythrocyte; **bZIP** basic leucine zipper domain; **CCM** cell-conditioned medium; **C/EBPs** CCAAT/enhancer binding proteins; **CFU** colony forming unit; **CFU-G** colony-forming unit-granulocyte; **CFU-GEMM** colony-forming unit granulocyte/erythrocyte/macrophage/megakaryocyte; **CFU-GM** colony-forming unit-granulocyte/macrophage; **CFU-M** colony-forming unit-macrophage; **CFU-Meg** colony-forming unit-megakaryocyte; **CFU-S** colony-forming unit-spleen; **CHT** caudal hematopoietic tissue; **CLP** common lymphoid progenitor; **CMP** common myeloid progenitor; **ConA** concanavalin A; **CRH** cytokine receptor homology; **CSFs** colony-stimulating factors; **CSF-1** colony-stimulating factor 1; **CSF-1R** colony-stimulating factor-1 receptor; **DCs** dendritic cells; **EGR/egr** early growth response; **EPO** erythropoietin; **GCSF** granulocyte colony-stimulating factor; **GFI1** growth factor independence 1; **GM-CSF** granulocyte/macrophage colony-stimulating factor; **GM-CSFR** granulocyte/macrophage colony-stimulating factor receptor; **GM-CSFRα** granulocyte/macrophage colony-stimulating factor receptor alpha chain; **GMP** granulocyte/macrophage progenitor; **HPCs** hematopoietic progenitor cells; **hpf** hours post fertilization; **HSCs** hematopoietic stem cells; **IL** interleukin; **IL-3Rα** interleukin-3 receptor alpha; **IRF** interferon response factor; **JAK** Janus family of protein tyrosine kinases; **Ks/Ka** ratio of synonymous to asynonymous nucleotide substitutions; **LMPPs** lymphoid-myeloid primed multipotent progenitors; **LPS** lipopolysaccharide; **LT-HSCs** long-term hematopoietic stem cells; **MCSF** macrophage colony-stimulating factor; **mCSF-1R** membrane-bound colony-stimulating factor-1 receptor; **MEP** megakaryocyte/erythroid progenitor; **MMP** matrix metalloprotease; **mpo** myeloperoxidase; **MPPs** multipotent progeni-

tors; **mpx** myeloid-specific peroxidase; **mRNA** messenger ribonucleic acid; **mSCF** membrane-bound stem cell factor; **multi-CSF** multi-lineage colony-stimulating factor; **NF-kB** nuclear factor kappa B; **NK** natural killer; **PBI** posterior blood island; **PBL** peripheral blood leukocytes; **PDGFR** platelet-derived growth factor receptor; **PGCs** primordial germ cells; **PKM** primary kidney macrophages; **PI3K** phosphatidylinositol-3-kinase; **PLCγ** phospholipase Cγ; **PLM** posterior lateral mesoderm; **PMA** phorbol esters; **Poly I:C** polyinosinic:polycytidylic; **RasGRP1** Ras guanine nucleotide releasing protein 1; **RBI** rostral blood island; **SCF** stem cell factor; **sCSF-1R** soluble colony-stimulating factor-1 receptor; **SOCS** suppressor of cytokine signaling; **sSCF** solube stem cell factor; **STATs** signal transducers and activators of transcription; **ST-HSCs** short-term hematopoietic stem cells; **TFs** transcription factors; **UTR** untranslated region;

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