Expressions of transcription factors in goldfish (Carassius auratus L.) macrophages and their progenitors

Barbara A. Katzenback a, Bahram V. Foroutanpay b, Miodrag Belosevic a,b,*

*Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada

a School of Public Health, University of Alberta, Edmonton, Alberta, Canada

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The development of macrophages is a highly regulated process requiring coordination amongst transcription factors. The presence/absence, relative levels, antagonism, or synergy of all transcription factors involved is critical to directing lineage cell fate and differentiation. While relative levels of many key myeloid transcription factors have been determined in mammalian macrophage differentiation, a similar set of studies have yet to be conducted in a teleost system. In this study, we report on the mRNA levels of transcription factors (cebpα, cjun, cmnb, egr1, gata1, gata2, gata3, lmo2, maβb, pal5, pu.1 and runx1) in sorted goldfish progenitor cells, monocytes, and macrophages from primary kidney macrophage cultures. The mRNA levels of runx1 and pu.1 were significantly higher, gata3 and pal5 mRNA levels were lower, in monocytes compared to progenitors, and the mRNA levels of cjun, gata1, gata2, maβb and pal5 were significantly decreased in macrophages compared to progenitor cells. The relative mRNA levels of the interferon regulatory factor family of transcription factors, irf1, irf2, irf5, irf7, irf8 and irf9 in sorted progenitors, monocytes and macrophages were also measured. In contrast to other irf family transcription factors examined, irf8 mRNA levels were increased in monocytes compared to progenitors by greater than three-fold, suggesting that irf8 is important for monopoiesis. Lastly, we show the differential regulation of myeloid transcription factor mRNA levels in sorted progenitor cells from 1, 2, or 3-day old cultures in response to the recombinant goldfish growth factors, rgCSF-1 and rgKITLA.

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1. Introduction

Macrophages possess both homeostatic and innate immune defense roles in metazoans, necessitating their continual production from hematopoietic precursors. The development of macrophages from progenitor cells, termed monopoiesis, occurs through the interplay between extracellular growth factors and intracellular transcription factors (Cantor and Orkin, 2002; Friedman, 2002; Ye and Graf, 2007; Zhu and Emerson, 2002). The key growth factors that regulate monopoiesis are colony-stimulating factor-1 (CSF-1) (Fixe and Praloran, 1997; Guilbert and Stanley, 1980; Stanley et al., 1997; Tushinski et al., 1982) and interleukin-34 (IL-34) (Chihara et al., 2010; Lin et al., 2008; Wei et al., 2010), which act through the CSF-1 receptor (CSF-1R) to regulate the survival, proliferation, and development of macrophages and their precursors (Hamilton, 1997; Pixley and Stanley, 2004). Although IL-34 has yet to be identified in teleosts, CSF-1 and CSF-1R have been shown to be important in monopoiesis of teleosts (Barreda et al., 2005; Herbomel et al., 2001; Honda et al., 2005; How et al., 1996; Mulero et al., 2008; Roca et al., 2006; Williams et al., 2002), and we have previously reported on the expression of CSF-1R on goldfish progenitor cells committed to the macrophage lineage (Katzenback and Belosevic, 2012b).

At the transcriptional level, distinct transcription factors act to direct cell fate decisions. The first major decision is the commitment of a hematopoietic progenitor cell (HPC) to a myeloid lineage over an erythroid lineage and is governed by the antagonism between PU.1/GATA1 (Burda et al., 2010), where high levels of PU.1 antagonize GATA1 and promote commitment along a myeloid lineage. Once directed along a myeloid lineage, the decision for an HPC to commit to a macrophage or neutrophil cell lineage is influenced by key transcription factors. For example, CSF-1 induced signaling in mouse bone marrow derived progenitor cells increases Egr1, an important transcriptional regulator in the development and differentiation of monocytes/macrophages (Carter and Tourtellotte, 2007; Krishnaraju et al., 2001). Egr1 is known to antagonize C/EBP-β, a member of the interferon regulatory factor family (IRF) family of transcription factors, has been implicated in determining macrophage cell fate over neutrophil cell fate during embryonic monopoiesis in the zebrafish...
2. Materials and methods

2.1. Fish

Goldfish (Carassius auratus L.) 10–15 cm in length were obtained from Aquatic Imports (Calgary, Alberta, Canada) and maintained according to the guidelines of the Canadian Council of Animal Care (CCAC-Canada). Fish were housed in tanks with a continuous flow water system at 20 °C and with a 14 h light/10 h dark period in the aquatic facilities in the Biological Sciences building at the University of Alberta. Fish were fed until satiated daily. Prior to handling, fish were sedated using tricaine methane sulfonate (TMS) solution of 40–50 mg/L of water.

2.2. Isolation and generation of goldfish primary kidney macrophage (PKM) cultures

The isolation and cultivation of goldfish kidney leukocytes in complete MGFL-15 medium containing 5% carp serum and 10% newborn calf serum was performed as previously described (Neumann et al., 1998, 2000). Cells were cultured at 20 °C in the absence of CO₂. Primary kidney macrophage (PKM) cultures were composed of three distinct cell populations consisting of early progenitors (R1 gated cells), monocytes (R3 gated cells), and mature macrophages (R2 gated cells) based upon flow cytometry, cytchemistry, and molecular and functional characterization studies.

2.3. Sorting of goldfish sub-populations from PKM cultures

To isolate goldfish progenitor cells (R1 gated cells), PKMs were isolated from day zero cultures (cell population transcription factor expression experiments), or were cultured for one, two, or three days (growth factor treatment experiments) at 20 °C in complete MGFL-15 medium. Cells were harvested and centrifuged at 230×g for 10 min, enumerated using a hemacytometer, and resuspended to 5–10×10⁶ cells/mL in complete MGFL-15 medium containing 2% penicillin/streptomycin (Pen/Strep) for cell sorting on a FACS Aria flow cytometer. Cells were sorted into tubes containing complete MGFL-15 medium containing 2% Pen/Strep. Monocytes (R3 gated cells) were sorted from day 3–4 PKM cultures, and macrophages (R2 gated cells) were sorted from day 6–8 PKM cultures.

2.4. Production and purification of recombinant goldfish CSF-1 (rgCSF-1)

The sequence for goldfish CSF-1 was cloned into the pcDNA3.1 mammalian expression vector (Invitrogen) and the plasmid used to transiently transfect CHO cells using the Turbofect (Fermentas) transfection reagent as previously described in (Katzenback et al., 2011). Briefly, following transfection, media were collected every 5–6 days for 2–3 weeks, cleared of suspension cells by centrifugation at 230×g for 10 min, and stored at 4 °C prior to purification of secreted rgCSF-1 fused to a 6× his tag. CHO cell-conditioned supernatants were adjusted to 20 mM imidazole and 100 mM HEPES, required for protein binding to beads. MagneHis (Promega) beads were used to purify rgCSF-1 using the 6× his tag according to manufacturers instructions. Buffer exchange from MagneHis elution buffer to 1× PBS was performed using iCON concentrators (Pierce) according to specifications. Protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Scientific) employing bovine serum albumin as the standard curve. The solution containing rgCSF-1 was filter sterilized (0.22 μm) and stored at 4 °C until use. The identity of the recombinant molecule was confirmed using mass spectrometry.

2.5. Production and purification of recombinant goldfish kit ligand a (rgKITLA)

The production, purification, and functional characterization of gKITLA, fused to a 6× his tag for purification, was performed as previously described in (Katzenback and Belosevic, 2009b). Briefly, the goldfish KITLA/pSECTag2B construct was transiently transfected into HEK293T cells using the TurboFect (Fermentas) transfection reagent according to manufacturer’s protocols. Cell-conditioned medium containing the recombinant protein were collected every 5 days, pooled and used for protein purification with NiNTA beads according to the manufacturers specifications and as described previously (Katzenback and Belosevic, 2009b). Protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Scientific) employing bovine serum albumin as the standard curve. The preparation containing the recombinant protein was filter sterilized (0.22 μm) and stored at 4 °C until use. The identity of the recombinant molecule was confirmed using mass spectrometry.
2.6. Treatment of goldfish R1 progenitor cells with recombinant growth factors

Day 1, 2, or 3 PKM cells were collected and R1 cells sorted as described in Section 2.3. Cells were washed 2 × in MGFL-15 and re-suspended to 5 × 10⁶ cells/mL. To each well of a 24-well plate, 0.5 mL of cell suspension was added, followed by 0.5 mL of either medium, 200 ng/mL of rgKITLA, 200 ng/mL of rgCSF-1, or a combination of 200 ng/mL of rgKITLA and 200 ng/mL of rgCSF-1. The resulting final concentrations of rgKITLA or rgCSF-1 were 100 ng/mL. Cells were harvested 0.5 h, 3 h, or 6 h post treatment.

2.7. RNA isolation, cDNA synthesis, and quantitative PCR (qPCR)

RNA was isolated using the RNA MicroPrep Kit (ZymoResearch) and reverse transcribed into cDNA using Superscript II enzyme (Invitrogen) according to the manufacturer’s protocol. Quantitative PCR for transcription factor expression was performed using the delta delta Ct method as described previously (Katzenback and Belosevic, 2009b). Primers for qPCR are listed in Supplementary Table 1. Values were normalized to the reference group (progenitors, medium treated progenitors) for each transcription factor.

2.8. Statistics

Data analyses were performed using the GraphPad 6.0 software. Statistical analyses of qPCR data were performed using the delta Ct values for all comparisons. Analyses of transcription factor mRNA levels in progenitors, monocytes, and macrophages (Figs. 1–5) were performed using a one-way ANOVA with a Dunnet’s multiple comparisons post hoc test, while a paired t-test was used for treatment of progenitor cells with growth factors (Figs. 6–8). A probability of \( P < 0.05 \) was considered significant; \( P < 0.08 \) was considered marginally significant.

3. Results

3.1. Early myeloid transcription factor mRNA levels in progenitors, monocytes, and macrophages

To examine the transcription factors expressed at distinct junctions of macrophage development, progenitor cells, monocytes, and macrophages were sorted from PKM cultures and the relative mRNA levels of a panel of transcription factors were determined. The relative mRNA levels of transcription factors involved in early myelopoiesis, runx1, cmyb and gata2 were examined in each of the macrophage sub-populations (progenitor cells, monocytes, and macrophages). The transcription factors runx1, cmyb and gata2 were chosen based on their involvement in progenitor cell survival and maintenance as previously described (Katzenback et al., 2011). The mRNA levels of runx1 were significantly higher in monocytes compared to that of progenitors by approximately 3-fold (Fig. 1A). However, runx1 mRNA levels in macrophages were not significantly different from that of progenitors (Fig. 1A). In macrophages, cmyb mRNA levels were significantly higher than that of progenitor cells, by 3-fold (Fig. 1B), while cmyb mRNA levels in monocytes were not different from that of the progenitor cells (Fig. 1B). The mRNA levels of gata2 tended to decrease with macrophage development, and were significantly lower in macrophages compared to progenitors (\( \sim 0.4 \) fold) (Fig. 1C).

3.2. Myeloid transcription factor mRNA levels in macrophage sub-populations

The transcription factors cebpα, cjun, egr1, mafb and pu.1 were chosen based on their role in myeloid development, as previously described (Katzenback et al., 2011). The mRNA levels of pu.1 were significantly higher in monocytes by approximately 8-fold compared to progenitor cells, while macrophages had approximately 2-fold higher mRNA levels of pu.1 compared to that of progenitor cells (Fig. 2A). When the mRNA levels of mafb, cjun, and egr1 were examined, a similar trend was seen among them; macrophages had significantly lower mRNA levels of mafb (Fig. 2B), cjun (Fig. 2C), and egr1 (Fig. 2D) in comparison to progenitor cells (<0.2 fold). Although no significant differences were observed in cebpα mRNA levels in the progenitor cell, monocyte and macrophage sub-populations, there was a general trend of decreasing mRNA levels with macrophage development (Fig. 2E).

3.3. Erythroid and lymphoid transcription factor mRNA levels in progenitors, monocytes, and macrophages

The transcription factors gata1 and lmo2, and gata3 and pax5, were chosen for their involvement in erythropoiesis and lymphopoiesis, as previously described (Katzenback et al., 2011). Furthermore, we have recently shown that the erythroid transcription...
factors, \textit{gata1} and \textit{lmo2} are up-regulated in goldfish progenitor cells in response to treatment with recombinant goldfish erythropoietin, further supporting the role of these transcription factors in erythroid development in teleosts (Katakura et al., 2013). For \textit{gata1} (Fig. 3A) and \textit{lmo2} (Fig. 3B) mRNA levels, no significant differences were observed amongst the progenitor cell, monocyte and macrophage sub-populations. However, the mRNA levels of the transcription factors involved in lymphopoiesis, \textit{gata3} (Fig. 4A) and \textit{pax5} (Fig. 4B), were both significantly lower ($P < 0.05$) in monocytes and macrophages compared to the mRNA levels in progenitor cells.

### 3.4. Interferon regulatory factors mRNA levels in progenitors, monocytes, and macrophages

Members of the interferon regulatory factor group of transcription factors have been shown to play a role in macrophage commitment and development in mammalian systems (Tamura et al., 2000; Yamamoto et al., 2011) and in monopoiesis in zebrafish using \textit{irf8} morphants (Li et al., 2011). Therefore, the relative mRNA levels of members of the IRF family were examined in the context of the goldfish macrophage sub-populations. In general, the mRNA levels of \textit{irf1}, \textit{irf2}, \textit{irf5} and \textit{irf7} decreased with macrophage development (Fig. 5A–D, respectively). In the case of \textit{irf1} (Fig. 5A) and \textit{irf7} (Fig. 5D), mRNA levels were significantly decreased in macrophages, but not monocytes, compared to that of the progenitor cells. The mRNA levels of both \textit{irf2} (Fig. 5B) and \textit{irf5} (Fig. 5C) were significantly lower in monocytes and macrophages compared to the progenitor cell group. In contrast, the mRNA levels of \textit{irf8} were significantly higher in monocytes by approximately 3-fold, but not macrophages, compared to that of the \textit{irf8} mRNA levels of the progenitor cell group (Fig. 5E). No significant differences were observed in the mRNA levels of \textit{irf9} amongst the progenitor cell, monocyte and macrophage sub-populations (Fig. 5F). A summary figure of the changes in all transcription factors examined during myelopoiesis, in relation to the progenitor cell population, is shown in Fig. 6.

### 3.5. Transcription factor mRNA levels in day 1, day 2, and day 3 sorted progenitor cells treated with recombinant goldfish growth factors

We next wanted to characterize the population of progenitor cells present at different time points in culture, and chose to sort progenitor cells from day 1, day 2 and day 3 PKM cultures for use in growth factor treatment experiments. These time points were chosen based on changes in transcription factor mRNA levels in sorted progenitor cells from different days of PKM culture from a previous study (Katzenback et al., 2011). Based on previous data, we believed the population of progenitor cells to change over time in culture with progenitor cells becoming committed to the macrophage lineage by day 2 of cultivation (Katzenback et al., 2011). The growth factors used in this experiment were rgCSF-1 and rgKITLA.
or a combination of rgCSF-1 and rgKITLA. Our laboratory has previously functionally characterized rgCSF-1 and rgKITLA. The recombinant goldfish CSF-1 has been showing to promote the proliferation and differentiation of progenitor cells and monocytes (Hanington et al., 2007), while rgKITLA promotes progenitor cell chemotaxis, proliferation, and survival (Katzenback and Belosevic, 2009).

We first examined the response of myeloid transcription factor mRNA levels (runx1, cebpα, cjune1, gata2, ef1a, and pu.1) in progenitor cells treated with rgCSF-1-R. The mRNA levels of cebpα and cjune1 were down-regulated in day 1 progenitor cells treated with rgCSF-1, whereas egr1 mRNA levels were significantly up-regulated in rgCSF-1 treated day 1 progenitor cells compared to that of the medium control at 0.5 h post treatment (Fig. 7A). However, by 3 and 6 h post treatment with rgCSF-1, the mRNA levels of cebpα, cjune1, and egr1 were not significantly different from that of the medium controls (Fig. 7A). No significant changes were observed in the mRNA levels of runx1, gata2, ef1a, or gata2 in day 1 progenitor cells treated with rgCSF-1 at any time point measured (not shown). When day 2 progenitor cells were treated with rgCSF-1, a marginally significant increase was observed in cebpα mRNA levels at 3 h post treatment compared to that of the medium control, but returned to basal levels by 6 h (Fig. 8A). Six hours post treatment of day 2 progenitor cells with rgCSF-1, a significant increase was observed in the mRNA levels of cjune1 and egr1 compared to those of the medium control (Fig. 8A). No significant changes were observed in the mRNA levels of runx1, gata2, ef1a, or gata2 in day 2 progenitor cells treated with rgCSF-1 at any time point (not shown). The mRNA levels of both runx1 and cebpα showed a general trend of down-regulation at 0.5 h and 3 h post rgCSF-1 treatment and were significantly down-regulated in day 3 progenitor cells treated with rgCSF-1 at 6 h (Fig. 9A). The mRNA levels of mafb were marginally increased in day 3 progenitor cells treated with rgCSF-1 by 6 h (Fig. 9A). No significant changes were observed in the mRNA levels of cjune1, gata2, or pu.1 in day 3 progenitor cells treated with rgCSF-1 at any time point (not shown).

We next assessed the regulation of transcription factor mRNA levels in progenitor cells treated with rgKITLA. In day 1 progenitor cells, there were significant decreases in runx1, cebpα and cjune1 mRNA levels by 6 h post rgKITLA treatment compared to the medium control (Fig. 7B). However, in day 2 progenitor cells, there was a marginal decrease in the mRNA levels of cebpα at 3 h, followed by an increase in the mRNA levels of cebpα, egr1, and mafb by 6 h post treatment with rgKITLA compared to the mRNA levels of the medium controls (Fig. 8B). A generalized decrease in the mRNA levels of cebpα, cjune1, and egr1 were observed in day 3 progenitor cells treated with rgKITLA at all time points (Fig. 9B). The mRNA levels of cjune1 and cebpα were decreased at 3 h, while only cebpα mRNA levels were significantly decreased by 6 h compared to the time matched medium controls (Fig. 9B).

Lastly, the treatment of progenitor cells with a combination of rgCSF-1 and rgKITLA was examined. Upon treating day 1 progenitor cells with the combination of growth factors, there was an initial decrease in the mRNA levels of runx1 and cebpα at 0.5 h compared to that of the time-matched medium controls (Fig. 7C). The mRNA levels of runx1 and cebpα in sorted day 1 progenitor cells were not significantly different from their respective time-matched medium controls (Fig. 8C). Treatment of day 2 sorted progenitor cells with the combination of rgCSF-1 and rgKITLA showed no significant change in transcription factor mRNA levels at 0.5 h and 3 h (Fig. 8C). However, at 6 h, the mRNA levels of cebpα and egr1 were significantly increased in day 2 progenitor cells compared to their respective time matched medium controls (Fig. 8C). Day 3 progenitor cells treated with rgCSF-1 and rgKITLA did not exhibit any significant changes in myeloid transcription factor expression until 6 h post treatment. At 6 h post treatment, the mRNA levels of runx1...
and cebp4 were marginally and significantly decreased, respectively, compared to the time-matched medium controls (Fig. 9C).

4. Discussion

The co-ordination of transcription factor up-regulation and down-regulation at distinct junctures of macrophage development is required for maintaining cellular stages and conversely for allowing cellular differentiation to proceed. To further our understanding of the transcriptional regulators that may be involved in the development of progenitor cells into monocytes and then into macrophages, we surveyed the relative mRNA levels of a panel of goldfish transcription factors using quantitative PCR. This study is the first, to our knowledge, to examine the differences in gene expressions of a panel of transcription factors between progenitors, monocytes and macrophages in teleosts.

Within our PKM culture system, we have previously shown endogenous CSF-1 to act through the CSF-1R found on progenitors, monocytes and macrophages to promote all aspects of macrophage development (Barreda et al., 2005; Hanington et al., 2009a; Katzenback and Belosevic, 2012b). As such, this system represents a unique model system in which to study cell populations at distinct junctures of teleost macrophage development. In general, the mRNA levels of the 18 transcription factors examined in the progenitor cell, monocyte and macrophage populations can be grouped into six patterns. The patterns in the mRNA levels are (1) no change between all sub-populations, (2) no change in monocytes, increase in macrophages, (3) increase in monocytes and macrophages, (4) increase in monocytes, no change in macrophages, (5) no change in monocytes and decrease in macrophages, and (6) decrease in monocytes and macrophages, relative to the progenitor cell population (Fig. 6).

From patterns in TF mRNA levels, we can begin to understand the transcriptional regulation required at distinct junctures of teleost macrophage development. Transcription factors from the first
Fig. 7. Changes in myeloid transcription factors in sorted day 1 progenitor cells in response to recombinant growth factors. Progenitor cells were sorted based on size and internal complexity using a FACS Aria flow cytometer and 2.5 × 10^5 cells treated with 100 ng/mL of recombinant growth factors for 1, 3 or 6 h prior to RNA isolation and cDNA synthesis. Relative mRNA levels of the myeloid transcription factors were determined using qPCR. The mRNA levels of each transcription factor were normalized to that of the medium-treated, time-matched control group, ef1a was used as the endogenous reference. Myeloid transcription factor mRNA levels in day 1 sorted progenitor cells treated with 100 ng/mL rgCSF-1 (A), 100 ng/mL rgKITLA (B), or a combination of 100 ng/mL rgCSF-1 and 100 ng/mL of rgKITLA (C). Mean ± standard error are shown (n = 3–4). Significance is denoted by (*) to show P < 0.05, and ($) is P < 0.08. P, progenitor cells; M, monocytes; MΦ, macrophages.

Fig. 8. Changes in myeloid transcription factors in sorted day 2 progenitor cells in response to recombinant growth factors. Progenitor cells were sorted based on size and internal complexity using a FACS Aria flow cytometer and 2.5 × 10^5 cells treated with 100 ng/mL of recombinant growth factors for 1, 3 or 6 h prior to RNA isolation and cDNA synthesis. Relative mRNA levels of the myeloid transcription factors were determined using qPCR. The mRNA levels of each transcription factor were normalized to that of the medium-treated, time-matched control group, ef1a was used as the endogenous reference. Myeloid transcription factor mRNA levels in day 2 sorted progenitor cells treated with 100 ng/mL rgCSF-1 (A), 100 ng/mL rgKITLA (B), or a combination of 100 ng/mL rgCSF-1 and 100 ng/mL of rgKITLA (C). Mean ± standard error are shown (n = 3–4). Significance is denoted by (*) to show P < 0.05, and ($) is P < 0.08.
pattern in which mRNA levels did not change amongst all three cell sub-populations included transcription factors involved in erythropoiesis (*gata1* and *lmo2*) (Brandt and Koury, 2009; Burda et al., 2010), granulopoiesis (*cebp* and *irf*). Although *cebp* mRNA levels did not significantly differ between the three sub-populations, there was a generalized decrease in mRNA levels coinciding with macrophage differentiation. Furthermore, we have previously isolated and characterized goldfish neutrophils (Katzenback and Belosevic, 2009a) and have demonstrated neutrophils to have significantly higher mRNA levels of *cebp* compared to kidney progenitor cells (Katzenback and Belosevic, 2012a), supporting the role of *cebp* in granulocyte versus macrophage differentiation (Zhang et al., 1997).

Falling within the second pattern, where an increase in mRNA level in the macrophage population was observed, was *cmyb*. In zebrafish, *cmyb* is a marker of definitive HSCs (Bertrand et al., 2008) and appears to have an evolutionarily conserved role in hematopoiesis (Soza-Ried et al., 2010). However, in the mammalian model system, c-Myb appears to be down-regulated in macrophages by Maf-B as a mechanism of inhibiting proliferation and allowing for differentiation, reviewed by (Sarrazin and Sieweke, 2004). Unlike the mammalian macrophages, goldfish macrophages appear to retain their proliferative ability in their terminally differentiated state (Barreda et al., 2000). Therefore, the up-regulation of *cmyb* and down-regulation of *mafb* (discussed later) in terminally differentiated macrophages may be a mechanism for goldfish macrophages to retain their proliferative ability, at least in vitro.

The mRNA levels of *pu.1* in monocytes increases by about 8 fold compared to the progenitor population, and remains elevated in macrophages by approximately 2.5 fold, comprising the sole transcription factor observed in the third pattern. The role of *pu.1* in myeloid cell development has been well documented in the mammalian system (Burd a et al., 2010), and in fish (Su et al., 2007), and thus the elevated levels of *pu.1* in goldfish monocytes and macrophages is not surprising. Recently, a *pu.1* like gene (*spi.1* like) was identified in zebrafish and shown to act downstream of *pu.1*, likely involved in terminal macrophage and neutrophil cell development (Bukrinsky et al., 2009). The *spi.1* like gene, a *pu.1* orthologue, although not examined here, may explain why *pu.1* mRNA levels decreased in macrophages compared to that of the monocyte sub-population. In the fourth pattern are *runx1* and *irf8*, in which their mRNA levels increase in monocytes, but not in macrophages compared to the progenitor cell population. The significant increase in *runx1*, *irf8* and *pu.1* mRNA levels in monocytes compared to the mRNA levels in progenitor cells suggests that these transcription factors may play a vital role in the developing monocyte population. RUNX1, PU.1 and CEBPα binding sites have all been identified in the regulation of CSF-1R mRNA expression (*fms* gene) in mice (Gangganahalli et al., 2005; Tagoh et al., 2002). Thus, the increase in *runx1* and *pu.1* in goldfish monocytes may be due to the increasing expression of CSF-1R that is observed during macrophage development (Barreda et al., 2004; Katzenback and Belosevic, 2012b). Furthermore, *irf8* has been shown to be involved in promoting macrophage development over neutrophil cell development in mammals (Wang and Morse, 2009; Yamamoto et al., 2011) and developing zebrafish (Li et al., 2011), thus the increase in *irf8* mRNA levels in monocyes is in agreement with monopoiesis of other vertebrate model systems.

In the case of *mafb*, *cjun*, *egr1*, *gata2*, *irf1* and *irf7* mRNA levels, there were no significant differences between the mRNA levels of progenitor cells and monocytes, but a significant decrease in the mRNA levels of the transcription factors in macrophages, comprising the fifth pattern of transcriptional regulation. These data
suggest that mafb, cjun, egr1, gata2, irf1 and irf7 are important during development, but once myeloid cells are terminally differentiated into mature macrophages, these transcription factors are no longer required. MafB, important for progenitor cell renewal (Aziz et al., 2009; Sarrazin et al., 2009), decreases in progenitors leading to PU.1 up-regulation, increased cell responsiveness to CSF-1 (Sarrazin et al., 2009) and monocyte/macrophage differentiation which then requires MafB expression (Aziz et al., 2009; Sarrazin et al., 2009). Similarly, GATA2 is also involved in progenitor cell maintenance and proliferation (Hosoya et al., 2010), and acts to inhibit monocyte/macrophage differentiation through PU.1 suppression (Maeda et al., 2010). The down-regulation of EGR, RUNX1, RUNX3, GATA2 and IRF7 transcription factors were also observed in monocyte to macrophage in vitro differentiation from healthy human donors (Liu et al., 2008). However, CEBPζ was up-regulated in macrophages compared to monocytes (Liu et al., 2008), which was not the case when we compared goldfish cebpa mRNA levels in monocytes and macrophages. In a study examining macrophage development in mice, they noted a similar increase in cebpa mRNA early during macrophage development, with a slight decrease in mature macrophages, while the mRNA levels of gata1, gata2, pu.1 and runx1 were decreased in mature macrophages compared to progenitor cells (Tagoh et al., 2002).

The last set of transcription factors that falls into pattern 6 are those whose mRNA levels decrease in monocytes and macrophages compared to that of the progenitor cell group. These transcription factors included gata3 and pan5, which are important for lymphopoiesis (Fuxa and Busslinger, 2007; Hosoya et al., 2010), as well as irf2 and irf5. In mammalian systems, an increase in irf5 mRNA has been documented in type 1 (M1) polarized macrophages that were differentiated from monocytes in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF) (Krausgruber et al., 2011). The down-regulation of irf5 in monocytes and macrophages compared to progenitor cells in goldfish is interesting as macrophage differentiation is driven by CSF-1. Therefore, it is possible that these in vitro derived PKMs represent type 2 (M2) polarized macrophages. It is interesting to speculate that if these macrophages are M2 polarized, many of the functional studies performed by our laboratory could be examining how M2 macrophages respond to pro-inflammatory stimuli. This represents an important area of study, particularly for cancer biology in which cancerous cells often create an immunosuppressive environment, often including M2 polarized cells as a mechanism of immune evasion, reviewed by (Hao et al., 2012).

Lastly, treatment of sorted progenitor cells from day 1, day 2, or day 3 of culture with the recombinant myeloid growth factors demonstrated the differential regulation of transcription factors by myeloid growth factors based on the day of progenitor cell isolation. In particular, the increase in mRNA levels of myeloid transcription factors (cebpa, cjun, and egr1) in day 2 sorted progenitor cells after 6 h of treatment with rGCSF-1, rGKTLA, or a combination of the two growth factors, suggests the exposure of these progenitor cells to endogenous growth factors produced by cells in the PKM cultures that may act to ‘prime’ the progenitor cells to become responsive to rGCSF-1 or rGKTLA. Unlike differentiated cells, progenitor cells require multiple growth factor signals to induce a response, reviewed in (Kondo, 2010). Regardless of the day of progenitor cell isolation, we did not observe a rapid (by 0.5 h) response in egr1 mRNA levels in goldfish progenitor cells treated with rGCSF-1. This is in contrast to experiments performed on isolated mouse bone marrow cells treated with mouse CSF-1, where CSF-1 induced a rapid and significant increase in Egr1 mRNA levels in mouse bone marrow isolates (Krishnaraju et al., 2001). We believe this may represent a differential regulation of CSF-1 signaling in fish macrophages in comparison to mouse macrophage precursors. Conversely, these data may signify the differences in the composition of the precursor pool isolated from the hematopoietic niche of fish or higher vertebrates such as mice.

This study focused on the transcriptional regulation of a panel of transcription factors in goldfish progenitors, monocytes and macrophages. While the majority of transcription factors selected for this study have no reported orthologues, the presence of orthologues in teleost fish, particularly for cytokines, suggests that there may be unidentified and uncharacterized orthologues of these transcription factors. Our knowledge on the role that transcription factor orthologues play in teleost myelopoiesis is limited, but suggests that sub-functionalization can occur between orthologues. Future studies on the protein levels of transcription factors in the cell sub-populations or knock-down of particular transcription factors in progenitors and monocytes using RNAi would provide important information on the role of the transcription factors in this study, and subsequently identified orthologues, in driving macrophage commitment and differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.devcel.2013.05.019.

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