Growth Factors of Lower Vertebrates

CHARACTERIZATION OF GOLDFISH (CARASSIUS AURATUS L.) MACROPHAGE COLONY-STIMULATING FACTOR-1*

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Colony-stimulating factor-1 (CSF-1) regulates mononuclear cell proliferation, differentiation, and survival. The functions of CSF-1 are well documented in mammals; however, little is known about CSF-1 biology in lower vertebrates. This is the first report on the identification and functional characterization of a fish CSF-1 molecule expressed highly in the spleen and in phorbol 12-myristate 13-acetate-stimulated monocytes. Goldfish CSF-1 is a 199-amino acid protein that possesses the required cysteine residues to form important intra-chain and inter-chain disulfide bonds that allow CSF-1 to form a functional homodimer and to interact with its high affinity receptor, CSF-1R. Recombinant goldfish CSF-1 formed a homodimer and bound to the soluble goldfish CSF-1R. The addition of the recombinant CSF-1 to sorted goldfish progenitor cells, monocytes, and macrophages induced the differentiation of monocytes into macrophages and the proliferation of progenitor cells, monocytes, and macrophages induced the differentiation of monocytes into macrophages and the proliferation of monocyte-like cells. The proliferation of these cells was abrogated by addition of an anti-CSF-1R antibody as well as the soluble CSF-1R. The addition of the recombinant CSF-1 to sorted goldfish progenitor cells, monocytes, and macrophages induced the differentiation of monocytes into macrophages and the proliferation of monocyte-like cells. The proliferation of these cells was abrogated by addition of an anti-CSF-1R antibody as well as the soluble CSF-1R. The ability of the soluble CSF-1R to inhibit CSF-1-induced proliferation represents a novel mechanism for the regulation of CSF-1 function.

From an evolutionary standpoint, macrophages and their functions are quite conserved; macrophage-like cells can be found in almost all multicellular organisms. Macrophage development is relatively unknown in almost all of these organisms with the exception of mammals. Until recently, mammals were the only group of organisms in which CSF-1 had been identified, and the suggestion that the CSF-1 pathway of macrophage development existed in organisms other than mammals was the identification of CSF-1 transcripts in the chicken (9, 10) and fish (11–13) as well as the identification of a CSF-1R in the goldfish (14), pufferfish (15), rainbow trout (16), zebrafish (17), and the gilthead sea bream (18).

Here we describe the identification and biological characterization of the first CSF-1 from a nonmammalian organism, the goldfish. Goldfish CSF-1 was encoded for by a 600-bp open reading frame that translates into a predicted peptide of 199 amino acids. Although it had only a 27% amino acid identity with human CSF-1, important cysteine residues required for formation of the CSF-1 homodimer were conserved. Goldfish CSF-1 was highly expressed in the spleen, and its expression was up-regulated in goldfish monocytes after treatment with phorbol esters (PMA). Recombinant goldfish CSF-1 induced differentiation and proliferation of sorted goldfish monocytes. The proliferative effect was abrogated by addition of an anti-CSF-1R antibody and/or soluble goldfish CSF-1R (sCSF-1R), we previously characterized (14).

EXPERIMENTAL PROCEDURES

Fish—Goldfish (Carassius auratus) were purchased from Mt. Parnell Fisheries Inc. (Mercersburg, PA) and maintained at the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were kept at 20 °C in a flow-through water system and fed to satiation daily with trout pellets. The fish were acclimated to this environment for at least 3 weeks prior to use in experiments. All of the fish ranged from 10 to 15 cm in length and whenever possible an equal number of both sexes was used.

Cultivation of Goldfish Macrophages—The culture medium (NMGFL-15) used has been described previously (12). Complete NMGFL-15 medium used for the cultivation of goldfish macrophages contained 5% carp serum and 10% newborn calf serum (HyClone, Logan, UT). Cultures were grown in the absence of CO₂ at 20 °C.

Isolation and Generation of Primary Kidney Macrophages from Goldfish—Isolation of goldfish kidney leukocytes and the generation of primary kidney macrophages were performed as...
Goldfish CSF-1

**TABLE 1**

| Primer                        | Sequence                        |
|-------------------------------|---------------------------------|
| Conserved CSF-1 sense         | GCCTCTTCCTACATCAGACACAT         |
| Conserved CSF-1 antisense     | AATCTTTTTCTGGAATTACACAGACAC     |
| Goldfish CSF-1 qPCR sense     | ACGACATTACGCACCCCAACAGCC        |
| Goldfish CSF-1 qPCR antisense | GCACAGGAGGAAGTAAAGCACTGACT      |
| Actin qPCR sense              | GCACGGCATCTGACACCTGAA           |
| Actin qPCR antisense          | GAAGGGCGGCCTCGGAAGTTA           |
| Goldfish CSF-1 pSeqTag2 sense | ATGACACACATATACGGCCCAACAGCC     |
| Goldfish CSF-1 pSeqTag2 antisense | ACAGACTCTCTGTTGGATGATAG         |

Described previously (12). Three distinct macrophage subpopulations are a feature of primary kidney macrophage cultures that represent macrophage subsets temporally arrested at distinct differentiation junctures in development: the early progenitor cells, the monocyte-like cells, and mature macrophages (19).

**DNA Sequencing and in Silico Analysis of Goldfish CSF-1**—The goldfish CSF-1 transcript was identified by homology-based PCR using the trout and known mammalian CSF-1 sequences as references. All primers used for sequencing, expression, and cloning of goldfish CSF-1 are shown in Table 1. From this fragment, RACE PCR (BD Biosciences) was performed following the manufacturer’s specifications to obtain the full open reading frame sequence of goldfish CSF-1 from which recombinant expression primers were designed. Sequencing was performed by first cloning the CSF-1 PCR amplicon into the PCR 2.1 TOPO vector (Invitrogen) and identifying the colonies that contained positive inserts by colony PCR using the vector-specific M13 primers (Invitrogen). Positive clones were isolated using the QIAquick PCR purification kit (Qiagen) and sequenced using a DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences) and a PE Applied Biosystems 377 automated sequencer. Single pass sequences were analyzed using Vector NTI (Invitrogen), and subsequent gene annotations were conducted using BLAST programs (www.ncbi.nlm.nih.gov/BLAST/).

Conserved CSF-1 motifs were identified in the putative amino acid sequence, and subsequent predictions were based on analytical tools provided in the ExPASy proteomics server. Sequence alignments were performed using ClustalX version 1.83.

**Quantitative PCR Analysis of CSF-1 Expression in Different Tissues**—Quantitative PCR analyses were carried out using the Applied Biosystems 7500 fast real time PCR equipment. The relative expression of goldfish CSF-1 in relation to β-actin in the kidney, spleen, liver, heart, gill, intestine, and brain was assessed using primers generated with the Primer Express software (Applied Biosystems). Thermocycling parameters were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Analyses of the relative tissue expression data for six fish (n = 6) were carried out using the 7500 Fast software (Applied Biosystems).

**Real Time PCR Analysis of Goldfish CSF-1 Expression in Sorted Macrophage Subpopulations**—Cultured goldfish kidney macrophages were sorted into early progenitor, monocyte, and macrophage subpopulations using a FACSCalibur flow cytometer (BD Biosciences) using a protocol we described previously (19), and the RNA was isolated immediately after sorting using TRIzol (Invitrogen) following the manufacturer’s specifications. First strand cDNA synthesis was done using the SuperScript II cDNA synthesis kit (Invitrogen) according to the manufacturer’s protocols. The sorted experimental groups each represent the combined data collected from six fish (n = 6). Statistical significance of the data obtained from the quantitative PCR was analyzed using a one-way analysis of variance, and results were deemed to be significant at p < 0.05.

**Design of Expression Constructs, PCR Amplification, and Cloning**—Goldfish CSF-1 was designed for expression using the pSeqTag2 mammalian expression system (Invitrogen). PCR amplification of the expression insert was performed as follows: 7 μl of the CSF-1 clone template was added to 76 μl of double distilled H2O, dNTPs (0.2 μl of each dATP, dCTP, dGTP, dTTP 100 mM solutions), 10 × PCR buffer (10 μl of 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl2, 0.01% (w/v) gelatin), expression primers and a 15:1 ratio of Taq-Pfu DNA polymerases (1 μl of 5 units/liter solution). PCR amplification was conducted in an Eppendorf Mastercycler gradient thermal cycler. The amplification program consisted of a 6-min hot start at 94 °C, followed by 28 cycles of 94 °C for 20 s, 59 °C for 20 s, 72 °C for 2 min, and a final elongation step of 72 °C for 7 min. Amplification was confirmed by agarose gel electrophoresis.

**Production of Recombinant CSF-1**—Plasmid DNA containing the CSF-1 expression construct was transformed into CHO cells (Invitrogen) for recombinant protein expression. 10 ng of plasmid DNA was transfected into CHO cells using 293fectin (Invitrogen) according to the manufacturer’s specifications. After transfection, cells were transferred to a 25-cm² culture flask and allowed to sit for 2 days before supernatants were collected and tested for recombinant protein by Western blot using an antibody specific for the His6 tag located on the C-terminal end of the recombinant protein. Cultures that were successfully transfected were placed in a 2-liter culture roller bottle and grown for 7 days at 37 °C after which the supernatants were collected.

**Purification of Recombinant CSF-1**—Supernatants collected from the transfected CHO cells were collected and dialyzed overnight against 1× phosphate-buffered saline and pooled together. Recombinant CSF-1 was purified from dialyzed culture supernatants using MagneHis beads (Promega) according to the manufacturer’s specifications. Purified proteins were eluted in a solution containing 100 mM HEPES and 500 mM KCl, pH 9.0, and then dialyzed overnight against 1× phosphate-buffered saline. Samples were then filter-sterilized in preparation for immunodetection and analysis of biological activity. Total protein concentration was determined using a bichinchoninic acid protein assay kit (Pierce) according to the manufacturer’s protocols. The presence of rCSF-1 in the purified sample was confirmed by Western blot, and the identity of the purified protein was ascertained to be goldfish CSF-1 by mass spectrometry. Briefly, proteins were separated by SDS-PAGE under reducing conditions using 12.5% polyacrylamide gels, transferred to 0.2-μm nitrocellulose membranes (Bio-Rad), and incubated overnight at 4 °C in the presence of the anti-His6 primary antibody. Membranes were subsequently washed, incubated with an alkaline phosphatase-conjugated mouse IgG monoclonal antibody, and developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.
Measurement of Macrophage Proliferation Induced by Purified rCSF-1—The effect of goldfish rCSF-1 on macrophage proliferation was assessed using the cell proliferation enzyme-linked immunosorbent BrdUrd colorimetric assay (Roche Applied Science). This technique was based on the incorporation of the pyrimidine analog, 5-bromo-2'-deoxyuridine (BrdUrd), into the DNA of proliferating cells. Macrophages were grown as described above and sorted using a FACS Calibur flow cytometer (BD Biosciences) based on size and complexity. Progenitor cells, monocytes, and macrophages were then counted and seeded at a density of $1 \times 10^4$ cells well$^{-1}$ in 96-well culture plates (Falcon). Cells were seeded in 50 µl of incomplete culture medium and treated with 50 µl of 50, 25, 10, 5, and 1 ng/ml rCSF-1 mixed with incomplete cell culture medium and incubated with BrdUrd labeling reagent at a concentration of 15 µM. Treatments were re-applied on days 1, 3, 5, and 7 in-between days that samples were collected. Blocking studies were done with the same rCSF-1 concentrations and included into the 50-µl treatment 10, 50, 100, or 250 ng/ml anti-CSF-1R antibody or 10, 25, and 50 ng/ml sCSF-1R. Preincubation with the anti-CSF1R antibody and the sCSF-1R was done over a period of 12 h at 4 °C. Cells were incubated with BrdUrd labeling reagent for 24 h, and then subsequent samples were taken every 2 days from the day 0 time point. The reaction was developed according to the manufacturer’s specifications, and absorbance was determined at 450 nm using a microplate spectrophotometer (Biotek). In control experiments the colorimetric reaction was found to be directly proportional to the number of proliferating macrophages in culture. Recordings from the nontreated cells were subtracted from the experimental groups to account for the endogenous production of growth factors by goldfish macrophages.

Flow Cytometric Analysis of Primary Goldfish Macrophage Cultures—Goldfish macrophages were sorted into progenitor cells, monocyte and macrophage subpopulations on day 4 post-isolation. They were seeded into 12-well plates at a concentration of $3 \times 10^6$ cells well$^{-1}$ in a volume of 3 ml of incomplete medium and exposed to treatments, including 50, 25, 10, 5, and 1 ng/ml macrophage cell culture medium (CCM), supernatants from untransfected control CHO cells treated in the same way as the rCSF-1, and cells alone. Treatments were re-applied on alternating days to data collection. Every 48 h after treatment the cells in each well were monitored using flow cytometric analysis using a FACS Calibur flow cytometer (BD Biosciences). Analysis was performed by measuring the forward (size) and side (internal complexity) scatter light patterns of the cells in each treatment group for eight different fish ($n = 8$).

Goldfish rCSF-1/sCSF-1R Binding Assay—Goldfish rCSF-1 and soluble CSF-1R were biotinylated using the EZ-Link biotinylation kit (Pierce) according to the manufacturer’s protocol. Effective biotinylation of each protein was confirmed using the EZ-Biotin quantitation kit (Pierce) according to the manufacturer’s protocol. To determine whether rCSF-1 formed a homodimer before interacting with its receptor and to see if rCSF-1 and the sCSF-1R interacted in vitro, 100 µg of each protein was incubated in de-ionized water, pH 7.5, for 60 min. The protein mixture was then cross-linked using bis(sulfosuccinimidyl) suberate (BS3) for 15 min and then run on a reducing SDS-polyacrylamide gel. The proteins were then transferred to nitrocellulose membrane (Bio-Rad) and probed using horse-radish peroxidase-conjugated avidin (Pierce). The blot was...
RESULTS

Analysis of Goldfish CSF-1 Transcript and Predicted Peptide Sequences—The expression of goldfish CSF-1 in tissues and in sorted progenitor cells, monocytes, and macrophages was assessed using quantitative real time PCR. Goldfish CSF-1 was strongly expressed in the spleen compared with other tissues (Fig. 1A). Nonstimulated sorted goldfish monocytes were observed to express CSF-1 almost 4-fold more than sorted progenitor cells or sorted macrophages. Interestingly, this expression was increased an additional 7-fold compared with that observed in progenitor cells and macrophages after treatment with 10 ng/ml PMA. PMA was found to significantly increase the expression of CSF-1 message in monocytes by almost 2-fold 12 h after treatment, but had no significant effect on the CSF-1 expression in progenitor cells or macrophages (Fig. 1B). The predicted goldfish CSF-1 peptide was 199 amino acids and featured a secretion signal peptide cleaved at amino acid 29 (Fig. 2A). The predicted goldfish CSF-1 amino acid sequence (GenBank™ accession number EU045335) shared its highest amino acid identity with a putative zebrafish CSF-1 (XP001343870) at 89% and a putative trout CSF-1 (CAD88593) at 65%. The amino acid identity between goldfish and human CSF-1 (BAD92189) was only 27%. Importantly, cysteine residues responsible for the formation of the intra-chain and inter-chain disulfide bonds required for functional CSF-1 in mammals were present in the goldfish sequence. Although these cysteine residues were not in the same positions as the cysteines of the human CSF-1, the distance between the residues was almost identical between the goldfish and human sequences (Fig. 2A). RACE PCR identified three nucleotide sequence variants that differed...
in the 3'-untranslated region (Fig. 2B). The most common 
variant sequence occurring in 43 of 60 3'-RACE clones 
was submitted to GenBank™. The full nucleotide sequence of all 
variants contained TATA box, poly(A) signal, and poly(A) 
tail (Fig. 2B).

**Goldfish rCSF-1 Binds to Recombinant Goldfish sCSF-1 Receptor**—One hundred μg of biotinylated goldfish rCSF-1 and 
sCSF-1R were run on an SDS gel in conjunction with rCSF-1 
and sCSF-1R cross-linked using BS3. The gel was transferred to 
nitrocellulose membrane and developed using horseradish per-
oxidase-conjugated avidin and ECL. The sCSF-1R ran at ~31 
kDa as a doublet likely resulting from differing glycosylation of 
the soluble receptor (Fig. 3A) and the rCSF-1 at ~26 kDa (Fig. 
3B). In the cross-linked sample of rCSF-1, two distinct bands 
were observed likely representing both monomeric rCSF-1 as 
well as the homodimeric form, which was ~50 kDa (Fig. 3C). In 
the sample containing cross-linked rCSF-1 and sCSF-1R, mul-
tiple bands were observed, and bands representing unbound 
rCSF-1 and sCSF-1R were seen at the predicted Mr, and a band 
representing homodimeric rCSF-1 was observed as well as a 
larger band at ~90 kDa, which represented sCSF-1R bound to 
the homodimeric form of rCSF-1.

**Goldfish rCSF-1-induced Differentiation of Goldfish Monocyte into Macrophages**—Recombinant goldfish CSF-1 was 
added to sorted goldfish progenitor cells, monocytes, and mac-
rophages 2 days post-cultivation at concentrations of 50, 25, 10, 
5, and 1 ng/ml. Cell growth was monitored each day by flow 
cytometry, and rCSF-1-treated cells were compared with cells 
treated with CCM (positive control), elution buffer, and non-
treated cultures. Progenitor cells and macrophages were not 
affected by the addition of rCSF-1. However, sorted monocytes 
were induced to differentiate into mature macrophages much 
earlier than those incubated in the presence of CCM. The rCSF-
1-induced transition of monocytes to macrophages was 
observed as early as day 2 post-treatment, which was more than 
3 days earlier than cultures treated with CCM (Fig. 4).

**Goldfish rCSF-1-induced Proliferation of Sorted Goldfish Monocytes and Macrophages**—50, 25, 10, 5, and 1 ng/ml of 
rCSF-1 were used to assess the capacity of goldfish rCSF-1 to 
induce proliferation of sorted goldfish progenitor cells, mon-
ocytes, and macrophages. rCSF-1 induced proliferation in sorted 
monocytes and macrophages but not progenitor cells. The pro-
liferative response was dose-dependent where 1 ng/ml rCSF-1 
induced the lowest response and 10–50 ng/ml induced a strong 
proliferative response. The proliferative response observed in 
the monocyte cultures was normalized by subtracting the val-
ues for nontreated cells from each experimental value for each 
fish. As shown in Fig. 5B, 10 ng/ml of rCSF-1 induced a signifi-
cantly higher proliferative response that the positive control 
(CCM) as determined by one-way analysis of variance (p < 
0.05). In contrast 10 ng/ml rCSF-1 did not induce a significant 
proliferative response in sorted mature macrophages, although 
a consistently higher proliferation of mature macrophages was 
observed from day 2 of cultivation onward (Fig. 5C). Although 
CCM induced a stronger proliferative response in sorted pro-
genitor cells when compared with any of the rCSF-1 treatment 
groups, the addition of 10, 25, and 50 ng/ml rCSF-1 induced a 
proliferative response in progenitor cell cultures starting on day 
4 post-addition of rCSF-1 (Fig. 5A). CSF-1 elution buffer (neg-
ative control) did not induce or inhibit the proliferative 
response in any of the experiments.

Preincubation of sCSF-1R with the rCSF-1 at a ratio of 50 
ng/ml sCSF-1R to 10 ng/ml rCSF-1 abrogated the proliferative 
response observed in the sorted monocyte and macrophage 
panels compared with those treated with rCSF-1 alone 
(Fig. 5, A–C). Addition of the sCSF-1R to the cultures treated 
with CCM inhibited proliferation, however not to the extent 
seen in the rCSF-1-treated cultures. Treatment of sorted pro-
genitor cells, monocytes, and macrophages with 50 ng/ml 
sCSF-1R alone had no significant effect on cell proliferation 
(Fig. 5, A–C).
The proliferative response induced by rCSF-1 in sorted monocytes and macrophages was also inhibited by preincubation of the sorted cells by the affinity-purified rabbit anti-CSF-1R IgG, which recognized the first two extracellular Ig binding domains of the CSF-1R. The addition of 10, 50, 100, or 250 ng/ml anti-CSF-1R IgG to sorted progenitor cells (Fig. 6A) and monocyte (Fig. 6B), or macrophage cultures (Fig. 6C) for
2 h at room temperature before addition of ligand (rCSF-1) resulted in a dose-dependent inhibition of cell proliferation. Maximal inhibition was observed after addition of 100 ng/ml anti-CSF-1R. Addition of 100 ng/ml anti-CSF-1R by itself to cells had no effect on cell proliferation when compared with untreated cells (Fig. 6, A–C).

DISCUSSION

In this study we described the identification and functional characterization of a fish CSF-1 molecule, which was the first description for this molecule in lower vertebrates. Goldfish CSF-1 has 199 amino acids and was significantly smaller than the mammalian CSF-1 isoforms (secreted glycoprotein or the secreted/matrix bound proteoglycan form of mammalian CSF-1) and similar to the membrane-bound glycosylated form (20). Interestingly, all of these mammalian forms were shown to be functional where the first 150 amino acids were required for proper folding and function (21).

Goldfish CSF-1 was most similar to zebrafish and trout CSF-1 molecules that have been identified recently. It shared the most sequence identity and structure with zebrafish CSF-1, which was the same size as goldfish CSF-1 and had an identical cysteine spacing pattern. Goldfish CSF-1 was highly expressed in the spleen, suggesting that the spleen may be a primary site of macrophage/monocyte development. Although the kidney appears to be the primary location of hematopoiesis in bony fishes (12, 19, 22, 23), it is possible that nondifferentiated cells enter the circulation and are directed down the final steps of the myeloid pathway in the spleen or by the CSF-1 synthesized in the spleen. When we examined the CSF-1 expression in sorted goldfish progenitor cells, monocytes, and macrophages, we found it to be highly expressed in the monocyte subpopulation. It has been demonstrated that fish monocytes/macrophages produce their own autocrine/paracrine growth factors (12, 24, 25), and the capacity of monocyte-like cells to produce CSF-1 was further evidence toward this hypothesis. Furthermore, mammalian CSF-1 has been shown to be synthesized by monocytes following activation with lipopolysaccharide or PMA (26). Similarly, the treatment of sorted goldfish progenitor cells, monocytes, and macrophages and subsequent treatment of these cells with PMA resulted in an increase in CSF-1 expression in the monocyte subpopulation.

The proliferative effects of goldfish rCSF-1 were blocked by treatment with an antibody generated against the first two immunoglobulin binding domains of the CSF-1R. These regions have been shown to be required for CSF-1 functioning in mammals (27), and in sufficient amounts the affinity-purified anti-CSF-1R IgG antibody abrogated proliferation of cells induced by rCSF-1. Interestingly, the proliferative effect induced by rCSF-1 was also abrogated by addition of a recombinant goldfish sCSF-1R. We have shown that the addition of sCSF-1R to actively growing goldfish macrophage cultures inhibited cell proliferation as measured by BrdUrd incorporation (14). The ability of sCSF-1R to dose-dependently inhibit proliferation induced by rCSF-1 and the ability of rCSF-1 to bind to sCSF-1R suggest a novel mechanism of self-regulation of macrophage development in fish.

Goldfish CSF-1 represents the first CSF-1 identified and characterized in nonmammalian organisms and is the first colony-stimulating factor to be functionally characterized in fish. Although goldfish CSF-1 is significantly smaller than mammalian CSF-1, it featured the required amino acids in the right positions to form a functional peptide. We also demonstrated that goldfish CSF-1 was functionally similar to the mammalian CSF-1 because it induced the differentiation and proliferation of fish mononuclear cells by binding to the CSF-1R.

Although goldfish CSF-1 appears to act in a very similar manner to mammalian CSF-1, it should be noted that its effects may not be regulated by similar mechanisms. Mammalian CSF-1 was shown to be rapidly removed from the circulation by sinusoidal macrophages, primarily by Kupffer cells in the liver. CSF-1 binds to the CSF-1R and the entire complex is internalized and destroyed. Thus, CSF-1 levels were shown to be regulated by the number of macrophages in the sinuses (28). We do not know whether this internalization process may be the regulation mechanism for CSF-1 in fish. However, we identified a soluble CSF-1 receptor (14) and showed that it bound CSF-1 with sufficient affinity to abrogate the proliferative effects mediated by the ligand. This novel soluble receptor regulation of CSF-1 function may be a different mechanism by which cells of the monocytic lineage in fish regulate their own development.

REFERENCES

1. Stanley, E. R. (1985) Methods Enzymol. 116, 564–587
2. Motoyoshi, K. (1998) Int. J. Hematol. 67, 109–122
3. Pampfer, S., Arceci, R. J., and Pollard, J. W. (1991) BioEssays 13, 535–540
4. Pixley, F. J., and Stanley, E. R. (2004) Trends Cell Biol. 14, 628–638
5. Guilbert, L. J., and Stanley, E. R. (1980) J. Cell Biol. 85, 153–159
6. Sherr, C. J., Ashmun, R. A., Downing, J. R., Obitsuka, M., Quan, S. G., Golde, D. W., and Roussel, M. F. (1989) Blood 73, 1786–1793
7. Sherr, C. J., Rettenmier, C. W., and Roussel, M. F. (1988) Cold Spring Harbor Symp. Quant. Biol. 53, 521–530
8. Hwang, Z. E., Myles, G. M., Brandt, C. S., Lioubin, M. N., and Rohrschneider, L. (1993) Mol. Cell. Biol. 13, 5348–5359
9. Oshibe, T., Kitamura, I., Tanaka, K., Baba, T., Kodama, H., Mukamoto, M., and Tsuji, S. (1999) J. Vet. Med. 46, 389–398
10. Shao, X., Kikuchi, K., Watari, E., Norose, Y., Araki, T., and Yokomuro, K. (1996) Reprod. Fertil. Dev. 8, 103–109
11. Belosevic, M., Hanington, P. C., and Barreda, D. R. (2006) Fish Shellfish Immunol. 20, 152–171
12. Neumann, N. F., Barreda, D., and Belosevic, M. (1998) Dev. Comp. Immunol. 22, 417–432
13. Barreda, D. R., and Belosevic, M. (2001) Fish Shellfish Immunol. 11, 169–185
14. Barreda, D. R., Hanington, P. C., Stafford, J. L., and Belosevic, M. (2003) Dev. Comp. Immunol. 29, 879–894
15. Williams, H., Brenner, S., and Venkatesh, B. (2002) Gene (Amst.) 295, 255–264
16. Honda, T., Nishizawa, T., Uenohe, M., Kohchi, C., Kuroda, A., Ootake, M., Nakanishi, T., Yokomizo, Y., Takahashi, Y., Inagawa, H., and Soma, G. (2005) Mol. Immunol. 42, 1–8
17. Parichy, D. M., and Turner, J. M. (2003) Development (Camb.) 130, 817–833
18. Roca, F. J., Sepulcre, M. A., Lopez-Castejon, G., Meseguer, J., and Mulero, V. (2006) Mol. Immunol. 43, 1418–1423
19. Neumann, N. F., Barreda, D. R., and Belosevic, M. (2000) Fish Shellfish Immunol. 10, 1–20
20. Shadle, P. J., Aldwin, L., Nitecki, D. E., and Koths, K. (1989) *J. Cell. Biochem.* **40**, 91–107
21. Koths, K. (1997) *Mol. Reprod. Dev.* **46**, 31–38
22. Kobayashi, I., Moritomo, T., Ototake, M., and Nakanishi, T. (2007) *Dev. Comp. Immunol.* **31**, 696–707
23. Kobayashi, I., Sekiya, M., Moritomo, T., Ototake, M., and Nakanishi, T. (2006) *Dev. Comp. Immunol.* **30**, 1034–1046
24. Hanington, P. C., Barreda, D. R., and Belosevic, M. (2006) *J. Biol. Chem.* **281**, 9963–9970
25. Hanington, P. C., and Belosevic, M. (2005) *Fish Shellfish Immunol.* **18**, 359–369
26. Becker, S., Warren, M. K., and Haskill, S. (1987) *J. Immunol.* **139**, 3703–3709
27. Stanley, E. R., Berg, K. L., Einstein, D. B., Lee, P. S., Pixley, F. J., Wang, Y., and Yeung, Y. G. (1997) *Mol. Reprod. Dev.* **46**, 4–10
28. Bartocci, A., Mastrogiannis, D. S., Migliorati, G., Stockert, R. J., Wolkoff, A. W., and Stanley, E. R. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6179–6183