A NOVEL HEMATOPOIETIC GRANULIN INDUCES PROLIFERATION OF GOLDFISH (Carassius auratus L.) MACROPHAGES*

Patrick C. Hanington¹, Daniel R. Barreda³, and Miodrag Belosevic¹,²

From the ¹Departments of Biological Sciences and ²Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9, and ³Department of Hematology and Oncology, University of Pennsylvania, Philadelphia, PA, 19104 USA

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Address correspondence to: Miodrag Belosevic, Department of Biological Sciences, CW-405 Biological Sciences Building, University of Alberta, Edmonton, AB T6G 2E9 Canada. Tel: 780 492-1266; Fax: 780 492-9234; E-mail: mike.belosevic@ualberta.ca

Granulins are a group of highly conserved growth factors that have been described from a variety of organisms spanning the metazoa. In this study, goldfish granulin was one of the most commonly identified transcript in the differential cross screening of macrophage cDNA libraries, and was preferentially expressed in proliferating macrophages. Unlike mammalian granulins, which possess 7.5 repeats of a characteristic signature of 12 cysteine residues, the goldfish granulin encoded a putative peptide possessing only one and one half cysteine repeats. Northern blot and real-time PCR analyses indicated that goldfish granulin was expressed only in the hematopoietic tissues of the goldfish, specifically the kidney and spleen, and activated peripheral blood mononuclear cells. We expressed granulin using a prokaryotic expression system and produced an affinity-purified rabbit anti-goldfish granulin IgG. Recombinant goldfish granulin induced a dose-dependent proliferative response of goldfish macrophages that was inversely related to the myeloid differentiation stage of the cells studied. The highest proliferative response was observed in macrophage progenitor cells and monocytes. This proliferative response of macrophages was abrogated by the addition of anti-granulin IgG. These results indicate that goldfish granulin is a growth factor that positively modulates cell proliferation at distinct junctures of macrophage differentiation.

First identified as small (6 kDa) peptides, granulins are produced by the proteolysis of a larger precursor molecule by leukocyte derived elastase activity [1-7]. The larger precursor is known by several names, including granulin/epithelin precursor [8], proepithelin [9], acrogranin [10], PC cell derived growth factor (PCDGF) [11] and progranulin. Granulins have a unique 12 cysteine motif that is arranged in 4 β-hairpins, stacked one upon another in a helical formation and connected via a central rod connected by disulphide bonds [12-14]. Structurally, granulins are distinct from most growth factors, with exception of the epidermal growth factor/transforming growth factor-alpha family [13]. In addition to the mammalian granulins of human [1,6], rat [2,12], mouse [10,15] and horse [16], granulin-like proteins have been identified in a number of non-mammalian organisms including the nematode Caenorhabditis elegans [17], the locust [18], the mussel [19], the marine worm Hediste diversicolor [20], and bony fishes [21-23]. Granulin-like motifs have also been identified in multiple thiol protease gene sequences from plants [24-25].

The mammalian progranulin genes are ubiquitously expressed in various tissues [9-10, 26-29], and have been detected in epithelial and hematopoietic cell lines [26-29] and neoplastic cells [30-36]. Progranulin was shown to be highly expressed in epithelial cells that exhibit rapid turnover, such as the columnar epithelium of the gastrointestinal tract [29], and the cells of the immune and nervous systems [21-22,37].

In general granulin gene sequences that encode for functional peptides are progranulin genes. There are a number of published granulin-like sequences identified in lower vertebrates as well as invertebrates (e.g. zebrafish, GenBank AF273479 and AF273480). Although a number
of granulin genes have been identified in lower vertebrates and invertebrates, many of the peptides encoded by these genes have yet to be functionally characterized.

We report on a unique granulin-like gene of the goldfish. Northern blot, real-time PCR and RT-PCR analyses revealed that this granulin gene was exclusively expressed in the hematopoietic tissues of the goldfish. Recombinant goldfish granulin induced a dose-dependent proliferation of primary goldfish macrophages in vitro, which was abrogated by an affinity-purified anti-granulin IgG. Our findings indicate that granulin was present in macrophage culture supernatants and that it promoted growth of cells at discrete stages of myeloid differentiation pathway.

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 three weeks prior to use in experiments.

Isolation of primary macrophages from goldfish and RNA isolation - Isolation of goldfish kidney leukocytes, and the generation of primary kidney macrophages (PKM) and peripheral blood mononuclear cells were performed as previously described [38-42]. The kinetics of PKM growth in culture were similar to those reported for mammalian macrophages derived from bone marrow cultures in the presence of conditioned medium from the L-929 fibroblast cell line [43]. Three distinct macrophage subpopulations are a feature of PKM cultures: the early progenitors, the monocytes and mature macrophages [44-45]. PKM cultures were incubated at 20°C until the cells were at a stage of active proliferation (proliferative phase) or a non-proliferative stage (senescence phase) typically 6 and 10 days post cultivation, respectively. PKM from proliferative and senescence phases were isolated, flash frozen using liquid nitrogen, and stored at -80°C until used. The mRNA for the two macrophage subpopulations was isolated using Trizol™ reagent (Gibco BRL) and the Oligotex mRNA isolation kit (Qiagen) according to the manufacturer’s specifications.

Generation of macrophage activating factor supernatants- Macrophage activating factor (MAF) supernatants were prepared using protocols described previously [41]. These supernatants contain a complex mixture of factors that have been functionally characterized and shown to induce antimicrobial responses of goldfish macrophages [41-42].

Construction of cDNA libraries of primary kidney macrophages of goldfish - Complementary DNA libraries were constructed from 2 μg of proliferative or senescence phase PKM poly (A)+ RNA by directional ligation of PKM cDNA into lambda ZAP bacteriophage using a ZAP cDNA synthesis kit, and the ZAP-cDNA Gigapack III Gold cloning kit (Stratagene), as described previously [46]. Non-amplified PKM proliferative and senescence phase cDNA libraries were screened using standard procedures described previously [46]. Following the tertiary PCR-based screen, individual clones were PCR-amplified, sequenced, confirmed to encode for a single-sized insert, and individually stored at 4°C in 500 μL of SM buffer and chloroform.

DNA sequencing and analysis - The PCR amplified clone inserts corresponding to each of the confirmed granulin positive clones were purified using the QIAquick PCR purification kit (Qiagen), and sequenced using a DYEnamic™ ET terminator cycle sequencing kit (Amersham Pharmacia) and a PE Applied Biosystems 377 automated sequencer. Sequences were analyzed using Genetool™ (Biotools) and subsequent gene annotations were conducted using BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/). Conserved motifs were identified, and predictions were based on analytical tools provided in the ExPASy proteomics server (http://www.expasy.org) [47]. Sequence alignments were performed using CLUSTAL X v1.83.

Real-time PCR analysis of granulin expression - Real-time PCR analysis was carried out using the Applied Biosystems 7500 fast real-time PCR
system. The relative expression of goldfish granulin in relation to β-actin was assessed using primers generated with the Primer Express software (Applied Biosystems). The primers used for expression analysis of goldfish granulin were: 5' TTGATGTTACTCATGGCAGCTCTT 3' and 5' GGCCCTGAGAGATCCATCATT 3'. The primers used for expression analysis of goldfish β-actin were: 5' GCACGCGACTGA CACTGAAG 3' and, 5' GAAGGCCGCTCCCGAGGTA 3'. Analysis of the relative tissue expression data from five fish was carried out using the 7500 Fast software (Applied Biosystems).

RT-PCR analysis of goldfish granulin expression in macrophages- Cultured PKM were sorted into early progenitor, monocyte and mature macrophage subpopulations using a FACS Calibur flow cytometer (Becton/Dickinson) as described previously [39, 44-45], and the RNA isolated immediately after sorting. First-strand synthesis was done using an oligo dT primer (Stratagene, La Jolla, CA), 2.5 μg total RNA according to manufacturer’s protocols. The primers used to amplify goldfish granulin by RT-PCR were: sense 5‘-AAGATGGTTCCAGTGTTGATGTTAC-3’, antisense 5‘-ACCCCACTGGCCGGCTGCTGT - 3’. northern blot analysis - Twenty five μg of total RNA was subjected to electrophoresis on a 1.5% agarose, 20% formaldehyde gel and transferred overnight to Genescreen Plus nylon membranes (NEN Research). Blots were screened using 200 ng of a goldfish granulin probe created using RT-PCR. The probe was singly labeled using α-32P-dCTP and purified using QIAquick gel extraction columns (Qiagen). Hybridization with the probe was allowed to proceed overnight at 42°C, and then washed 3 X with 2x SSC, 0.1% SDS for 5 min. each, and 0.1x SSC, 0.1% SDS 3X for 20 minutes. Blots were then exposed to Kodak X-OMAT film and stored at -80°C for 24 hours before being developed.

Prokaryotic expression of goldfish granulin - Goldfish granulin was expressed using a prokaryotic protein expression system. PCR amplification of the protein expression construct insert was performed as follows: 7 μL of the granulin clone template was added to 76 μL ddH2O, dNTPs (0.2 μL of each dATP, dCTP, dGTP, dTTP 100 mM solutions), 10x PCR buffer (10 μL of 100 mM Tris-HCl pH8.3, 500 mM KCl, 15 mM MgCl2, 0.01% (w/v) gelatin), expression primers (2.4 μL of each 20 μM solution: sense 5’-CACCCCTCATGGCAGCTCTTGTAG – 3’ antisense 5’-ACGGGGTTGT TTACTTAC – 3’, and a 15:1 ratio of Taq/Pfu DNA polymerases (1 μL of 5 unit solution). PCR amplification was conducted in an Eppendorf Mastercycler Gradient thermal cycler. Amplification was confirmed by agarose gel electrophoresis.

The granulin amplicon was cloned into the pET SUMO TA expression vector (Invitrogen) and transformed into chemically competent TOP10 E. coli (Invitrogen) according to the manufacturer’s specifications. Cells were plated onto LB-Ampicillin (100 μg/mL) plates and incubated overnight at 37°C. Randomly selected colonies were amplified by PCR and positive clones were grown overnight in 5 mL of LB medium containing 100 μg/mL. Ampicillin and plasmids were isolated using a QIAPrep Spin Miniprep kit (Qiagen). Once positive clones were isolated, restriction digests followed by gel electrophoresis verified the presence of insert and vector DNA. Plasmids were sequenced, as described above, in order to confirm that inserts were ligated into the expression vector in the proper orientation and in frame. Sequence data were analyzed using Genetool (Biotools).

Production of recombinant granulin - Plasmid DNA containing the granulin expression construct was transformed into BL21 Star™ (DE3) One Shot® E. coli (Invitrogen) for recombinant protein expression. 10 ng of plasmid DNA was transformed into the bacteria which was then grown overnight at 37°C in LB medium containing 50μg/mL kanamycin. Induction of recombinant protein expression was performed in a pilot expression experiment by the addition of IPTG according to the manufacturer’s protocols. The expression of recombinant granulin was evaluated after 1, 2, 4, and 6 h post-induction with IPTG. Individual samples were then analyzed by SDS-PAGE and Western blotting for the presence of recombinant protein expression.
For large-scale expression and purification of the target proteins, 50 mL of LB medium containing 100 μg/mL carbenicillin was grown overnight at 30°C with shaking to an O.D.600 of ~1.0 to 2.0. Ten milliliters of this culture was then inoculated into 250 mL of LB (100 μg/mL carbenicillin) and a total of 4 flasks were prepared (1 L total). Cultures were incubated until mid-log phase of growth was achieved followed by the induction of target protein expression with 0.1 mM IPTG. Cultures were then grown for 2 h prior to the purification of the recombinant molecules.

Recombinant granulin was engineered to contain a N-terminal 6xHis tag to facilitate subsequent detection and purification. Bacteria were removed by centrifugation at 2000 x g and supernatants were collected. Granulin was purified from 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 imidazole, and then dialyzed overnight against 1X PBS. Protein samples were then filter-sterilized in preparation for immunodetection and analysis of biological activity. Total protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce) according to the manufacturer’s protocols.

**Immunodetection of recombinant goldfish granulin** - rgfGrn was used as a source of antigen for rabbit immunizations. The primary immunization was performed by combining an equal volume of purified recombinant granulin (100 μg), with 750 μL of Freund’s Complete Adjuvant. Booster injections were done exactly as the primary immunizations but substituted with Freund’s Incomplete Adjuvant. The IgG fraction was purified by precipitation using saturated ammonium sulphate, solubilization of precipitate in PBS, and purification using a HiTrap Protein A HP column (Amersham) according to the manufacturer’s protocol. Fractions containing IgG were pooled and filter-sterilized (0.22 μm filter; Millipore). The specificity of the antibody was determined by immunoblot using as a target rgfGrn under native and denaturing conditions.

rgfGrn and native goldfish granulin (4X concentrated macrophage culture supernatants) were detected by immunoblot analysis using an anti-6x His mAb (Invitrogen), or with affinity-purified rabbit anti-goldfish rgfGrn IgG. Briefly, proteins were separated by SDS-PAGE under reducing conditions using 12.5% polyacrylamide gels, transferred to 0.2 μm nitrocellulose membranes (BioRad), and incubated overnight at 4°C in the presence of the primary antibody. Membranes were subsequently washed, incubated with an horseradish peroxidase-conjugated monoclonal antibody (mAb), and developed using the ECL Advance™ Western Blotting Detection Kit (Amersham Biosciences) according to the manufacturer’s specifications.

**Induction of proliferation of macrophages by recombinant granulin** - PKM cultures were established, distinct differentiation stages sorted by FACS, and seeded at a density of 1 x 10^4 cells well^−1 in 96-well culture plates (Falcon). Cells were seeded in 50 μL of complete culture medium and treated with 50 μL of 5, 50, 100, 250 and 500 ng of recombinant goldfish granulin in incomplete cell culture medium and incubated for 52 hr at 20°C. Fifteen μL/well of BrdU labeling reagent (Roche) was added and cells were incubated for an additional 24 h at 20°C. The reaction was developed according to the manufacturer’s specifications and optical densities determined at 450 nm using a microplate spectrophotometer (Biotek). The colorimetric reaction was directly proportional to the number of proliferating PKM in culture (data not shown). The induction of macrophage proliferation by rgfGrn was determined after addition of different amounts (1, 10, 50, 100, 300, 500 ng) of anti-rgfGrn to cultures.

**RESULTS**

The most common transcript identified in differential cross screening of proliferative and senescence phase goldfish macrophage cDNA libraries was granulin (Fig. 1). Thirty one partial granulin-like transcripts were identified, and all exhibited higher expression in proliferating macrophages. All of the transcripts were sequenced and were found to be identical. The fully sequenced cDNA transcript of goldfish granulin is 947 nucleotides in length with an open reading frame of 477 nucleotides. The predicted protein is 159 amino acids long and had 18
conserved cysteine residues, 12 of which represent a full granulin cysteine motif common for all known granulin proteins. The remaining 6 cysteine residues make up one half of this motif (Fig. 2). The granulin sequence has been submitted to GenBank (Accession No. DQ369750).

The predicted goldfish granulin protein possessed conserved amino acids found in granulins spanning the metazoans. Granulins have been identified in mammals, fish, insects, bivalves and nematodes. The amino acid sequence alignment of goldfish granulin and other known fish granulins of carp, zebrafish and goldfish intestine show highly conserved cysteine rich motifs (Fig. 3B). Goldfish granulin was most similar to carp granulin 3, with an amino acid identity of 56%. Of all the granulins analyzed, goldfish granulin shared the highest identity with other fish granulins (Fig. 3A), which was supported by phylogenetic analysis that grouped the goldfish granulin in close proximity to carp granulins 2 and 3 (Fig. 4). Phylogenetic analysis also suggested that all fish granulins shared distinct features that separated them from the granulins of mammals. Although the granulin proteins identified in carp and from goldfish intestine have no corresponding mRNA transcript sequences, zebrafish granulins 1, 2 and zebrafish hybrid granulin, had similar transcript organization to that of goldfish granulin.

The expression of goldfish granulin transcript was analyzed by Northern blot, RT-PCR, and real time PCR. Analysis of transcript expression in the heart, brain, spleen, kidney, gill, liver and intestine revealed that goldfish granulin was expressed primarily in the kidney and the spleen (Fig. 5A & B). Real time PCR and RT-PCR analyses of granulin expression were also done using non-activated and activated macrophages, and sorted goldfish macrophage subpopulations. Goldfish granulin expression was upregulated in activated macrophages and activated peripheral blood mononuclear cells compared to non-activated controls (Fig. 6A & B). Interestingly, goldfish granulin was primarily expressed in monocyte subpopulation, with lower expression evident in mature macrophages and the early progenitor cells (Fig. 6C & D).

To examine the effect(s) of granulin on fish macrophage development in vitro, we generated recombinant goldfish granulin (rgfGrn) using a prokaryotic expression system. The ability of rfgGrn to induce a proliferative response of goldfish macrophages was tested by adding different amounts (5ng – 500ng) of the rfgGrn to newly established cultures of sorted early progenitor cells, monocytes and mature macrophages. The proliferation assays were done using eight separate PKM cultures established from individual fish (n=8). All optical density values were normalized to those of untreated control cells. The proliferation responses depended on the differentiation stage of macrophages treated with recombinant granulin. For example, rfgGrn induced significant proliferative response in progenitor cells (3-fold increase over crude CCM by day 8) (Fig. 7A) and lower but significant proliferation in monocytes cultures (1.6-fold increase over CCM by day 8) (Fig. 7B). In contrast, the mature macrophage subpopulation, did not proliferate in the presence of rfgGrn (Fig. 7C). No increase in proliferation of cultured macrophages was observed in the presence of the vector control (Fig. 7 and Fig. 8A).

The rfgGrn was used to generate an affinity-purified anti-rfgGrn rabbit IgG. The anti-6His (Fig.8B-1) and anti-rfgGrn IgG recognized rfgGrn, (21kD; granulin plus additional vector sequence), as well as an unknown bacterial protein of approximate Mr of 31kD (Fig. 8B-2). Interestingly, the immunoblot analysis using anti-rfgGrn IgG as a probe showed that the native goldfish granulin was present in goldfish macrophage culture supernatants, indicating that the molecule was secreted by actively growing macrophages (Fig 8B-3). The addition of known amounts of anti-rfgGrn IgG abrogated the proliferative response of macrophages in a dose-dependent manner (Fig. 8C).

DISCUSSION

In this study, we report on a novel granulin gene of the goldfish. Granulin was the most common transcript identified during differential cross-screening of the goldfish macrophage proliferative and senescence phase libraries [47]. Granulins were first purified from the extracts of human inflammatory cell exudates, and from rat bone marrow [1]. To date, seven granulin

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peptides (A to G) have been characterized [9-10, 26-27], and it has been shown that they are generated following proteolytic cleavage of progranulin [7]. The two main differences between goldfish granulin and mammalian progranulin, were: (a) the goldfish granulin gene encoded for a much smaller protein (159 aa); and (b) unlike mammalian progranulin which has been shown to be ubiquitously expressed in different tissues, the goldfish granulin expression was limited to hematopoietic tissues (kidney and spleen) and blood mononuclear cells. Furthermore, goldfish granulin was found to be differentially expressed in different macrophage subpopulations, and found to promote growth of macrophages; this was inversely related to their stage of maturation/differentiation.

The presence of granulin proteins in hematopoietic tissues of the carp has been reported; granulin-1 which was found to be mainly in extracts of the spleen, and granulin 1, 2 and 3 which were present in extracts of the head kidney [21]. Furthermore, antibodies generated against carp granulin-1 appeared to recognize mononuclear cells in the head kidney of carp [21-22]. Sequence data of zebrafish granulins (GenBank AF273479 and AF273480) suggests that this fish species possesses two genes that encode granulin proteins. Similar to the goldfish granulin, zebrafish granulin 1 and 2 possess one and one half cysteine repeats, which may be the possible orthologs to goldfish granulin. [49]

Since the expression of goldfish granulin was upregulated in proliferating macrophages, and granulin was present in macrophage culture supernatants, we hypothesized that granulin may play a role in cell proliferation. Indeed, the recombinant granulin induced a significant and dose-dependent proliferative response in early progenitor and monocyte subpopulations in vitro, indicating that this molecule may contribute to the regulation of goldfish macrophage hematopoiesis.

Progranulin was shown to be the only growth factor capable of inducing the proliferation of R-cells in the absence of IGF-1 and platelet derived growth factor [56], through the activation of the p44/42 mitogen-activated protein kinase (MAP kinase) and the phosphatidylinositol 3-kinase (PI3 kinase) pathways and induction of cyclin D1 and cyclin B. Interestingly, these pathways are involved in the signaling cascade for IGF-1 and thus may be the reason why progranulin can act in place of IGF-1 [8, 57-58]. Progranulin was shown to participate in inflammatory responses by inducing cellular migration during wound healing [4, 59-60]. Although the multi-functional nature of the progranulin was well characterized, a receptor for progranulin has yet to be identified.

Progranulin can not only exert its biological effects as an intact protein, but also can generate multiple functions as a result of proteolytic cleavage and production of functional smaller granulin peptides. For example, Epithelin 1/Granulin A (Epi1/GrnA) was shown to induce the proliferation of murine keratinocytes as well as rat kidney cells NRK-SA6 in the presence of transforming growth factor β. However, Epi1/GrnA was also shown to inhibit the DNA synthesis, and thus proliferation of the epidermoid cell line A431 and the human colon carcinoma cell line HCT116 [2, 9]. Interestingly, Epithelin 2/Granulin B (Epi2/GrnB) was shown to antagonize the proliferative effects of Epi1/GrnA, as well as having growth inhibitory effects on A431 cells, albeit not to the same extent as Epi1/GrnA [2, 9].

The structure, distribution and function of the goldfish granulin transcript identified in this study, set it apart from known mammalian granulins. Its obvious association with the hematopoietic organs of the goldfish and upregulation in cells that are undergoing proliferation, suggests that it may be an important growth factor during hematopoiesis in goldfish. Furthermore, the upregulation of granulin expression after activation of macrophages and high expression in monocytes suggests that goldfish granulin, like mammalian granulin, may be involved in inflammation and wound repair events. Whether goldfish granulin can modulate the inflammation and wound healing events is currently under investigation in our laboratory.
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FOOTNOTES

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4 Abbreviations: PKM – primary kidney macrophages, CCM – cell conditioned medium, FACS – fluorescence activated cell sorting, IPTG - isopropyl-beta-D-thiogalactopyranoside, ELISA – enzyme linked immuno-absorbant assay, rgfGRN – recombinant goldfish granulin, IGF-1 – insulin-like growth factor 1, MAP kinase – mitogen activated protein kinase, Epi1 – epithelin 1, Grn1 – granulin 1.

FIGURE LEGENDS

Fig. 1. Macroarray analysis of the differential expression of fourteen distinct granulin transcripts during proliferation (P) and senescence (S) phases of cultured primary macrophages of the goldfish. Numbers indicate different granulin transcripts whose expression was consistently up regulated during the proliferation phase of macrophages.

Fig. 2. cDNA sequence of goldfish granulin with the predicted amino acid translation of the open reading frame. The cysteine residues composing the one and one half granulin motif are underlined (Gen Bank accession No. DQ369750).

Fig. 3. (A) Amino acid alignment of the conserved cysteine area of Xenopus [Xgm (AAV26493)], human (hGRN (AAH105777)), zebrafish (zfGRN, zfGRN1, zfGRN2), goldfish intestinal granulin peptide (gfGRNi), carp (cGRN1, cGRN2, cGRN3), C. elegans (C.elGRNa (CAB54304), C.elGRNb (CAB54305)), and goldfish granulin (gfGRN). (B) Amino acid alignment of the known granulin sequences of fish. zfGRN1 – zebrafish granulin 1 (AAK58708), gfGRN1 – goldfish granulin identified from intestine (AAB47075), cGRN1 – carp granulin 1 (AAB26496), gfGRN – goldfish granulin, cGRN3 – carp granulin 3 (AAB26498), cGRN2 – carp granulin 2 (AAB26497).

Fig. 4. Phylogenetic tree of selected granulin peptides. Goldfish granulin groups closely to carp granulins 3 and 2, which are closely associated with the zebrafish granulins 1, 2 and hybrid as well as carp granulin 1 and the goldfish granulin identified from intestinal exudates. All of the progranulin peptides from Xenopus, human, mouse and rat group closely and all are out grouped by a granulin-like peptides identified in C. elegans. The tree was bootstrapped 10000 times to ensure accuracy. Abbreviations are the same as Fig.3.

Fig. 5. Northern blot (A) of goldfish granulin transcript expression in various tissues (L=liver, K=kidney, H=heart, G=gill, S=spleen, l=intestine, Br=brain). Kidney and spleen show the highest level of transcript expression. 18S ribosomal RNA was used as a loading control. (B) Real-time PCR analysis of granulin expression in different tissues of the goldfish. The data are from five independent experiments (n = 5). Fold difference relative to β-actin expression.

Fig. 6. Real-time (B, D) and RT-PCR (A, C) analysis of granulin expression in goldfish macrophages. A1 and A2 represent granulin expression in non-activated and LPS/MAF-activated goldfish macrophages, respectively. A3 and A4 represent granulin expression in non-activated and LPS/MAF-activated peripheral blood mononuclear cells, respectively. C1, C2 and C3
represent granulin expression in progenitor cells, monocytes and macrophages, respectively. β-actin was used as a loading control in RT-PCR analyses. The data for real time PCR are from five independent experiments (n = 5). RT-PCR data are from a representative experiment of five that were performed.

Fig. 7. The induction of macrophage proliferation by recombinant goldfish granulin (rgfGrn). Sorted progenitor cells (A), monocytes (B) and macrophages (C) were treated with 500 ng of rgfGrn or 500 ng of rgfGrn and 300 ng of anti-rgfGrn antibody and analyzed for their ability to proliferate using a BrdU assay. The OD 450 values were normalized to cells alone controls. The data are from 8 independent experiments (n = 8).

Fig. 8. (A) The proliferative response of goldfish macrophage progenitor cells after addition with known amounts of recombinant goldfish granulin (rgfGrn) compared to cell conditioned medium (CCM) and vector only controls. (B) Immunoblot analysis of rgfGrn probed with anti-6His antibody (B1), rgfGrn probed with anti-rgfGrn IgG (B2), and concentrated macrophage culture supernatant (4X) probed with rgfGrn IgG (B3). (C) The proliferative response of goldfish macrophage progenitor cells exposed to 250 ng of rgfGrn mixed with different amounts of anti-rgfGrn antibody. The data are from 8 independent experiments (n = 8).
Figure 1
FIGURE 2

```
1  acgggtgctagagaattcgccagacgcagcagcagcagaaaaagctataacagcagagaccagacagcagcag
    PVLMLMLMAALVADEPHMDL
76  cca gtt atg tta ctc atg gca gct ctt gta gct gca gat gac cca agt atg gat ctc
   SGPLESDSAVSIIIFCDAST
136  tca ggc cca tta gag tct gac agt ggc tct gtt tct atc atc ttc tgt gat gct tct act
   TCPSTCTCRRSPFGIWYCCP
196  aca tgt cct agc gga aca aag tgc tgt cgt tct ctt tgt att tgg tac tgc tgc cca
   FSNGQCCRPDRGHRCHCRHGYHC
256  ttc tca atg ggt cag tgc aag gtt gna cgc cat tgc tgt cgt cat ggt tct cag tgc
   DASSTLCRLGWLKLPSAEP
316  gat ggc tca tgg acc ctt tgt tgt tgt agg ggg tgg tgg aag ctt cca ctc cct gct ggt gag ccg
   AKAIOPQSVPVIDQALKWK
376  gcc acc aag gct atc cag aac ctt cag tct gtt ccc att gac cag gct ctt aag tgg aag
   SETSEYHCDGNLYCSTEQFC
436  agc gag act gat ctt gtt cat tgt gat gga aat ctc tac tgc tca act gag cag ttc tgt
   CKTAAGQWGCCEMNML
496  tgc aag aca gca ggc ggc cag tgt ggt tgc tgc aat gag atg tgt tgg taa gtaaacaacccc
559  cgtgactttggtgcaggtttttaaaagacttaccaaggtgtaagtttgccacacttcccagatctttcccccttt
639  tgttaattttgtgttatgacactctattagtgtcagcactgtatatcttatataatctatcactccccacttttgggtcatgta
719  ttttaatggaactgttttaaacttttgggtgtcagccgttctttgctttactattctctgtttgctttctgtttgctttctgttt
799  ggaattttaaaaactgtcctaatattacatgtgcaattttactctttttaacctcaaggtgtcaacagcatttttcagaaaaaaacactttaaatagatcctacagcttcacactttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Figure 4
Figure 5

A.

B.
Figure 7

A.  

B.  

C.  

Days post treatment
A novel hematopoietic granulin induces proliferation of goldfish (Carassius auratus L.) macrophages
Patrick C. Hanington, Daniel R. Barreda and Miodrag Belosevic

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