Cloning of Medaka glial cell-derived neurotrophic factor (GDNF) and its receptor GFRα1

LIM CHIAT KOO
(B.Sc. (Hons.), NUS)

A THESIS SUBMITTED

FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

NATIONAL UNIVERSITY OF SINGAPORE

2006

ACKNOWLEDGEMENTS

I would like to sincerely thank A/P Hong Yun Han for rendering much help and effort in the supervising of the project. His patience and kindness have provided me with the much needed encouragement in my dispirited days of my research.

I am also very grateful to Madam Veronica Wong for all the excellent logistic and technical help that she had given me. She had been a great asset to the lab and many of the experiments would not have gone smoothly without her. I would also like to give thanks to Madam Deng Jia Rong for her assistance in all matters related to the aquarium and fish management.

Gratitude must be extended to all my lab mates, especially Li Zhendong, Rao Feng, Chen Tiansheng, Li Mingyou, Qin Lianju and Lu Wenqing for giving me invaluable guidance in my experiments. It was a joy to work in the lab and I am indeed privileged to have all of you as my labmates.

TABLE OF CONTENTS

SUMMARY

Glial cell line-derived neurotrophic factor (GDNF) signaling through its receptor GFRα1 plays an important role in many biological processes including cell survival, proliferation and differentiation. The aim of this project was to isolate and characterize both Gdnf and Gfrα1 from the medaka fish (*Oryzias latipes*). A full length cDNA for the medaka *gfrα1* was obtained by RT-PCR followed by RACE. It is 2551 bp long and has an open reading frame of 1401 bp for 466 amino acids. The predicted protein shares the best homology to the known vertebrate GFRα1 proteins although several domains distinctive to the gene do not appear to be well conserved. A cDNA of 762 bp for a putative medaka Gdnf of 253 amino acids was similarly obtained and sequence analysis across species indicates that it is generally well conserved in the medaka. Expression analysis suggests the presence of a currently unidentified binding ligand to Gfrα1 in the medaka brain and that Gdnf might also play a slightly different role in the medaka.

(166 words)

LIST OF ABBREVIATIONS

A_{al}	$A_{aligned}$ Spermatogonia
A_{pr}	A_{paired} Spermatogonia
A_s	A_{single} Spermatogonia
AA	Amino Acids
ARTN	Artemin
cDNA	Complementary DNA
EB	Ethidium Bromide
ERM	Embryo Rearing Medium
FRS2	Fibroblast Growth Factor Receptor Substrate 2
GCM	Germ Cell Culture Medium
GDNF	Glial Cell Derived Neurotrophic Factor
GFL	GDNF-family ligand
GFRα1	GDNF Family Receptor-α
GPI	Glycosylphosphatidylinositol
IPTG	Isopropylthio-β-D-galactoside
LB	Luria-Bertani
MES	Medaka Embryonic Stem cell line
NCAM	Neural Cell Adhesion Molecule
NRTN	Neurturin
PCR	Polymerase Chain Reaction
PSPN	Persephin
RACE	Rapid Amplification of cDNA ends

RT-PCR	Reverse Transcription – Polymerase Chain Reaction
SG3	Spermatogonial cell line
SHC	Src-Homologous and Collagen-like protein
SSCs	Spermatogonial Stem Cells
TFIID	Transcription Factor II D
TGF-β	Transforming Growth Factor-β
X-gal	5-bromo-4-chloro-3-idoldyl-β-D-galactoside

LIST OF FIGURES

LISTS OF TABLES

CHAPTER 1: INTRODUCTION

1.1 Spermatogonial Stem Cells (SSCs)

Differentiation of germ cells in the testis originates from a constantly renewed small pool of stem cells, termed SSCs located at the basement membrane of the seminiferous tubules (Oakberg 1971; De Rooij 1973). According to the model proposed by Huckins (1971) and Oakberg (1971), these stem cells are represented only by a discrete sub-population of type A spermatogonia cells or more specifically, A_{single} (A_s) spermatogonia and numbered no more than 0.03% of the total number of germ cells (Tegelenbosch and de Rooij 1993). Depending on the signals produced by the surrounding Sertoli cells, A_s spermatogonia will undergo mitosis to either renew themselves by forming two single stem cells or differentiate into A_{paired} (A_{pr}) spermatogonia that remain connected by an intracellular bridge. The A_{pr} spermatogonia will then divide into chains of four $A_{aligned}$ (A_{al}) spermatogonia which themselves, will further divide into chains of 8, 16 and up to, although rarely, 32 cells. Upon reaching this stage, the spermatogonia will give rise to more differentiated germ cells such as A1–A4 spermatogonia, type B spermatogonia, and spermatocytes which will then ultimately undergo meiosis (Fig. 1) to form mature sperms. Although $A_{s,}$ A_{pr} and A_{al} are sometimes collectively referred to as undifferentiated spermatogonia, (Lin *et al.* 1993; De Rooij and Grootegoed 1998; De Rooij *et al.* 1999; Meng *et al.* 2000), the A_s spermatogonia are regarded as the true stem cells of spermatogenesis (Huckins 1971; Oakberg 1971; Lok *et al.* 1982; De Rooij 1998). Due to the increasing amount of contradicting evidence in recent years, the A_0 model put forth by Clermont and Bustos-Obregon (1968) and

Clermont and Hermo (1975) shall not be discussed in this paper. For review, see De Rooij and Grootegoed (1998).

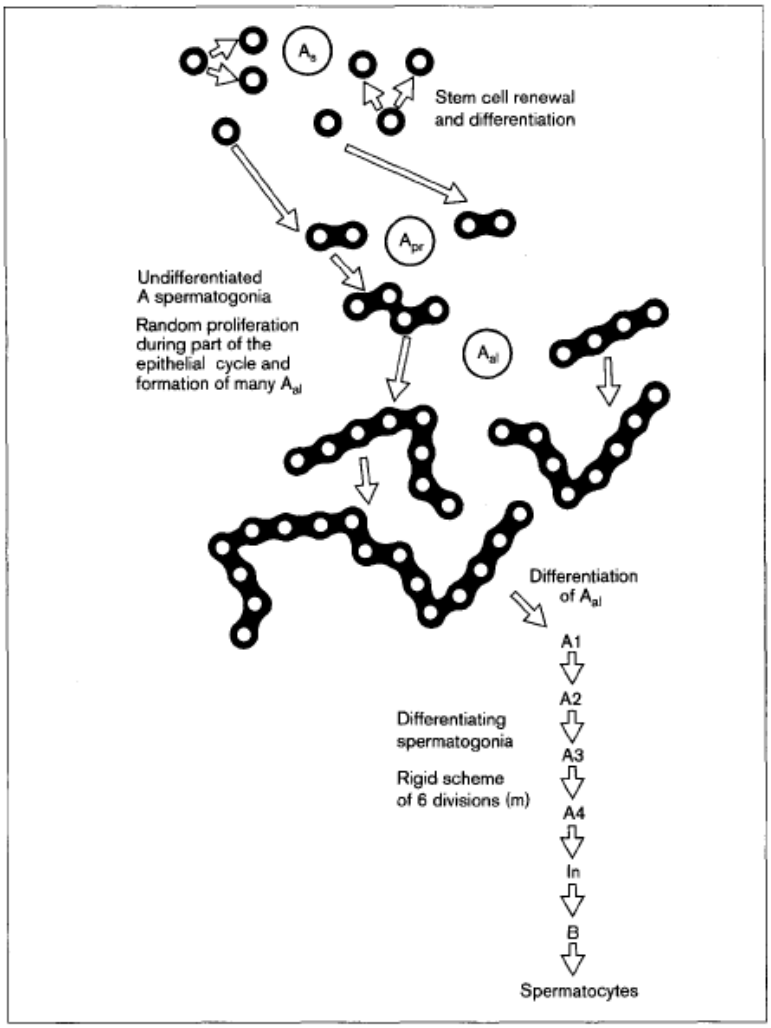


Fig. 1 Schematic diagram of spermatogonial multiplication and stem cell renewal.

This scheme probably applies to all mammals except for humans (De Rooij 1983). Stem cells (A_s) proliferate, renewing the stem cell pool and also producing undifferentiated A type paired spermatogonia (A_{pr}) which are joined together by intercellular cytoplasmic bridges. Further divisions of A_{pr} produce chains of aligned spermatogonia (A_{al}). These differentiate through six mitotic divisions into A1, A2, A3, A4, Intermediate (In), and B spermatogonia to become primary spermatocytes. (De Rooij and Grootegoed 1998)

1.2 Glial Cell Derived Neurotrophic Factor (GDNF)

GDNF is the founding member of a family of structurally related molecules, of which there are currently four members: GDNF, neurturin (NRTN) (Kotzbauer *et al.* 1996), persephin (PSPN) (Milbrandt *et al.* 1998), and artemin (ARTN) (Baloh *et al.* 1998). Together, these factors form the GDNF-family ligand (GFL), a distinct subgroup of the transforming growth factor-β (TGF-β) superfamily. Despite low amino-acid sequence homology, GDNF and other structurally characterized members of the TGF-β superfamily have similar conformations (Ibanez 1998); they all belong to the cystine-knot protein family, and they function as homodimers.

First isolated in 1993 and identified to be a potent survival factor for midbrain dopaminergic neurons *in vitro* (Lin *et al.* 1993), GDNF was soon discovered to have a much wider role in development. It supports the survival of several neuronal populations including motor neurons (Henderson *et al.* 1994; Oppenheim *et al.* 1995; Yan *et al.* 1995), central noradrenergic neurons (Arenas *et al.* 1995), cerebellar Purkinjie neurons (Mount *et al.* 1995), peripheral sensory and sympathetic neurons (Buj-Bello *et al.* 1995), autonomic neurons in peripheral ganglia (Ebendal *et al.* 1995; Trupp *et al.* 1995) as well as dopaminergic neurons (Beck *et al.* 1995; Tomac *et al.* 1995), making it a good candidate for treatment of dopaminergic neuron or motor neuron diseases such as Parkinson's disease and amyotrophic lateral sclerosis. More recently, it was also discovered to stimulate the proliferation of clusters of A_s spermatogonia and A_{pr} spermatogonia, *in vivo* (Meng et al. 2000; Yomogida et al. 2003) and *in vitro* (Nagano et al. 2003; Kubota et al. 2004; Hofmann et al. 2005), hence establishing its central role in dictating the cell fate of SSCs.

1.3 GFL-induced activation of the protein tyrosine kinase, RET requires GDNF Family Receptor-α (GFRα)

Unlike typical members of the TGF-β superfamily, GFLs do not signals through receptor serine-threonine kinase. Instead, it signals through a receptor complex formed by the receptor tyrosine kinase RET and a novel class of protein, known as GDNF Family Receptor-α (GFRα), which are linked and localized to the lipid rafts of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. (For review, see Airaksinen and Saarma 2002). Lipid rafts are lipid micro-domains constituted of sphingolipids and cholesterol within the plasma membrane and play important roles in cellular signaling. Recent evidences have indicated that lipid rafts are crucial for abundant biological events including growth factor-receptor signaling, cellular adhesion, synaptic transmission and membrane associated proteolysis (Brown and London 1998; Tooze *et al.* 2001).

GDNF binds to RET via GFRα1 (Jing *et al.* 1996) while NRTN, ARTN and PSPN use GFRα2, GFRα3 and GFRα4 as the preferred ligand-binding receptors respectively (Treanor *et al.* 1996; Baloh *et al.* 1997; Buj-Bello *et al.* 1997; Creedon *et al.* 1997; Jing *et al.* 1997; Sanicola *et al.* 1997; Baloh *et al.* 1998; Enokido *et al.* 1998)., although alternative ligand-coreceptor interaction also appears to occur in culture. Studies have shown, at least in several occasions that NRTN is capable of signalling via GFRα1 in human (Baloh *et al.* 1997), mice (Widenfalk *et al.* 1997; Golden *et al.* 1998), rat (Creedon *et al.* 1997)and chicken (Homma *et al.* 2000), although GFRα2 is its preferred receptor. ARTN had also been known to exhibit such similar behaviour (Fig. 2) (For review, see Sariola and Saarma, 2003).

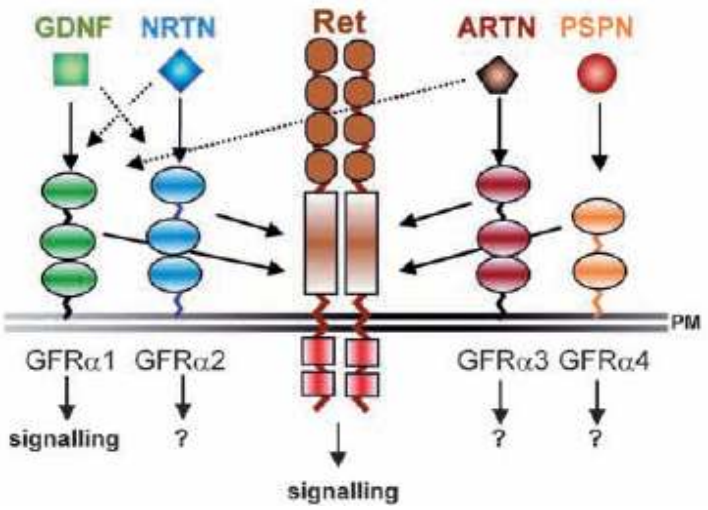


Fig. 2 GDNF-family ligand interaction with their receptors.

All GFLs activate RET tyrosine kinase via different GFRα receptors. Solid arrows indicate the preferred functional ligand-receptor interactions, whereas dotted arrows indicate putative crosstalk. GFRα proteins are attached to the plasma membrane through a GPI-anchor and consist of three (GFRα4 has only two) globular cysteine-rich domains joined together by adapter sequences. (Modified from Sariola and Saarma, 2003)

Upon stimulation by GDNF, the anchored GFRα1 will recruit RET to the lipid rafts (Fig. 3A-B) and such localization is thought to be critical for effective downstream signalling. Any disruption to such localization would lead to acute attenuation in intracellular signaling events including neuronal differentiation and survival, even if RET is phosphorylated after GDNF stimulation (Tansey *et al.* 2000). So, the lipid rafts seem to compartmentalize signalling molecules on both sides of the plasma membrane, which allows them to interact with each other and prevents interactions with proteins that are excluded from the rafts.

However, Ibanez and his colleagues soon demonstrate that unbound soluble GFRα1 can too recruits RET to lipid rafts (Fig. 3C) and mediates intracellular signaling events, albeit with delayed kinetics (Paratcha *et al.* 2001). In addition, they also illustrate that RET which moves to the lipid rafts upon stimulation by GDNF triggers the signal

through FRS2 (fibroblast growth factor receptor substrate 2) while those that moves to the outside of the rafts trigger the signal through SHC (Src-homologous and collagen-like protein) (Fig. 3B). Since SHC and FRS2 share the same tyrosine 1062 docking site in RET (Coulpier *et al.* 2002), this imply that differences in GDNF signalling through RET could lead to dramatically different cellular response although the mechanisms that bring the complex of GDNF, soluble GFRα1 and RET to rafts, and prolong signalling, are unclear at the moment.

Alternatively, GDNF could also signal independently of RET, by utilizing the neural cell adhesion molecule (NCAM) in collaboration with GFRα1. (Paratcha *et al.* 2003). This binding will activate Fyn, a member of the Src family of cytoplasmic tyrosine kinases (Panicker *et al.* 2003), although it seems highly unlikely that this alternate pathway is involved in SSCs development at the moment (Fig. 4).

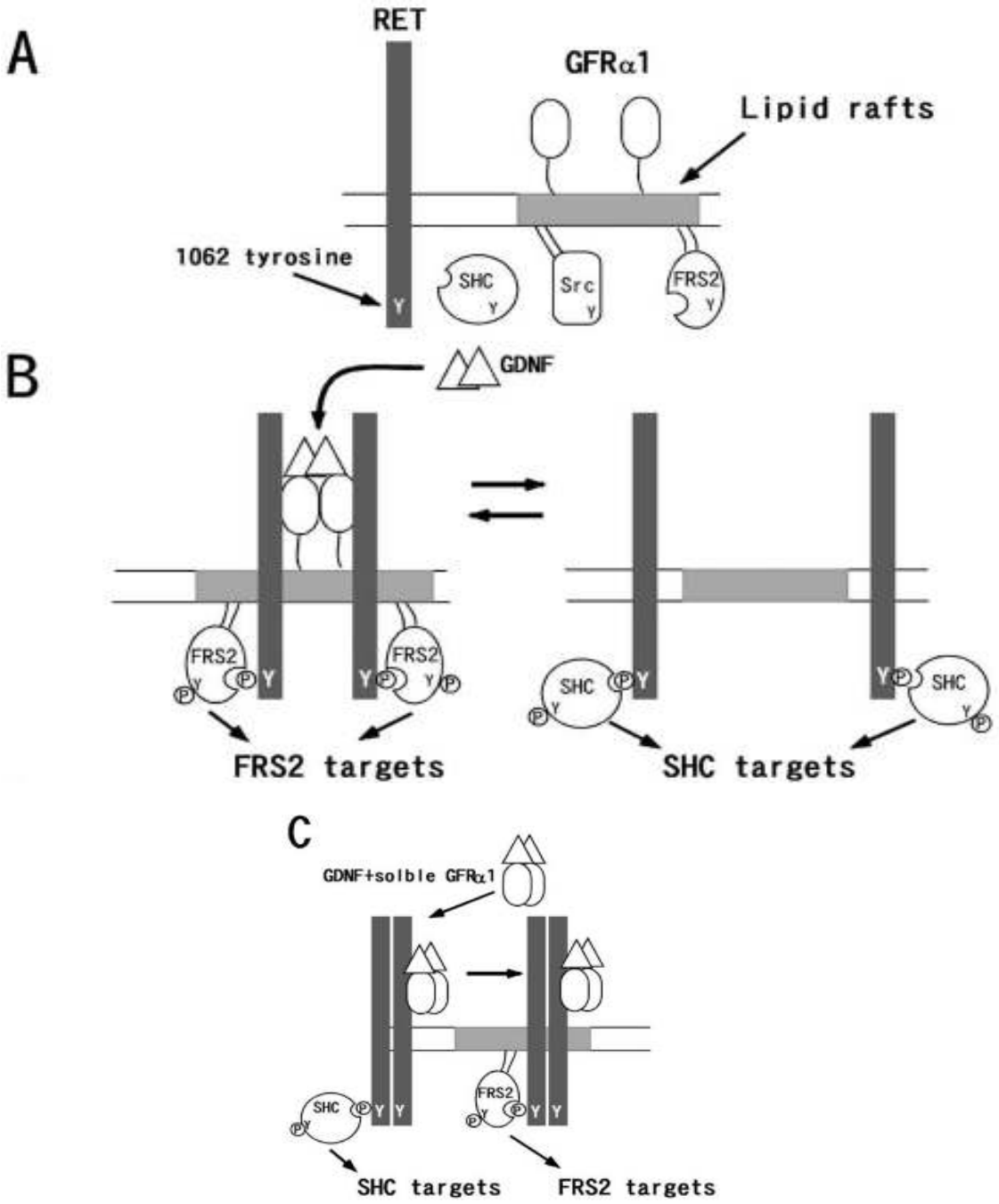


Fig. 3 Schematic illustration of initial signaling events mediated by the binding of GDNF to RET receptor complex in lipid rafts.

(A) GFRα1 is anchored in the lipid rafts, while RET is located in the outside of lipid rafts in the inactive form. (B) GFRα1 recruits RET to lipid rafts upon the binding of GDNF to GFRα1 and the recruitment of RET to the lipid rafts results in the dimerization and activation of RET. RET which moved to lipid rafts following GDNF stimulation triggers the signals through SNT/FRS2, while activated RET located outside of the rafts trigger the signal through SHC. (C) Soluble GFRα1 with GDNF also recruits RET to lipid rafts and mediate intracellular signaling events in inside and outside of lipid rafts. (Modified from Ichihara, 2004)

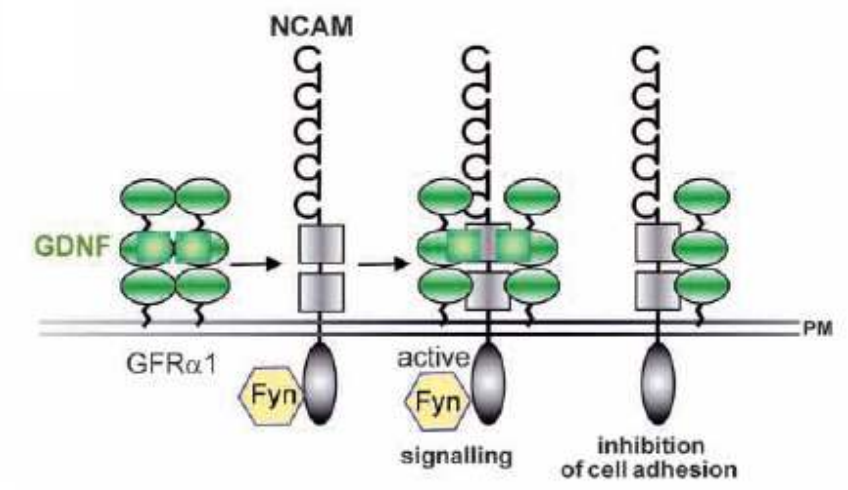


Fig. 4. Schematic illustration of GDNF and GFRα1 signalling through NCAM.

GDNF can signal independently of RET and utilizes NCAM instead. In this case, a Src-like kinase known as Fyn will be activated instead of FRS2 and SHC. (Modified from Sariola and Saarma 2003).

1.4 Medaka as a Model Organism

Medaka (*Oryzias latipes*) is a small (3cm to 4cm) egg-laying freshwater fish that is found primarily in Japan, Korea and China. It has a short generation time of 2 to 3 months, and a short life-span of 2 years. Hardy and prolific, it can survive a wide range of temperatures (4°C to 40°C) and easily induced to spawn in captivity; when kept at an optimum temperature between 25°C to 28°C, spawning can be induced simply by light cycles (12hr light and 12hr dark).

Medaka can lay up to 30 to 50 eggs daily. Transparent and synchronous in development, the eggs can then be staged under dissecting microscope to study early developmental process, fertilization and embryology. In addition, being eurythermal in nature, early embryos can be maintained at temperatures as low as 4°C to slow down their development for up to 3 months. This will be useful for transplantation and microinjection experiments. Sperm can also be stored for stock preservation.

With the ease of breeding and low susceptibility to common fish diseases, the maintenance of the medaka is easy, cheap and not space consuming, making medaka an ideal animal model source for carrying out research experiments.

However, it is the major advances in recent years that make medaka an increasingly popular candidate as a model organism. Firstly, the establishment of the medaka embryonic stem cell-line (MES) had provided scientist with a unique tool for introducing targeted or random genetic alterations through gene replacement, insertional mutagenesis, and gene addition due to the possibility of *in vitro* selection for the desired, recombinant genotype (Hong *et al.* 1998). Secondly, the successful generation of the see-through medaka model with transparent body through out adult life (Fig. 5A) had allowed convenient, noninvasive studies of morphological and molecular events that occur in internal organs in the later stages of life (Wakamatsu *et al.* 2001). Thirdly, the Medaka Genome Sequencing Project started in mid 2002 has already achieved a current status of draft assembly covering 91-99% of the genome and once completed, this comprehensive database will provide future investigator a powerful mean to identify and map genes rapidly. Finally, the establishment of a normal medaka fish spermatogonial cell line (SG3) capable of test tube production (Fig. 5B) (Hong *et al.*, 2004) will offer researchers a unique opportunity to study spermatogenesis *in vitro* and develop new approaches to germline transmission.

The major advantages and unique features of the medaka fish are summarized in Table 1, in comparison with the other 2 common fish models, zebrafish and *Fugu*. The evolutionary relationship between medaka and various other fish models is illustrated in Fig. 6. For additional details, see Wittbrodt *et al*. 2002 and Shima and

Mitani 2004, as well as to the 'Medakafish homepage' curated by H. Hori (http://biol1.bio.nagoya-u.ac.jp:8000/).

Table 1 Biological characteristics and availability of experimental tools in three teleost species (Ishikawa 2000).

Biological Characteristics	Zebrafish	Medaka	Pufferfish
Genome size (Mb)	1700	800	400
Chromosome number of 2n	50	48	42
Sex determination	-	XY type	-
Life cycle	3 months	3 months	-
Outdoor breeding	No	Yes	Yes
Crossing in lab	Yes	Yes	No
Linkage map	Yes	Yes	No
Information on sequenced genes, mapped genes and DNA markers	Much	Much	Much
Genetic information on wild populations	None	Good	None
The number of inbred strains	0	Many	0
Cryopreservation of spermatozoa	Yes	Yes	No
Transgenic fish	Yes	Yes	No
Chimeric fish	Yes	Yes	No
Gynogenesis	Yes	Yes	No
ES-like cells	No	Yes	No
Active transposable elements	No	Yes	No

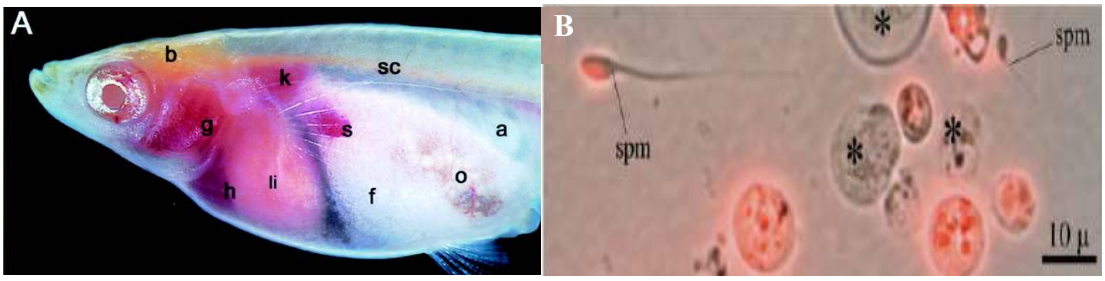


Fig. 5 Recent advancement in medaka fish.

(A) Picture of the unique see-through fish (Wakamatsu *et al.* 2001). (B) Sperm from in vitro spermatogenesis (Hong *et al.* 2004).

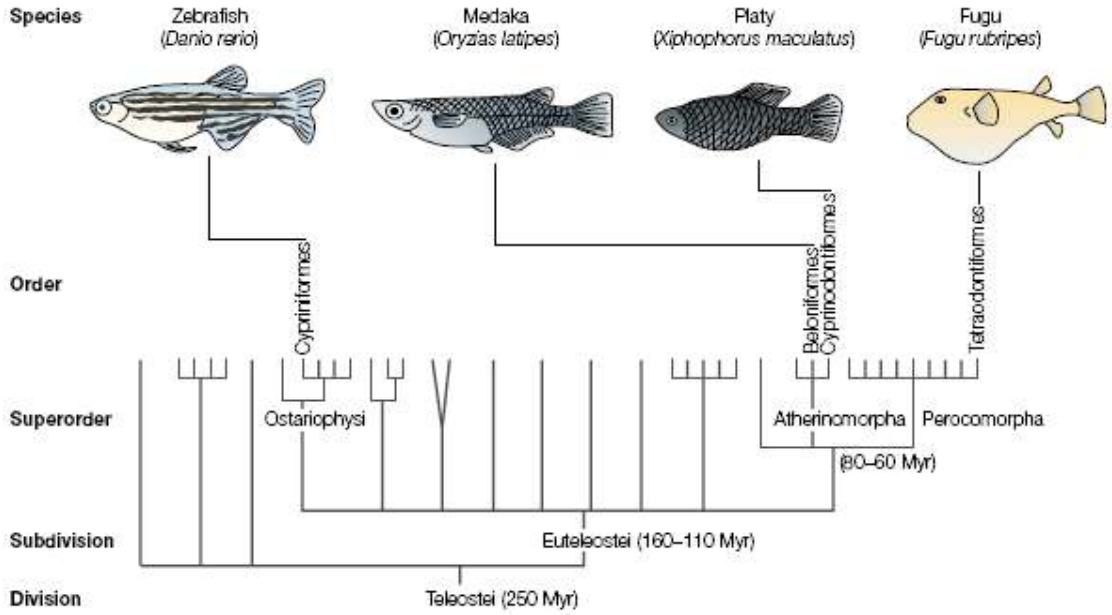


Fig. 6 Evolutionary relationships between fish models.

This evolutionary tree illustrates that the last common ancestor of medaka and zebrafish lived more than 110 million years ago. Notably, medaka is a much closer relative to *Fugu* than it is to zebrafish, or than zebrafish is to *Fugu* (Wittbrodt *et al.* 2002).

1.5 Goals

While full recapitulation of spermatogenesis has been achieved *in vitro* (Hong *et al.* 2004), the exact mechanism underlying the mitotic proliferation and differentiation of SSCs in medaka is still unclear. The germ cell culture medium (GCM) used for sustaining the SG3 cell line includes a plethora of molecules extracted from the 7-day-old medaka embryos, and identification of specific growth factors in the medium involved in spermatogenesis remains elusive. It would be interesting to see if the GDNF-GFRα1-RET signalling pathway observed in other vertebrates to be conserved in the medaka.

Hence, the aim of this study was to clone and characterize both Gdnf and Gfrα1 from the testis of the medaka fish via RT-PCR and RACE (Rapid Amplification of cDNA ends), so as to set the stage for future investigation into the GDNF-GFRα1-RET signalling pathway in medaka SSCs. The expression patterns of the genes in various tissues and embryonic stages will also be presented

CHAPTER 2: MATERIAL AND METHODS

2.1 Fish Maintenance and Collection of embryos

Medaka (*Oryzias latipes*) was maintained in household aquarium and was fed with *Artemia nauplii* twice daily. Temperature is maintained from 26°C to 29°C and under an artificial photoperiod of 14 hour light and 10 hour darkness. No attempts was made to separate the males and females, and the ratio of males to female kept in each tank is roughly 2 : 3.

Embryos were collected in the morning and transferred into ERM (Embryo Rearing Medium) before incubating it at 27°C. One liter of ERM has 1.0 g NaCl, 0.03 g KCl, 0.04 g $CaCl_2 \bullet 2H_2O$, 0.163 g $MgSO_4 \bullet 7H_2O$ and 10 ml of Methylene blue. The medium is changed daily and any embryos found dead will be discarded.

2.2 RNA isolation from organs and embryos

Total RNA was extracted from several organs including, liver, intestines, muscle, brain, eyes, testis and ovary of the medaka fish with Trizol Reagent (Invitrogen) under the conditions suggested by the manufacturer. The RNA was dissolved in RNase-free water by passing the solution a few times through a pipette tip and was incubated at 60°C for 10 min. The RNA samples were then stored at -80°C.

For embryos, the developmental stages were checked under microscope daily and RNA was extracted only from embryos at development stage 2, 7, 10, 22, 39 and 40 as determined according to Iwamatsu (2004). Conditions for extraction are similar to the organs.

2.3 cDNA synthesis

Total RNA isolated from tissues and embryonic stages were used to synthesize first strand cDNA and then served as template for PCR amplifications. cDNA synthesis was carried out with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega, USA) and 18-mer oligo dT according to the Manufacturer's protocol. Before cDNA synthesis, total RNA samples were treated by RNase-free DNase to eliminate any possible genomic or transfected DNA contaminations.

For RACE (Rapid Amplification of cDNA Ends), the cDNA template was synthesized according to the BD SMART™ RACE cDNA Amplification Kit User Manual and 1 μg of RNA was used per cDNA synthesis reaction. The synthesize cDNA was then diluted with 40ul of nuclease-free water for subsequent PCR reactions.

2.4 cDNA cloning of medaka Gdnf and Gfrα1 by RT-PCR and RACE

Polymerase Chain Reaction (PCR) was performed with the designed primers to amplify a specific DNA fragment using *Taq* polymerase (Fermentas, USA) in a thermocycler. PCR was performed following general protocols in 25ul for detection and 100ul for scaling up. Normal reactions were carried out as:

Reaction mixture:

14.85 μl	PCR-Grade water
2.5 μl	10x PCR Buffer with (NH4)2SO4
2 μl	MgCl2 (25 mM)
2.5 μl	dNTP Mix (2 mM)
1 μl	Template DNA (20ng)
1 μl	Primer1 (5 mM)
1 μl	Primer2 (5 mM)
0.15 μl	*Taq* DNA polymerase (Fermentas)

PCR was run for 25 to 40 cycles at 94°C for 15 sec, 56°C for 15 sec and 72°C for 60 sec.

For RACE, PCR was carried out according to manufacturer's specification with slight modifications. 4% DMSO was added to the reaction mixture and PCR was run for 35 cycles at 94°C for 20 sec, 68°C for 20 sec and 72°C for 4 mins instead.

Reagents and enzymes needed were purchased from Fermentas and thermocyclers from Applied Biosystem.

2.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate and detect differentially molecular weighted DNA and RNA fragments. Nucleotide fragments were separated by molecular filtering effect and visualized upon binding with ethidium bromide (EB) under UV light. According to molecular size of DNA fragments to be separated, agarose concentration may vary from 0.7%-2.0%. 1×TAE was used as electrophoresis buffer. Equipments used were ReadyAgarose^{TM} Precast Gel System (Bio-Rad, USA). DNA ladder (Promega, Fermentas) was added for estimating the molecular size of PCR products.

50×TAE	2M	Tris-acetic acid
	10mM	EDTA (pH8.0)
6×gel loading buffer	0.25 (w/v)	Bromophenol blue
	0.25 (w/v)	Xylene cyanol
	30%	glycerol

2.6 Recovery of DNA fragments from agarose gel

After electrophoresis, the desired gel band was excised, placed in a 1.5 ml Eppendorf tube and its weight determined. Gel purification was performed using Ultraclean^{TM} 15 DNA purification kit (Mobio, USA) and under conditions stipulated by the manufacturer. The concentration of the recovered DNA was determined using a Shimadzu UV-160A spectrophotometer and then stored at 4°C until further use.

2.7 Ligation of DNA fragment into pGEM-vector

After recovery of DNA fragment by gel extraction, the PCR products were ligated into pGEM-T Easy Vector (Promega) for cloning and sequencing. The pGEM-T Easy Vector multiple cloning sites is flanked by recognition sites for the restriction enzyme *Eco*R1 which allows single enzyme digestion for the release of insert. The ligation reaction mixture was set up according to manufacturer's recommendations with slight modifications:

5 µl	2x Rapid Ligation Buffer
0.8 µl	pGEM-T Easy Vector (40 ng)
3.2 µl	DNA fragment
1 µl	T4 DNA Ligase (3 Weiss units/µl)

The reaction was mixed by pipetting and incubated overnight at 4°C.

2.8 *E.coli* transformation

Transformation is a procedure to introduce circular plasmids into bacteria for the purpose of amplification. The tubes containing the ligation reaction were centrifuged to collect contents at the bottom of the tube. 2 µl of ligation reaction was transferred to a

1.5-ml microcentrifuge tube on ice. DH5α competent cells from -80°C storage prepared using the $MgSO_4$ method (Nishimura *et al.* 1990) were thawed in an ice bath for about 5 min and the content of the tubes were mixed gently by flicking. 50 µl of competent cells were added to the respective ligation reaction and the mixture were flicked gently and placed on ice for 30 min. The cells were then heat shocked for 90 secs in a water bath at 42°C, and the tubes were immediately returned to the ice for 2 min. 800 µl of Luria-Bertani (LB) medium was added to the tubes containing the cells transformed with ligation mixture and incubated for 45 mins at 37°C. During the incubation period, 30 µl of X-gal and 10 µl of IPTG were added to LB ampicillin (100µg/ml) agar plate for blue-white selection. After 1.5 h, the cells were pelleted at 3,000 rpm for 5 min, resuspended in 100 µl of LB medium and then plated. The plates were incubated at 37°C for 12 to 16 hours.

After incubation overnight, the LB plates were stored at 4°C for 30 min to facilitate blue-white screening. Individual colonies were then picked and incubated for 14-16 h in 4 ml of LB medium.

LB medium (20g/l)	10 g	Tryptone
	5 g	Yeast extract
	5 g	NaCl
LB agar powder (40g/l)	10 g	Tryptone
	5 g	Yeast extract
	10 g	NaCl
	15 g	Agar
IPTG (20% w/v)	0.8 M	Isopropylthio-β-D-galactoside
X-gal solution (2% w/v)	20 mg/ml	5-bromo-4-chloro-3-idoldyl-β-D-galactoside

2.9 Digestion of DNA with EcoRI

Plasmid DNA was incubated at 37°C for 1-2 hours in a reaction mixture containing 1 µl Buffer, 3 µl plasmid DNA, 5 µl H_2O and 1 µl of *EcoRI* (Invitrogen, USA). The digested plasmid DNA was then visualized on a 1% agarose gel under UV light.

2.10 DNA sequencing and analyses

Upon confirmation of positive inserts by test digestion, DNA sequencing was performed using Big Dye Terminators v3.1 Cycle Sequencing Kit (ABI PRISM, USA). The total sequencing reaction is 5 µl and consist 1 µl DNA template, 1.5 H_2O, 1 µl of M13 primer (3.2 pmol), 1.5 µl Big Dye (v3.1). The PCR cycling condition was: 94°C for 10 sec, 50°C for 5 sec and 60°C for 1 min. The product was purified with 20 µl ethanol/sodium acetate solution from a stock solution consisting of 3 µl of 3M sodium acetate (pH 4.6), 62.5 µl of non-denatured 95% ethanol and 14.5 µl of deionized water. The tubes were vortexed briefly and left at room temperature for 15 min to precipitate the extension products. The tubes were centrifuged at maximum speed for 20 min and the supernatants removed immediately. 500 µl of 70% alcohol was added to wash the pellets and mixed briefly. The tubes were placed in the microcentrifuge in the same orientation and spun for 5 min at maximum speed. The supernatant was then aspirated carefully and the samples left to dry under room temperature for about 15 mins.

12 µl of High Dye (ABI PRISM, USA) was added and reaction mixture was vortexed briefly. The samples were transferred to a 96 well plate for sequencing reaction using ABI 3100 automated DNA sequencers. Nucleotide sequences obtained were processed and analyzed with commercial software DNAMAN and Vector NTI.

2.11 Expression Analysis of tissues and embryos

After cDNA synthesis, expression of Gdnf and Gfra1 transcript was analyzed according to the PCR conditions stipulated above for 35 cycles. β-Actin was used as calibrations (28 cycles).

2.12 Medaka Genome Database

The medaka genome database is retrieved from http://dolphin.lab.nig.ac.jp/medaka and the data provided freely by the National Institute of Genetics and the University of Tokyo for use in this publication only

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Isolation and characterization of medaka Gdnf.

As sequence alignments across several vertebrate species revealed significant amino acid conservation in GDNF, a tblastn was performed using the mouse homologue as a query against the medaka genome database. From the blast results, we were able to identify the putative medaka Gdnf to Scaffold 77, and after careful analysis of the genome sequence, two pairs of putative primers were designed; Forward 1, 5'–ATGAA-GTATGGGATGTTTTGGC-3'; Reverse 1, 5'-CTCATGTCCAGTGTTTGGTCAA-3'; Forward 2, 5'-GACGAAGAGCCACTGTTCCAGCGCAAGG-3'; Reverse 2, 5'-CTGG-TATCCCAGCCCCAAATCCGTCACA-3' (Fig. 7).

Using the primers, part of the gene was successfully isolated and the medaka *gdnf* was found to contain a putative ORF (Open Reading Frame) of 762 bp, encoding for a protein of 253 aa (amino acids) residues (Fig. 7) and a predicted molecular weight of 30 kDa. The partially isolated Gdnf was then blast with our internal EST (Expressed Sequence Tag) database (data not shown) and a leader sequence of approximately 749 bp was uncovered (Fig. 7). The 5' UTR was subsequently confirmed by two additional primers (data not shown); Exon 1, 5'-CTGCTTTTCAGTGCAGTTGGAGC-3'; Exon 2, 5'-CATACACCAGATAAAGTGTGGATCAG-3'.

Similar to the mice (Matsushita *et al.* 1997) and rat cDNA (accession number. AJ011432), an additional in frame ATG codon 36 bp upstream of the predicted start codon was also discovered. Although direct experimental evidence, at least in the case of mice (Matsushita *et al.* 1997) had demonstrated that this ATG codon is not the translation

start point, it is unclear if this is also true in the medaka. The rest of the ATG detected upstream are out of frame and yield short peptide sequences not exceeding 70 aa. Hence, the ORF predicted initially is likely to be correct.

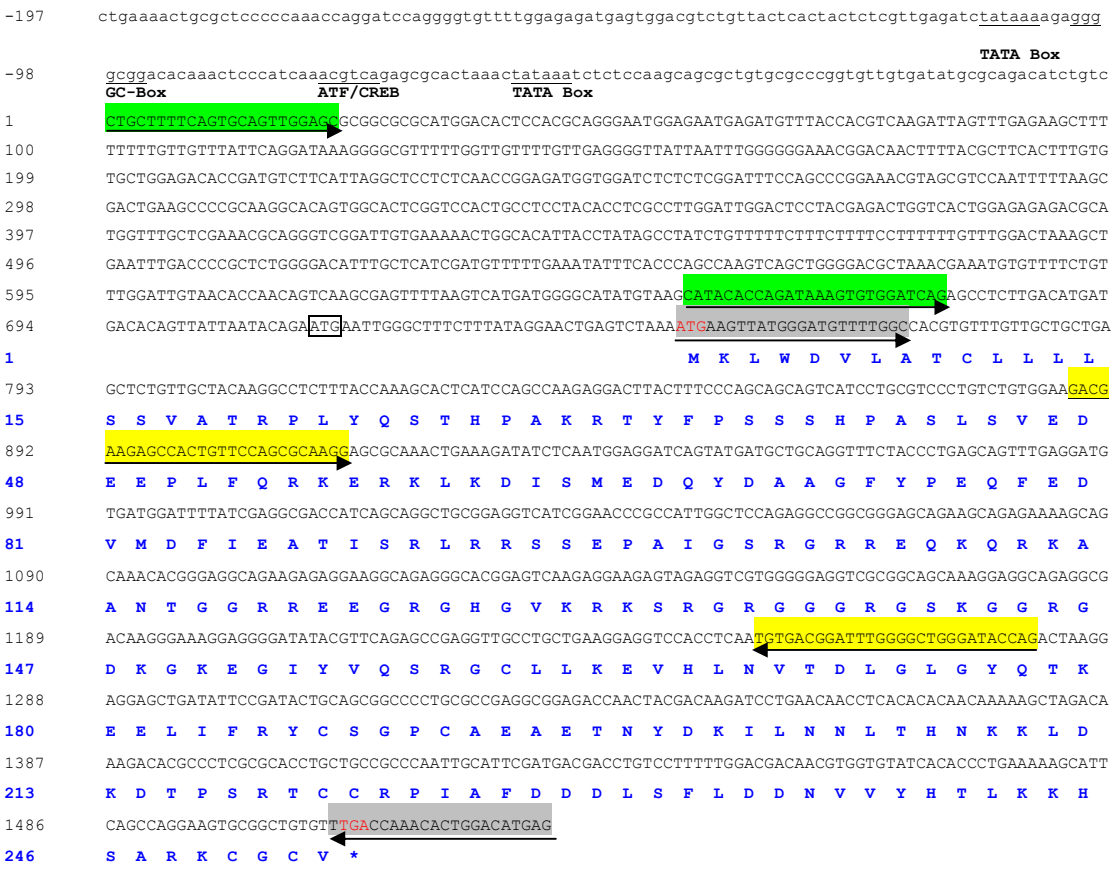


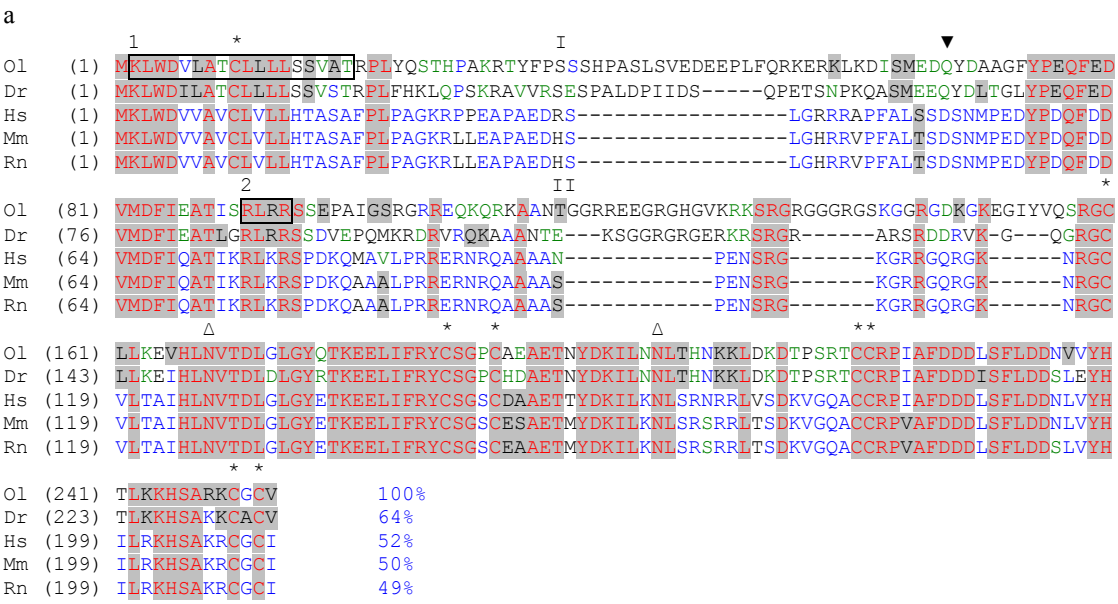
Fig. 7 Nucleotide sequence and deduced amino acid sequence of medaka *gdnf*.

The genomic sequence is depicted in lowercase, the cDNA in uppercase and the deduced amino acid protein sequence in blue and boldface. The four putative promoter elements are underlined and its corresponding identity listed underneath. The ATG indicated by a box is unlikely to be the translation start point although it is capable of yielding a polypeptide 265 aa long. The start and stop codon of *gdnf* is more likely to be the red ATG and TGA, respectively instead. The primers used to clone the *gdnf* CDS is highlighted in grey while those used for mRNA expression analysis in yellow. The primers in green are obtained only after blasting the cloned CDS with our internal EST database. The arrow indicates the direction of the primers.

A scan through the genomic sequence immediately preceding the predicted 5' end of the cDNA revealed 3 distinct putative promoter elements; a GC-Box, a CREB site and surprisingly, two TATA box, all localized within 150 bp upstream of the predicted exon 1. It is difficult to determine the true binding site for TFIID (Transcription Factor II D), although both TATA box might well be functional, with each of them responding to only a certain distinct set of transcriptional signals. Interestingly, the promoter sequence of the human homologue also contains a TATA box, a GC box and a CREB site although it has two additional NF-A1-like binding sites (Grimm *et al.* 1998). Taken together, it is very likely that the transcriptional control mechanism in medaka *gdnf* to be very similar to the human *Gdnf*.

Although sequence alignment using Vector NTI shows that the medaka Gdnf only shares 64%, 52%, 50% and 49% homology with its zebrafish, human, mice and rat counterpart respectively, all functionally identified motifs found within other vertebrates appear to be well conserved in the medaka (Fig. 8a). The consensus sequence RXK/RR for proteolytic processing by furin (van de Ven *et al.* 1990) in the constitutive secretion pathway predicted to release the mature GDNF is located between Arg^{91} and Arg^{94} and the potential secretion signal from Lys^{2} to Thr^{19}. In addition, all eight cysteine residues and two putative conserved N-linked glycosylation sites appears conserved (Fig. 8a). Sequence comparison by BLAST did not reveal significant homology to any other known proteins. The phylogenetic tree of Gdnf proteins from medaka and other organisms created by DNAMAN is illustrated in Fig 8b.

a



b

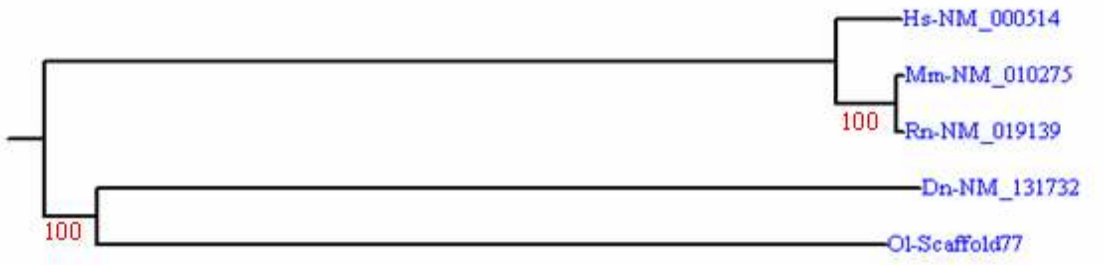


Fig. 8 Sequence comparison of medaka Gdnf and its orthologues

(a) The first and second exons are indicated as I and II respectively while ▼ demarcate exon-intron boundaries. Box 1 indicates potential secretion signal while Box 2 indicates the consensus sequence for proteolytic processing. The N-linked glycosylation sites are indicated by Δ and asterisks mark the conserved cysteine residues. Percent identity values between medaka Gdnf and its homologs are indicated at the end. (b) Phylogenetic tree of Gdnf proteins from medaka and other organisms. Bootstrap values at branching are given. The corresponding accession number is indicated after the species name. Ol, *Oryzias latipes;* Dr, *Danio rerio*; Hs, *Homo sapiens*; Mm, *Mus Musculus*; Rn, *Rattus norvegicus*.

While the medaka *gdnf* has four exons (Table 2), its CDS is made up of only two. The first two exons are part of its 5' UTR and like its human counterparts, the start codon is located at exon 3 (Grimm *et al.* 1998). However, in humans, there seems to be an additional level of control in the regulation of GDNF expression through the alternate use of exons; exon 1 and 2 are mutually exclusive and hence, human GDNF has only 3 exons (Fig. 9).

Table 2 Structure of *gdnf* with the predicted size of exons, introns and junction sequences.

Exon No.	Position in Scaffold 77	Exon 5'- 3' end	Exon Size (bp)	Intron 5'-3' end	Intron Size (bp)
1	1194182-1194832	CTGCTTTTCA-CATATGTAAG	651	gtaaggatgg-gtgttcacag	557
2	1195390-1195461	CATACACCAG-ATGAATTGGG	72	gtacgtttaa-tctttcacag	111
3	1195573-1195800	CTTTCTTTAT-GAGGATCAGT	228	gtaagcagac-tgccttttag	3404
4	1199205-1201900	ATGATGCTGC-ACGAAAGAGT	2696?		

The exact size of exon 4 is currently unclear as the 3' UTR of *gdnf* had yet to be fully characterized.

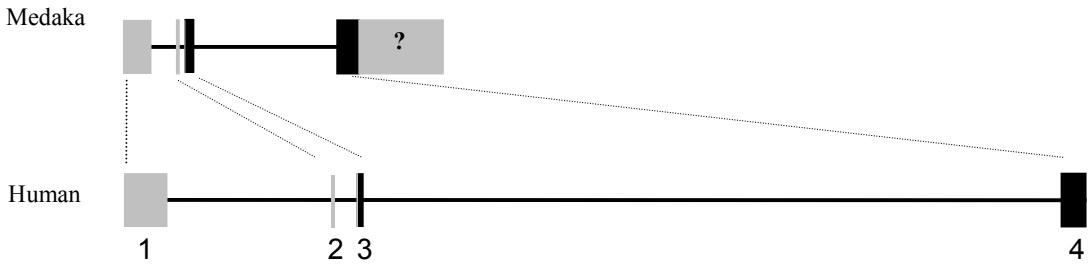


Fig. 9 Schematic diagram of medaka *gdnf* gene structure in comparison to human.

Untranslated exons are showed in grey box, translated exon in filled box and introns as solid lines. The human *Gdnf* gene has two reported isoforms; the longer isoform (Accession Number: NM_000514) utilize exon 1 while the shorter isoform (NM_199231) utilize exon 2. In medaka however, both exons appears to be included in the *gdnf* cDNA and not mutually exclusive. Diagram is drawn to scale except for the last medaka exon where the size is undetermined.

3.2 Isolation and characterization of the medaka Gfrα1.

Likes its ligand, a tblastn was also performed using the mouse homologue as a query. Similarly, two pairs of primers were designed from the scaffold; Forward (RACE), 5'-GACATCTTCCGCTTGGCTCCCATCAT-3'; Reverse (RACE), 5'-ACTCCACTGTA-GGCTTCATTGAGGGTCG-3'; Forward (Nested-RACE) 5'-GGGTGTCTGCCTCTGA-GGTCTGCAAC-3'; Reverse (Nested-RACE), 5'-TTGCTGGTAAAGTCCAACAGTC-GTGA-3'.

After obtaining the partial sequence of Gfrα1 through RT-PCR, the 5' and 3' end of the mRNA was subsequently determined by RACE. The mRNA was 2551 bp long and translation of the nucleic acid in six frames locates the start codon to the 251st nucleotide; the rest of the ATG codons failed to yield peptide longer than 150 aa. Hence, the open reading frame of Gfrα1 is predicted to be 1401 bp long, encoding for 466 aa and with a theoretical molecular weight of 52 kDa (Fig. 10). The sequence was further verified by the following primers; Forward, 5'-AAAGCAGAGGAATAAAGAGAAGT-G-3'; Reverse, 5'-TTAATGTATAAAAATGATA-CAAAGCAT-3'.

Similarly to Gdnf, a scan through the genomic sequence was performed and two promoter elements were detected upstream of exon 1; A CREB site and one putative NF-A1-like binding sites were localized at -300 and -581 bp, respectively. No TATA box was detected although its presence might be masked by ten unresolved nucleotide sequence localized at -85 to -75 region of the genomic sequence (Fig 10). Transcriptional control of the gene is not well understood in human although the putative promoter elements identified might hint at a control mechanism similar to GDNF.

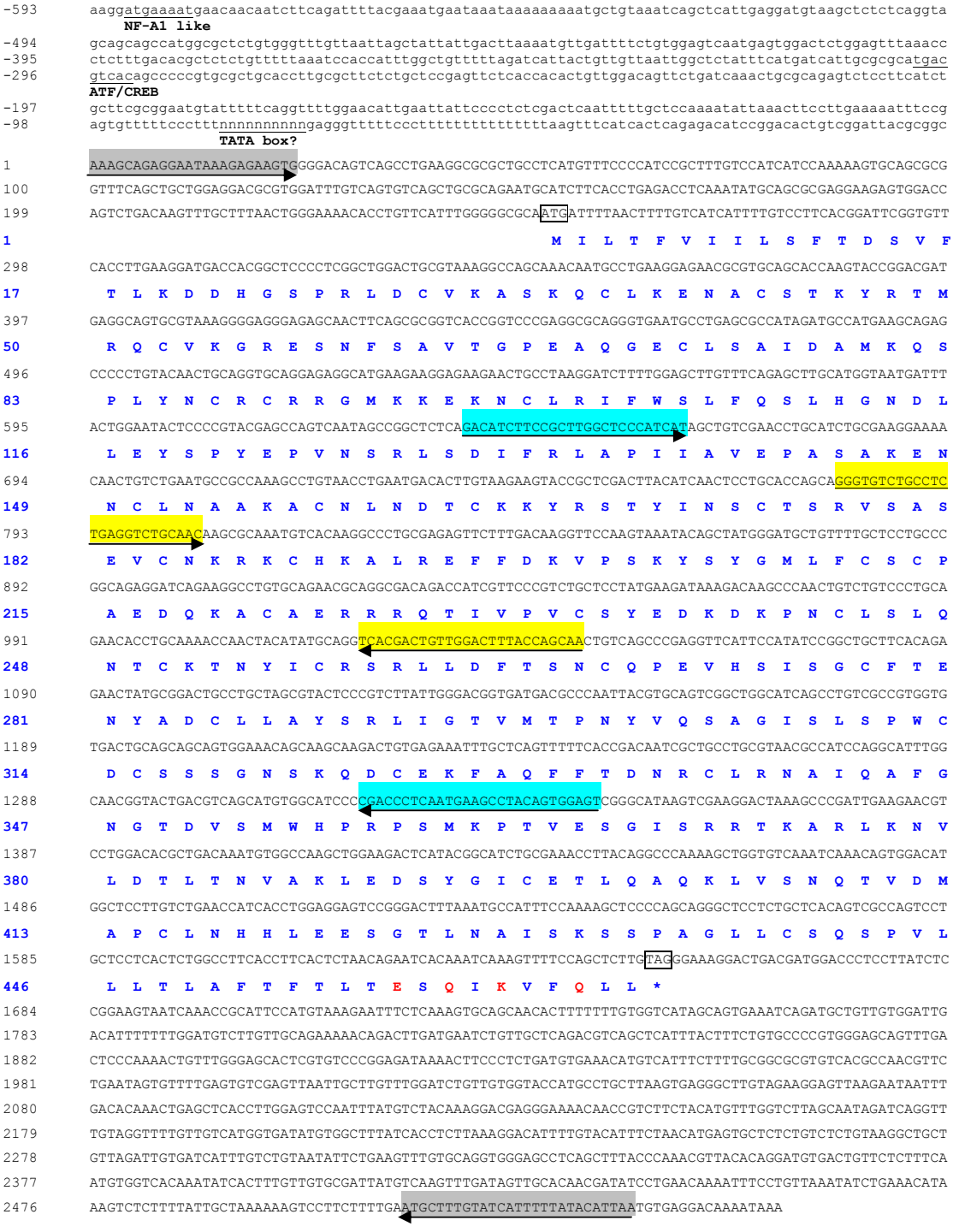

NF-A1 like
ATF/CREB
TATA box?

Fig. 10 Nucleotide sequence of the PCR product and deduced amino acid sequence of medaka Gfrα1.

The genomic sequence is depicted in lowercase, the cDNA in uppercase and the deduced amino acid protein sequence in blue and boldface. The two putative promoter elements are underlined and its corresponding identity listed underneath. The presence of a TATA box remains debatable due to ten unresolved nucleotide sequence at the -85 to -75 region of the genomic sequence. The boxed ATG and TGA are the predicted start and stop codon of *gfrα1*, respectively. The four amino acids in red at the C-terminal are hydrophilic residues found on a supposing stretch of hydrophobic residues. The primers used for *gfra1* cDNA cloning are highlighted in grey while those used for mRNA expression analysis in yellow. Putative primers initially designed from the medaka genome draft database are highlighted in cyan. The arrow indicates the direction of the primers.

A search of the NCBI database using the BLAST algorithm reveals that the medaka gene is most similar to other Gfrα1 homologues. Sequence alignment of amino acid sequence using vector NTI reveals medaka Gfrα1 shares 62% and 61% homology with the zebrafish paralogue pair, Gfrα1a and Gfrα1b, respectively while a lower identity value of 56%, 55% and 53% with human, mice and rat orthologues respectively (Fig. 11a).

Unlike medaka Gdnf, not every functionally identified motifs found within other vertebrates appear to be well conserved in the medaka Gfrα1. The three characteristic functional domain of GFRα1, namely the N-terminal hydrophobic domain representing a secretory signal peptide, GPI signal peptide and the C-terminal hydrophobic region could not be determined in the medaka as the corresponding regions shows poor homology in comparison. This is especially prominent at the C-terminal end where four polar amino acids, Glu^{457}, Gln^{459}, Lys^{461} and Gln^{464} were located in a supposing stretch of hydrophobic residues (Fig. 10). However, the number of cysteine residues in the receptor, like other homologues, remains constant at 31 although the last residue, Cys^{439} fails to align properly to its counterparts. Three putative N-glycosylation sites also appear to be conserved. The phylogenetic tree of Gfrα1 proteins from medaka and other organisms is illustrated in Fig 11b.

a


Ol GFRa1 (1) --------MILTFVIILSFTDSVFTLKDDHGSP----RLDCVKASKQCLKENACSTKYRTMRQCVKGRESNFSAVTGPEA
Dr GFRa1a (1) -------MFIAATYIILPLLDVLLSAEESYFSSSN--RLDCVKANELCLKEPGCSSKYRTMRQCVAGRESNFSMATGMEA
Dr GFRa1b (1) MDLNKATMIFATFWIMFPVLDLVHFSKADAIAQSRSVRLDCVRAHEQCLGKYGCSTKYRTMRQCVAGFTGNFSMKGEPEA
Rn GFRa1 (1) -------MFLATLYFALPLLDLLMSAEVSGGDR-----LDCVKASDQCLKEQSCSTKYRTLRQCVAGKETNFSLTSGLEA
Hs GFRa1 (1) -------MFLATLYFALPLLDLLLSAEVSGGDR-----LDCVKASDQCLKEQSCSTKYRTLRQCVAGKETNFSLASGLEA
Mm GFRa1 (1) -------MFLATLYFVLPLLDLLMSAEVSGGDR-----LDCVKASDQCLKEQSCSTKYRTLRQCVAGKETNFSLTSGLEA
Ol GFRa1 (69) QGECLSAIDAMKQSPLYNCRCRRGMKKEKNCLRIFWSLFQSLHGNDLLEYSPYEPVNSRLSDIFRLAPIIA----VEPAS
Dr GFRa1a (72) KDECRLVLDALKQSPLYNCRCKRGMKKEKNCLRIYWGIYQHLQGNDLLEDSPYEPVNSRLSDIFRLAPIYS----GEPAL
Dr GFRa1b (81) QDECRNAIESMKQSPLYDCKCRRGMKKEKNCLRIFWSIYQSLQANDLLEDSPYEPVNSRLSDIFRLAPIIS----GEAAF
Rn GFRa1 (69) KDECRSAMEALKQKSLYNCRCKRGMKKEKNCLRIYWSMYQSLQGNDLLEDSPYEPVNSRLSDIFRAVPFISDVFQQVEHI
Hs GFRa1 (69) KDECRSAMEALKQKSLYNCRCKRGMKKEKNCLRIYWSMYQSLQGNDLLEDSPYEPVNSRLSDIFRVVPFISDVFQQVEHI
Mm GFRa1 (69) KDECRSAMEALKQKSLYNCRCKRGMKKEKNCLRIYWSMYQSLQGNDLLEDSPYEPVNSRLSDIFRAVPFISDVFQQVEHI
Ol GFRa1 (145) AKENNCLNAAKACNLNDTCKKYRSTYINSCTSRVSASEVCNKRKCHKALREFFDKVPSKYSYGMLFCSCPAEDQKACAER
Dr GFRa1a (148) AKENNCLNAAKACNLNDTCKKYRSAYISPCTSRVSTAEVCNKRKCHKALRQFFDKVPPKHSYGMLYCSCPLGDQSACSER
Dr GFRa1b (157) TKDNNCLNAAKACNLNDTCKKYRSLYISPCTSRVSTTEVCNKRKCHKALRQFFDKVPPKHSYGMLFCSCPSGDHSACSER
Rn GFRa1 (149) SKGNNCLDAAKACNLDDTCKKYRSAYITPCTTSMSN-EVCNRRKCHKALRQFFDKVPAKHSYGMLFCSCR---DIACTER
Hs GFRa1 (149) PKGNNCLDAAKACNLDDICKKYRSAYITPCTTSVSN-DVCNRRKCHKALRQFFDKVPAKHSYGMLFCSCR---DIACTER
Mm GFRa1 (149) SKGNNCLDAAKACNLDDTCKKYRSAYITPCTTSMSN-EVCNRRKCHKALRQFFDKVPAKHSYGMLFCSCR---DVACTER
Ol GFRa1 (225) RRQTIVPVCSYEDKDKPNCLSLQNTCKTNYICRSRLLDFTSNCQPEVHSISGCFTENYADCLLAYSRLIGTVMTPNYVQS
Dr GFRa1a (228) RRQTIVPACSYEDKERPNCLTLQVSCKTNYICRSRLADFFTNCQPEPLSLSGCLKENYADCLLSYSGLIGTVMTPNYIRS
Dr GFRa1b (237) RRQTIVPACSYEDKEKPNCLSLQASCKTNYICRSRLADFLTNCQPEARSISGCLKENYADCLLAYSGLIGTVMTPNYLRA
Rn GFRa1 (225) RRQTIVPVCSYEERERPNCLSLQDSCKTNYICRSRLADFFTNCQPESRSVSNCLKENYADCLLAYSGLIGTVMTPNYVDS
Hs GFRa1 (225) RRQTIVPVCSYEEREKPNCLNLQDSCKTNYICRSRLADFFTNCQPESRSVSSCLKENYADCLLAYSGLIGTVMTPNYIDS
Mm GFRa1 (225) RRQTIVPVCSYEERERPNCLNLQDSCKTNYICRSRLADFFTNCQPESRSVSNCLKENYADCLLAYSGLIGTVMTPNYIDS
Ol GFRa1 (305) AGISLSPWCDCSSGGNSKQDCEKFAQFFTDNRCLRNAIQAFGNGTDVSMWHPRP-SMKPTVESGISR--RTKARLKNVLD
Dr GFRa1a (308) PKISVSPFCDCSSGGNSKEECDRFTEFFTDNACLRNAIQAFGNGTDVSVWHPMPPVQTTTSMTTPSQRARDKDRSPNAIE
Dr GFRa1b (317) PGISVSPWCDCSNGGNGKAECDKFTEFFTNNRCLRNAIQAFGNGTDVGVWQPQPPIMSTPADPYTPP--KGRDRTSNALD
Rn GFRa1 (305) SSLSVAPWCDCSNGGNDLEDCLKFLNFFKDNTCLKNAIQAFGNGSDVTMWQPAPPVQTTTATTTTATFRVKNKPLGPAGSE
Hs GFRa1 (305) SSLSVAPWCDCSNGGNDLEECLKFLNFFKDNTCLKNAIQAFGNGSDVTVWQPAFPVQTTTATTTTALRVKNKPLGPAGSE
Mm GFRa1 (305) SSLSVAPWCDCSNGGNDLEDCLKFLNFFKDNTCLKNAIQAFGNGSDVTMWQPAPPVQTTTATTTTAFRIKNKPLGPAGSE
Ol GFRa1 (382) TLTNVAKLED----SYGICETLQAQKLVSNQTVDMAPCLNHHLEESGTLNAISKSSP---AGLLCSQSPVLLLTLAFTFT
Dr GFRa1a (388) PATHINHLNPADNSLYQFCGNIQAQKKKTNNTIDVL-CVDPQIDDPSSSNTISKNSSPFQMTLSGLSSQLLLLATSLHCI
Dr GFRa1b (395) DPTLTNDLDSNADHLYSFCGSLQAQKLKSNVTLDVL-CVDQQLNDPSSFNAITRSS----TSAVCLVDWTVLLLLSLLSI
Rn GFRa1 (385) NEIPTHVLPP--------CANLQAQKLKSNVSGSTHLCLSDSDFGKDGLAGASSHITTKSMAAPPSCSLSSLPVLMLTAL
Hs GFRa1 (385) NEIPTHVLPP--------CANLQAQKLKSNVSGNTHLCISNGNYEKEGLG-ASSHITTKSMAAPPSCGLSPLLVLVVTAL
Mm GFRa1 (385) NEIPTHVLPP--------CANLQAQKLKSNVSGSTHLCLSDNDYGKDGLAGASSHITTKSMAAPPSCGLSSLPVMVFTAL
Ol GFRa1 (455) LTESQIKVFQLL 100%
Dr GFRa1a (467) FTPVML------ 62%
Dr GFRa1b (470) LHLDHLTASQTV 61%
Rn GFRa1 (457) AALLSVSLAETS 56%
Hs GFRa1 (456) STLLSLTETS-- 55%
Mm GFRa1 (457) AALLSVSLAETS 53%

b

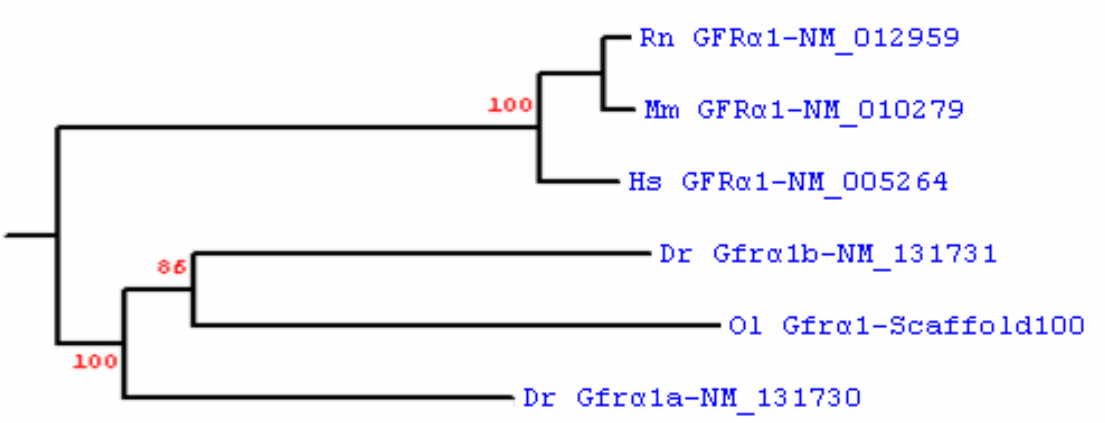

Rn GFRα1-NM_012959
Mm GFRα1-NM_010279
Hs GFRα1-NM_005264
Dr Gfrα1b-NM_131731
Ol Gfrα1-Scaffold100
Dr Gfrα1a-NM_131730
100
86
100

Fig. 11 Nucleotide and Amino Acid Sequences of the Gdnf Receptor.

(a) The nucleotide sequence of the medaka Gfrα1 cDNA and predicted translation product are shown. The exons are numbered by Roman numerals while ▼ demarcate exon-intron boundaries. Potential N-glycolsylation sites are marked with Δ and conserved cysteine residues are indicated with an asterisk. The last cysteine residue denoted by ● does not align well with the other homologues. The three characteristic functional domain of GFRα1, namely the N-terminal secretory signal peptide, GPI signal peptide and C-terminal hydrophobic region are underlined. However, the corresponding domains in medaka are currently undetermined as they do not appear to be conserved. (b) Phylogenetic tree of Gfrα1 proteins from medaka and other organisms. Bootstrap values at branching are given. The corresponding accession number is indicated after the species name while in the case of medaka, the scaffold number is given instead. Ol, *Oryzias latipes;* Dr, *Danio rerio*; Hs, *Homo sapiens*; Mm, *Mus Musculus*; Rn, *Rattus norvegicus*.

A BLAST search of the medaka genome database identified the isolated *gfrα1* to Scaffold100. In addition, the blast result also shows significant homology to Scaffold290, with a returned score of 82 Bits and an E-value of $5e^{-13}$, suggesting the possibility of a Gfrα1 paralogue in medaka although it could also well belong to any other member of the Gfrαs family. However, it seems unlikely that the paralogue, if present, is expressed in the testis as RT-PCR using other primers failed to reveal any other distinctive sequence.

Medaka Gfrα1 contains 10 exons (Table 3) and like its ligand, there are no splicing variants. On the other hand, human GFRα1 has two isoforms; the longer isoform has 11 exons while the shorter one has one less (Fig. 12).

Table 3 Structure of *gfrα1* with the predicted size of exons and introns and junction sequences.

Exon No.	Position in Scaffold 100	Exon 5′- 3′ end	Exon Size (bp)	Intron 5′-3′ end	Intron Size (bp)
1	975105-975391	AAAGCAGAGG-TCCTTCACGG	287	gtaagtgaat-ttgattgtag	239
2	975631-975927	ATTCGGTGTT-AGCTTGCATG	297	gtaggtttga-tgccctgcag	2381
3	978309-978392	GTAATGATTT-ATCATAGCTG	84	gtaagagttc-tttgtctcag	15425??
4	993818-993996	TCGAACCTGC-CTTTGACAAG	179	gtaatttttg-aaaaatcaag	230
5	994227-994399	GTTCCAAGTA-ACATATGCAG	173	gttagactta-ttttttaaag	739
6	995139-995248	GTCACGACTG-CGTCTTATTG	110	gtaagtttat-tcctctgcag	1475
7	996724-996858	GGACGGTGAT-CGCTGCCTGC	135	gtaagtaaca-tttcatgtag	364
8	997223-997407	GTAACGCCAT-AACCTTACAG	185	gtgatctgaa-gtcccctcag	2400
9	999808-999855	GCCCAAAAGC-TCCTTGTCTG	48	gtaagtccgg-gtttttccag	2922
10	1002778-1003817	AACCATCACC-TATACATTAA	1040		

The size of intron 3 is not determined as the medaka genomic sequence is currently still incomplete.

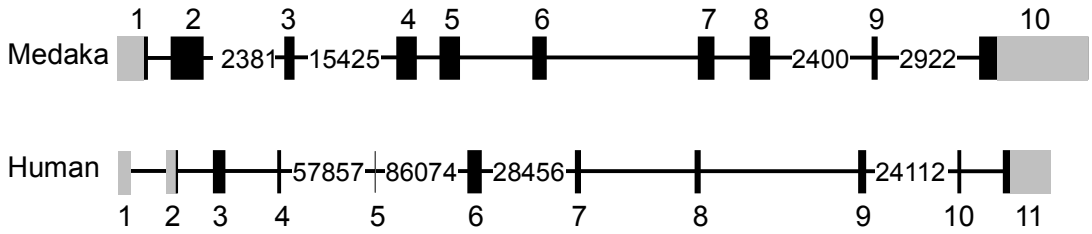


Fig. 12 Schematic diagram of medaka *gfrα1*gene structure in comparison to human.

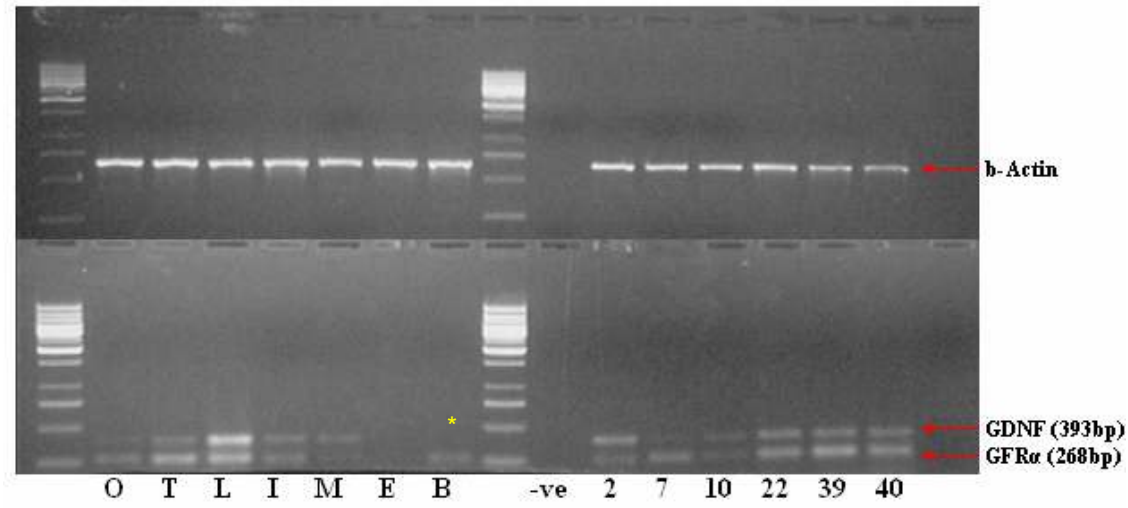
Untranslated exons are showed in grey box, translated exon in filled box and introns as solid lines. Like its ligand, the human GFRα1 has two isoforms; the longer isoform (Accession Number: NM_005264) has 11 exons while exon 5 is missing in the shorter isoform (NM_145793) Diagram is drawn to scale except for the introns whose size is given.

3.3 Expression Analysis

The *gdnf*- and/or *gfra1*-deficient mouse models are known to be neo-lethal (Naughton *et al.* 2006) and it appears that in medaka, both genes are also imperative to embryonic development. Both transcripts were present in all of the six embryonic stages investigated (Fig 13a) and expression was detected as early as the blastodisc stage. Although expression level steadily declines after that, a surge was detected at stage 22 and was maintained until hatching (Fig 13a). Such high level and virtually ubiquitous expression of the genes almost certainly point to a central role played by the Gdnf/Gfrα1 signalling pathway in the development of medaka embryos.

Gdnf expression also corresponds to Gfrα1 in tissues examined by RT-PCR, albeit in different levels (Fig. 13a). The disparity in expression is greatest in the brain where the ligand could only be faintly detected even after increasing the number of PCR cycles to 40 (Fig 13b). As the difference is so huge, it appears likely that another ligand belonging to the GFL family might be responsible for Gfrα1 signalling in the medaka brain. NRTN and ARTN are likely candidates (Fig. 2) although we could not entirely discount the possibility of a GDNF paralogue. Interestingly, an analysis of expression in zebrafish also failed to detect the presence of Gdnf in the brain (Shepherd *et al.* 2001). Hence, it seems very likely that the ligand-receptors crosstalk observed in other organism might also occur, at least in the case of medaka brain. Taken together, it is probable that GDNF might have acquired the ability to support the survival of several neuronal populations in the brain only very recently in evolution.

A



B

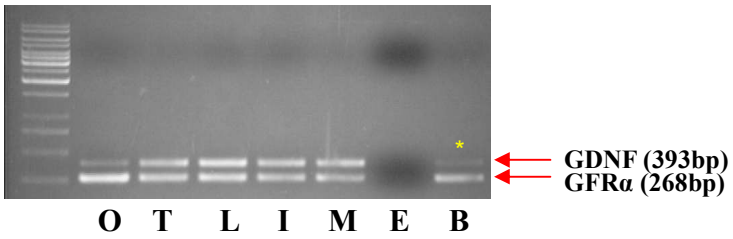


Fig. 13 Expression analysis of *gdnf* and *gfrα1* transcripts.

a) Both Gdnf and Gfrα1 were subjected to 35 PCR cycles across all tissues and embryonic stages studied. Expression of both transcripts was detected during stage 2, suggesting a maternal mRNA source. Expression then decrease steadily until a surge was detected at Stage 22, which is approximately equal to day one post-fertilization. Except for the brain, ligand expression coincides with that of the receptor in all of the tissues investigated. b) Faint Gdnf expression was detected in the brain only after the number of PCR cycles was increased to 40 (denoted by a yellow *).
b-Actin was used as calibrations (28 cycles) in the experiments. Number denotes the stage of the embryo while the size of the bands indicated in parentheses is determined through sequencing. Developmental stages of embryos are numbered according to Iwamatsu, 2004. O, ovary; T, Testis; L, Liver; I, Intestines; M, Muscle; E, Eyes; B, Brain; -ve, Negative Control;

CONCLUSION

The isolated Gdnf gene is 762 bp long and encodes for a putative protein of 253 aa. A Blast with the medaka EST and genomic database reveals that the full length cDNA is comprised of four exons and separated by three introns. In addition, sequence analysis also illustrates that most of the functional identified motifs of the gene are well conserved in medaka and transcriptional control likely to be similar to human.

The full length cDNA for the medaka *gfrα1* obtained by RACE is 2551 bp long and has a putative ORF of 1401 bp for 466 aa. The predicted protein shares the best homology to the known vertebrate GFRα1 proteins although several domains distinctive to the gene do not appear to be well conserved. Blast results revealed the gene has ten exons and the possibility of a paralogue in medaka.

Both genes are co-expressed throughout embryonic development and in most of the adult tissues studied. However, there is a huge disparity in the expression level of Gdnf compared to its alleged receptor in the brain. This suggests the presence of another binding ligand to Gfrα1 in the medaka brain and also probably, a different role for Gdnf in the medaka fish.

In conclusion, we report the successful isolation and characterization of two medaka genes, *gdnf* and g*frα1*. However, further study would be needed to determine if the GDNF-GFRα1-RET signalling pathway, like in other vertebrates, are also conserved in the medaka.

REFERENCES

Airaksinen MS, Saarma M (2002) The GDNF family: signalling, biological functions and therapeutic value. *Nat Rev Neurosci* **3**, 383-94.

Arenas E, Trupp M, Akerud P, Ibanez CF (1995) GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. *Neuron* **15**, 1465-73.

Baloh RH, Tansey MG, *et al.* (1997) TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret. *Neuron* **18**, 793-802.

Baloh RH, Tansey MG, *et al.* (1998) Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex. *Neuron* **21**, 1291-302.

Beck KD, Valverde J, Alexi T, Poulsen K, Moffat B, Vandlen RA, Rosenthal A, Hefti F (1995) Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature* **373**, 339-41.

Brown DA, London E (1998) Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol* **14**, 111-36.

Buj-Bello A, Adu J, Pinon LG, Horton A, Thompson J, Rosenthal A, Chinchetru M, Buchman VL, Davies AM (1997) Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. *Nature* **387**, 721-4.

Buj-Bello A, Buchman VL, Horton A, Rosenthal A, Davies AM (1995) GDNF is an age-specific survival factor for sensory and autonomic neurons. *Neuron* **15**, 821-8.

Clermont Y, Bustos-Obregon E (1968) Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted "in toto". *Am J Anat* **122**, 237-47.

Clermont Y, Hermo L (1975) Spermatogonial stem cells in the albino rat. *Am J Anat* **142**, 159-75.

Coulpier M, Anders J, Ibanez CF (2002) Coordinated activation of autophosphorylation sites in the RET receptor tyrosine kinase: importance of tyrosine 1062 for GDNF mediated neuronal differentiation and survival. *J Biol Chem* **277**, 1991-9.

Creedon DJ, Tansey MG, Baloh RH, Osborne PA, Lampe PA, Fahrner TJ, Heuckeroth RO, Milbrandt J, Johnson EM, Jr. (1997) Neurturin shares receptors and signal transduction pathways with glial cell line-derived neurotrophic factor in sympathetic neurons. *Proc Natl Acad Sci U S A* **94**, 7018-23.

De Rooij DG (1973) Spermatogonial stem cell renewal in the mouse. I. Normal situation. *Cell Tissue Kinet* **6**, 281-7.

De Rooij DG (1983) Proliferation and differentiation of undifferentiated spermatogonia in the mammalian testis. In *Stem cells. Their Identification and Characterization*. Ed Potten CS. Edinburgh: Churchill Livingstone; pp. 89-117

De Rooij DG (1998) Stem cells in the testis. *Int J Exp Pathol* **79**, 67-80.

De Rooij DG, Grootegoed JA (1998) Spermatogonial stem cells. *Curr. Opin. Cell Biol.,* **10**, 694-701.

De Rooij DG, Okabe M, Nishimune Y (1999) Arrest of spermatogonial differentiation in jsd/jsd, Sl17H/Sl17H, and cryptorchid mice. *Biol Reprod* **61**, 842-7.

Ebendal T, Tomac A, Hoffer BJ, Olson L (1995) Glial cell line-derived neurotrophic factor stimulates fiber formation and survival in cultured neurons from peripheral autonomic ganglia. *J Neurosci Res* **40**, 276-84.

Enokido Y, de Sauvage F, Hongo JA, Ninkina N, Rosenthal A, Buchman VL, Davies AM (1998) GFR alpha-4 and the tyrosine kinase Ret form a functional receptor complex for persephin. *Curr Biol* **8**, 1019-22.

Golden JP, Baloh RH, Kotzbauer PT, Lampe PA, Osborne PA, Milbrandt J, Johnson EM, Jr. (1998) Expression of neurturin, GDNF, and their receptors in the adult mouse CNS. *J Comp Neurol* **398**, 139-50.

Grimm L, Holinski-Feder E, Teodoridis J, Scheffer B, Schindelhauer D, Meitinger T, Ueffing M (1998) Analysis of the human GDNF gene reveals an inducible promoter, three exons, a triplet repeat within the 3'-UTR and alternative splice products. *Hum Mol Genet* **7**, 1873-86.

Henderson CE, Phillips HS*, et al.* (1994) GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* **266**, 1062-4.

Hofmann MC, Braydich-Stolle L, Dym M (2005) Isolation of male germ-line stem cells; influence of GDNF. *Dev Biol* **279**, 114-24.

Homma S, Oppenheim RW, Yaginuma H, Kimura S (2000) Expression pattern of GDNF, c-ret, and GFRalphas suggests novel roles for GDNF ligands during early organogenesis in the chick embryo. *Dev Biol* **217**, 121-37.

Hong Y, Liu T, Zhao H, Xu H, Wang W, Liu R, Chen T, Deng J, Gui J (2004) Establishment of a normal medakafish spermatogonial cell line capable of sperm production in vitro. *Proc Natl Acad Sci U S A* **101**, 8011-6.

Hong Y, Winkler C, Schartl M (1998) Production of medakafish chimeras from a stable embryonic stem cell line. *Proc Natl Acad Sci U S A* **95**, 3679-84.

Huckins C (1971) The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. *Anat Rec* **169**, 533-57.

Ibanez CF (1998) Emerging themes in structural biology of neurotrophic factors. *Trends Neurosci* **21**, 438-44.

Ichihara M, Murakumo Y, Takahashi M (2004) RET and neuroendocrine tumors. *Cancer Lett* **204**, 197-211.

Ishikawa Y (2000) Medakafish as a model system for vertebrate developmental genetics. *Bioessays* **22**, 487-95.

Iwamatsu T (2004) Stages of normal development in the medaka Oryzias latipes. *Mech Dev* **121**, 605-18.

Jing S, Wen D*, et al.* (1996) GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. *Cell* **85**, 1113-24.

Jing S, Yu Y*, et al.* (1997) GFRalpha-2 and GFRalpha-3 are two new receptors for ligands of the GDNF family. *J Biol Chem* **272**, 33111-7.

Kotzbauer PT, Lampe PA, Heuckeroth RO, Golden JP, Creedon DJ, Johnson EM, Jr., Milbrandt J (1996) Neurturin, a relative of glial-cell-line-derived neurotrophic factor. *Nature* **384**, 467-70.

Kubota H, Avarbock MR, Brinster RL (2004) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc Natl Acad Sci U S A* **101**, 16489-94.

Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**, 1130-2.

Lok D, Weenk D, De Rooij DG (1982) Morphology, proliferation, and differentiation of undifferentiated spermatogonia in the Chinese hamster and the ram. *Anat Rec* **203**, 83-99.

Matsushita N, Fujita Y, Tanaka M, Nagatsu T, Kiuchi K (1997) Cloning and structural organization of the gene encoding the mouse glial cell line-derived neurotrophic factor, GDNF. *Gene* **203**, 149-57.

Meng X, Lindahl M*, et al.* (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* **287**, 1489-93.

Milbrandt J, de Sauvage FJ*, et al.* (1998) Persephin, a novel neurotrophic factor related to GDNF and neurturin. *Neuron* **20**, 245-53.

Mount HT, Dean DO, Alberch J, Dreyfus CF, Black IB (1995) Glial cell line-derived neurotrophic factor promotes the survival and morphologic differentiation of Purkinje cells. *Proc Natl Acad Sci U S A* **92**, 9092-6.

Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brinster RL (2003) Maintenance of mouse male germ line stem cells in vitro. *Biol Reprod* **68**, 2207-14.

Naughton CK, Jain S, Strickland AM, Gupta A, Milbrandt J (2006) Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. *Biol Reprod* **74**, 314-21.

Nishimura A, Morita M, Nishimura Y, Sugino Y (1990) A rapid and highly efficient method for preparation of competent Escherichia coli cells. *Nucleic Acids Res* **18**, 6169.

Oakberg EF (1971) Spermatogonial stem-cell renewal in the mouse. *Anat Rec* **169**, 515-31.

Oppenheim RW, Houenou LJ, Johnson JE, Lin LF, Li L, Lo AC, Newsome AL, Prevette DM, Wang S (1995) Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature* **373**, 344-6.

Panicker AK, Buhusi M, Thelen K, Maness PF (2003) Cellular signalling mechanisms of neural cell adhesion molecules. *Front Biosci* **8**, d900-11.

Paratcha G, Ledda F, Baars L, Coulpier M, Besset V, Anders J, Scott R, Ibanez CF (2001) Released GFRalpha1 potentiates downstream signaling, neuronal survival, and differentiation via a novel mechanism of recruitment of c-Ret to lipid rafts. *Neuron* **29**, 171-84.

Paratcha G, Ledda F, Ibanez CF (2003) The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell* **113**, 867-79.

Sanicola M, Hession C*, et al.* (1997) Glial cell line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins. *Proc Natl Acad Sci U S A* **94**, 6238-43.

Sariola H, Saarma M (2003) Novel functions and signalling pathways for GDNF. *J Cell Sci* **116**, 3855-62.

Shepherd IT, Beattie CE, Raible DW (2001) Functional analysis of zebrafish GDNF. *Dev Biol* **231**, 420-35.

Shima A, Mitani H (2004) Medaka as a research organism: past, present and future. *Mech Dev* **121**, 599-604.

Tansey MG, Baloh RH, Milbrandt J, Johnson EM, Jr. (2000) GFRalpha-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. *Neuron* **25**, 611-23.

Tegelenbosch RA, de Rooij DG (1993) A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. *Mutat Res* **290**, 193-200.

Tomac A, Lindqvist E, Lin LF, Ogren SO, Young D, Hoffer BJ, Olson L (1995) Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. *Nature* **373**, 335-9.

Tooze SA, Martens GJ, Huttner WB (2001) Secretory granule biogenesis: rafting to the SNARE. *Trends Cell Biol* **11**, 116-22.

Treanor JJ, Goodman L*, et al.* (1996) Characterization of a multicomponent receptor for GDNF. *Nature* **382**, 80-3.

Trupp M, Ryden M, Jornvall H, Funakoshi H, Timmusk T, Arenas E, Ibanez CF (1995) Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. *J Cell Biol* **130**, 137-48.

van de Ven WJ, Voorberg J, Fontijn R, Pannekoek H, van den Ouweland AM, van Duijnhoven HL, Roebroek AJ, Siezen RJ (1990) Furin is a subtilisin-like proprotein processing enzyme in higher eukaryotes. *Mol Biol Rep* **14**, 265-75.

Wakamatsu Y, Pristyazhnyuk S, Kinoshita M, Tanaka M, Ozato K (2001) The see-through medaka: a fish model that is transparent throughout life. *Proc Natl Acad Sci U S A* **98**, 10046-50.

Widenfalk J, Nosrat C, Tomac A, Westphal H, Hoffer B, Olson L (1997) Neurturin and glial cell line-derived neurotrophic factor receptor-beta (GDNFR-beta), novel proteins related to GDNF and GDNFR-alpha with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. *J Neurosci* **17**, 8506-19.

Wittbrodt J, Shima A, Schartl M (2002) Medaka--a model organism from the far East. *Nat Rev Genet* **3**, 53-64.

Yan Q, Matheson C, Lopez OT (1995) In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons. *Nature* **373**, 341-4.

Yomogida K, Yagura Y, Tadokoro Y, Nishimune Y (2003) Dramatic expansion of germinal stem cells by ectopically expressed human glial cell line-derived neurotrophic factor in mouse Sertoli cells. *Biol Reprod* **69**, 1303-7.