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Accumulation of cells expressing macrophage colony-stimulating factor receptor gene in the ovary of a pregnant viviparous fish, *Neoditrema ransonnetii* (Perciformes, Embiotocidae)

Kazuki Ueda [a], Erina Saito [a], Kaoru Iwasaki [a], Shigeyuki Tsutsui [a], Aoi Nozawa [b], Kiyoshi Kikuchi [b], Osamu Nakamura [a, *]

[a] *School of Marine Biosciences, Kitasato University, Kanagawa, 252-0373, Japan*
[b] *Fisheries Laboratory, the University of Tokyo, Shizuoka, 431-0214, Japan*

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A B S T R A C T

Macrophage colony-stimulating factor receptor (M-CSFR), a member of the group of type III protein tyrosine kinase receptors, is expressed primarily by monocyte/macrophage lineage cells. In order to describe the distribution of macrophages at the maternal-fetal interface in *Neoditrema ransonnetii*, a viviparous fish species, M-CSFR cDNA was sequenced. Two sequences were obtained: NrM-CSFR1 (4381 bp, encoding 980 amino acids), and NrM-CSFR2 (3573 bp, encoding 1016 amino acids). Both the genes were expressed in the ovary of pregnant females. *In situ* hybridization revealed that a number of cells that were positive for NrM-CSFR1 and/or NrM-CSFR2 populated the ovigerous lamellae of the ovary during pregnancy. Following parturition, M-CSFR-positive cells disappeared from the subepithelial region of ovigerous lamellae, and were localized in perivascular tissues. These results suggest the role of M-CSFR-positive cells, which appear to be macrophages, in *N. ransonnetii* during pregnancy.

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1. Introduction

Viviparity is a mode of reproduction by which internal fertilization is followed by fetal development within the maternal organ. Viviparous species are widely distributed among vertebrates with the exception of birds and agnathan fish. In teleosts, approximately 500 viviparous species have been reported [27].

Viviparity raises an immunological conundrum, in that during pregnancy, two genetically distinct individuals must coexist. A strict allorecognition mechanism, based primarily on the interaction between T cell receptor (TCR) and major histocompatibility complex (MHC) molecules, has developed in vertebrates. This mechanism induces potent cytotoxic activity against individuals, tissues, or cells that express non-self MHC molecules on their surface. Therefore, viviparous vertebrates are confronted with a conflict between intra-maternal fertilization and gestation, and the immune response against spermatozoa and fetuses expressing paternal MHC molecules. To evade this conflict, viviparity requires highly sophisticated mechanisms that modulate the immune response at the maternal–fetal interface.

A large number of studies have addressed this conundrum, and revealed multiple mechanisms that contribute to successful pregnancy in mammalian species. These include suppression of human leucocyte antigen (HLA) class I expression on the trophoblast and elevated expression of a non-classical MHC molecule, HLA-G, instead [16]; and, secretion of immune-modulating substances such as interleukin-10 (IL-10), transforming growth factor beta (TGF-β), and prostaglandin E_2 (PGE_2) [12,22].

Maternal immune cells that populate the tissues at the maternal–fetal interface are highly specialized in their composition and function. In humans, these cells are primarily natural killer (NK) cells and macrophages. Small numbers of T cells and dendritic cells and, very rarely, B cells, are also found [4,24]. Decidual macrophages are thought to contribute to an immunosuppressive environment at the maternal–fetal interface because they secrete multiple immunosuppressive agents such as IL-10, PGE_2, and tryptophan metabolites, which suppress T cell proliferation. Decidual macrophages also contribute to vascular remodeling

* Corresponding author.
E-mail address: naosamu@kitasato-u.ac.jp (O. Nakamura).

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through the release of pro-angiogenic factors such as vascular endothelial growth factor and matrix metalloproteinases [14].

In contrast to what is known for mammals, immune-modulating mechanisms that facilitate gestation in non-mammalian viviparous vertebrates remain largely unexplored. Fish have a highly developed immune system that can recognize allografts and induce potent cytotoxic activities [9] [5]). Therefore, immune-modulating mechanisms supporting pregnancy must exist in viviparous fish.

Embiotocid fish, also called surfperch, are a representative viviparous fish species that have a long gestation period (more than six months), and full dependence of fetal growth on maternally delivered nutrients. In *Neoditrema ransonnetii* on the coast of Iwate, a northern part of Japan, internal fertilization occurs between December and January. During gestation in this species, fetuses take up maternally-supplied ovarian cavity fluid (OCF), and are born between late July and August. OCF appears to play significant roles in pregnancy, not only by supplying nutrients but also by modulating immune responses. We have previously demonstrated that OCF of *N. ransonnetii* suppresses both proliferation of lymphocytes [18], and cytotoxic activity of renal leucocytes [28]. These findings indicate that OCF contains immune-modulating molecules that may be indispensable to pregnancy success. For example, PGE_2 contained in OCF can suppress lymphocyte proliferation [18]. Other molecules contained within OCF, and the sources of these molecules, are yet to be identified, but ovarian macrophages may play a crucial role.

In order to explore distribution of macrophages in the ovary of a pregnant viviparous fish, we obtained cDNA sequences of macrophage colony-stimulating factor receptor (M-SCFR), also known as CSF1R or CD115, in *N. ransonnetii*. M-CSFR is a member of the type III protein tyrosine kinase receptor group, which also includes platelet-derived growth factor receptor (PDGFR), proto-oncogene protein Kit [29], and FLT3/flk2 (fms-like tyrosine kinase). M-CSFR is a receptor for M-CSF, a protein required for the differentiation and development of monocytes and macrophages [20]. IL-34 is the alternative ligand for M-CSFR, recently identified in mammals and birds [6,13].

It is thought that, in fish, M-CSFR is primarily expressed by monocyte/macrophage cell lineages [11,17].

In the current study, we observed that a large number of M-CSFR-positive cells accumulated beneath the epithelia of ovigerous lamellae in pregnant *N. ransonnetii*. This massive accumulation of cells disappeared following parturition, indicating that macrophages contribute to sustaining pregnancy, probably through the release of immune-modulating substances.

2. Materials and methods

2.1. Fish

Fish were caught by angling at Okirai Bay, Iwate, Japan, and kept in tanks with running seawater under natural environmental conditions. Fish were fed *ad libitum* with a commercial marine fish diet (Misaki, Nihon Nosan Kogyo, Japan).

2.2. cDNA sequencing and analysis

One pregnant female was anesthetized with 2-phenoxyethanol and a laparotomy was performed and the head kidney removed. Total RNA was obtained from this tissue using RNA extraction reagent (ISOGEN2, Nippon Gene) according to the manufacturer's instructions. cDNA was synthesized from 0.5 μg of total RNA, using SMART™ RACE cDNA Amplification Kit (Clontech).

Degenerate primers for M-CSFR were designed based on sequences obtained from other fish species. To clone the cDNA of M-CSFR, the degenerate primer pair M-CSFR-F (5′-CCNTAYAAY-GARAARTGGGA-3′) and M-CSFR-R (5′-CCRAARTCRCADATYTTNGC-3′) was used. Amplification was carried out using SP-Taq polymerase (Labo Pass) under the following conditions: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 50 °C for 30 s, then 72 °C for 30 s; and, 72 °C for 7 min. The amplicon was ligated into a vector (pGEM-T easy, Promega), introduced into competent cells, and sequenced using Big-Dye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems) and a DNA sequencer (3100-Avant, Applied Biosystems). Based on the obtained sequence, specific primers were designed and paired with the degenerate primers designed from sequences obtained from other fish; PCR was carried out in order to clone the extended cDNA sequences. Finally, 5′ and 3′ RACE PCR was carried out, using specific primers and an adapter primer (NUP-A, Clontech). All primer sequences are listed in the supplementary data (Table A.1).

2.3. Gene expression analysis

In June, approximately one month prior to parturition, three pregnant females, each weighing around 40 g, were euthanized with an overdose of 2-phenoxyethanol. Blood was collected and a laparotomy performed, and the following tissues were collected: brain, thymus, skin, gill, liver, spleen, fore gut, hind gut, head kidney, trunk kidney, and ovary. OCF was collected using a pipette, and centrifuged. The precipitates were recovered as ovarian cavity leucocytes (OCLs).

RNA extraction and cDNA synthesis were carried out as described in section 2.2 above. Genomic DNA of *N. ransonnetii* was also prepared from the ovary using NucleoSpin® Tissue (Macherey-Nagel). Reverse transcription PCRs for NrM-CSFR1 and NrM-CSFR2 were performed using specific primer pairs for each transcript, which were located on non-conserved positions. The primer sequences and PCR cycles used in the current study are listed in the supplementary data (Table A.1).

2.4. In situ hybridization

Sense and antisense DNA probes for NrM-CSFR1 and NrM-CSFR-2 were designed in non-conserved regions of these transcripts (Table A.2). Digoxigenin-conjugated DNA probes were synthesized by a commercial manufacturer (Operon Technologies).

Ovaries were obtained from pregnant (sampled in July) and post-pregnant (sampled in September) fish, following anesthesia with 2-phenoxyethanol. Tissues were fixed in 4% paraformaldehyde at 4 °C overnight, dehydrated using a series of ethanol baths, replaced with xylene, embedded in paraffin, and sectioned into 10-μm slices.

Paraffin sections were dewaxed by incubating in Auto Dewaxer (Pharma) at 105 °C, then rinsing three times for 10 s with Auto Alchole (Pharma). Sections were then immersed in Universal Buffer (Pharma) for 10 s three times, and then in 1x Immuno/DNA Buffer (Pharma) for 10 s three times. Sections were incubated for 12 min at 37 °C with Protease K solution (Wako Pure Chemicals), diluted 1.25 μg/ml in PBS, then washed with 1 Immuno/DNA Buffer three times for 10 s before hybridization at 50 °C overnight with 1 μg/ml digoxigenin-labeled DNA probes diluted with cRNA Probe Diluent (Pharma). After washing with 2x saline-sodium citrate (SSC) buffer three times (each at 42 °C for 20 min) and dipping in NaCl-Tris-EDTA (NTE) buffer (5 mM NaCl, 2 mM Tris–HCl, 2 μM EDTA) for 5 min at 37 °C, sections were treated with RNase (Nippon Gene) solution (10 μg/ml in NTE buffer) for 15 min. Sections were then washed in NTE buffer at 37 °C for 5 min, and six times in 0.1x SSC (each at 42 °C for 10 min). Following immersion in 1x Immuno/DNA

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