Systematic Study on the Interaction among GH/PRL Family Hormones with Their Receptors and the Role of PRLR1 in Zebrafish Development

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Abstract

In fish, growth hormone (GH), prolactin (PRL) and somatolactin (SL) are members of a gene family of polypeptide hormones which share homology in protein sequence and structure. To date, numerous functions have been attributed to this family of hormones such as growth, immune response, protein metabolism and ion regulation. The biological functions of GH/PRL are mediated through binding of the ligands on their respective receptors. It is believed that this gene family arose as the result of multiple gene duplications and subsequent divergent evolution, co-evolving with their corresponding receptors. Despite the above mentioned similarities in their structures, their cognate receptors and their signaling mechanisms, important differences among this gene family of polypeptide hormones can be recognized in their biological functions.

Bioinformatic searching on the zebrafish genome indicates that there are five members of this hormone family (namely GH, SLα, SLβ, PRL1 and PRL2) and four receptors (namely GHR1, GHR2, PRLR1 and PRLR2). However, it should be noted that these ligands and receptors are only named according to their sequence homology with those in other species. There is so far no systematic study to unravel the relationship among the ligands and receptors. The last point is particularly relevant as some of the ligands and receptors are duplicated in the fish genome. In addition, there is much controversy regarding whether one of the two GHRs is in fact the receptor for SL. A systematic study on the interaction among the ligands and receptors in zebrafish would help to resolve these issues.

In the present study, the luciferase reporter assay, His-tag pulldown assay and signaling pathway activation were employed to investigate the interaction among the ligands and their receptors. It was shown that recombinant zebrafish GH, PRL1 and PRL2 could only interact with their cognate receptors, i.e. GHR1, GHR2, PRLR1 and PRLR2 respectively. In comparison, zebrafish SLα and SLβ could neither interact with GHR1, GHR2, PRLR1 and PRLR2 in the binding study, nor could these two SLs activate the receptor-mediated downstream signaling and transcriptional activities of the four receptors in zebrafish. These data argue against the hypothesis that GHR1 is the SL receptor.

The role of PRLR in early development of zebrafish was also explored. Whole mount in situ hybridization (WISH) study showed that PRLR1 was mainly expressed in the pancreas and pronephric duct, while PRLR2 was expressed in the pronephric duct only. In the PRLR1 morpholino (MO) knockdown embryos, the yolk extension (YE), the formation of which was reported to be associated with pronephric duct development, disappeared at 24 hours post fertilization. This phenotype could not be observed in the PRLR2 MO knockdown or control embryos. Real time quantitative RT-PCR and WISH data revealed that several genes expressed in the pronephric duct were up or down-regulated. The protein expression pattern of pronephric duct marker atp1a1 was also affected in the embryos injected with PRLR1 MO. In addition, histological studies showed that structure of the pronephric duct was destroyed in the PRLR1 MO embryos. These results suggest that PRLR1 plays an important role in the development of the pronephric duct in zebrafish embryos.

摘要

在鱼类中，生长激素（GH），催乳素（PRL）和生长催乳素（SL）在蛋白质序列和结构上具有高度的相似性，它们因此组成了一个多肽激素家族,即 GH/PRL 激素家族。研究表明这个激素家族参与了各种生理功能，比如生长，免疫反应，蛋白质新陈代谢和离子调控等等。这些激素的生理功能是通过它们相对应的受体介导实现的。目前普遍的观点认为这些激素是由同一个原始基因经过多次的复制以及随后的分化而来的。虽然这些激素在结构上的相似度很高，但是它们在生理功能上还是存在着一些差异。

我们通过对斑马鱼（*Danio rerio*）基因组分析发现它的基因组上存在 5 个 GH/PRL 家族激素（包括 GH， SLα，SLβ， PRL1 和 PRL2）和 4 个相对应的受体(包括 GHR1， GHR2， PRLR1 和 PRLR2)，但是需要特别指出的是这些激素和受体仅仅是根据进化树分析（Phylogenetic analysis）来命名的，还缺乏实验上的证据。目前还没有人对这个家族的激素和受体之间的相互关系进行系统化的研究。另外，有人认为 GHR1 实际上是 SL 的受体，而 GHR2 才是真正的 GH 受体。这个猜测在学术上存在着很大的争议。

本论文利用了一系列的实验手段来研究以上提出的问题。 首先我利用了细菌蛋白质表达纯化系统制备了相应的重组斑马鱼激素和受体蛋白。 His 标签结合实验（His-tag pulldown assay）证实了 GH，PRL1 和 PRL2 只能结合到各自的受体，而 SLα和 SLβ则不能够结合任何一个受体。荧光素酶报告基因实验

（Luciferase reporter assay）和下游信号蛋白激活的检测实验（Detection of signaling pathway activation）也充分证实了 SLα和 SLβ不能激活 GHR1 介导的信号通路以及下游启动子活性。这些实验证实了至少在斑马鱼中，GHR1 并不是 SL 的受体，真正的 SL 受体的发现有待于进一步的研究。

以此同时，我也研究了 PRLR1 在斑马鱼早期发育中的作用。利用整胚原位杂交技术（WISH）我发现 PRLR1 和 PRLR2 都在斑马鱼的原肾管（pronephric duct）中表达。 PRLR1 Morpholino 沉默（MO knockdown）的斑马鱼胚胎的卵黄延长（YE）不能正常发育， 而 PRLR2 MO 沉默和对照的斑马鱼都没有出现这种表型。进一步的 WISH 和整胚原位免疫荧光研究 （whole mount *in situ* immunostaining）表明在 PRLR1 MO 注射下， 在原肾管中表达的标记基因（包括 pax2a，slc12a3 和 atp1a1）的表达量减少了。组织染色分析也表明 PRLR1 MO 的注射导致斑马鱼的原肾管结构遭到破坏。因此，本实验证实了 PRLR1 对于斑马鱼原肾管的早期发育具有很重要的作用。

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aa	Amino acid
ACTH	Adrenocorticotropic hormone
ADH	Antidiuretic hormone
Ap	ampicillin
AP-1	Activating Protein-1
BSA	Bovine serum albumin
C	Cysteine
CHD	Cytokine receptor homology domain
cv	Cardinal vein
da	Dorsal aorta
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
dpf	Day post fertilization
ECD	Extracellular domain
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
FSH	Follicle-stimulating hormone
GH	Growth hormone
GHR	Growth hormone receptor
GHR1	Growth hormone receptor 1
GHR2	Growth hormone receptor 2
gl	Glomerulus
Grb2	growth factor receptor-bound protein 2
GST	Glutathione S-transferase
HAART	Highly aggressive anti-retroviral therapy
hpf	Hour post fertilization
ICD	Intracellular domain
IGF	Insulin-like factor
IGFBP	Insulin-like factor binding protein
IL-2	Interleukin-2
IPTG	Isopropylthio-β-D-galatoside
IRS-1	Insulin-receptor substrate-1
JAK	Janus kinase
kDa	Kilodalton
LB	Luria-Brertani
LH	Luteinizing hormone
MAPK	mitogen-activated protein kinase
MeOH	Methanol
MO	Morpholino
MSH	Melanocyte-stimulating hormone
MYA	Million years ago

N	Asparagine
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PL	Placental lactogen
PLC	Phospholipase-C
PLR	Placenta lactogen receptor
PRL	Prolactin
PRLR	Prolactin receptor
PRLR1	Prolactin receptor 1
PRLR2	Prolactin receptor 2
Pr-RP	Prolactin-releasing hormone peptide
pt	Pronephric tubules
PTU	1-phenyl-2-thiourea
RT-PCR	Reverse Transcription–Polymerase chain reaction
SDS	Sodium dodecyl sulfate
SH2	Src-homology 2
SHC	Src homology 2 domain containing transforming protein
SIE	Sis-inducible element
SL	Somatolactin
SLa	Somatolactin α
SLβ	Somatolactin β
SLR	Somatolactin receptor
Sos	Son-of-sevenless
Stat	Signal transducers and activators of transcription
TMD	Transmembrane domain
WGD	Whole genome duplication
WISH	Whole mount in situ hybridization
Y	Tyrosine
YE	Yolk extension

Chapter 1

General introduction

The vertebrate pituitary, which is located at the base of the brain, is a pea-sized "master" endocrine gland as it is capable of secreting a variety of important hormones to regulate the functions of other endocrine glands such as thyroid gland, thymus, adrenal gland and gonads (Dorton, 2000). In human, the pituitary is composed of two distinctive regions, viz. anterior pituitary (adenohypophysis) and posterior pituitary (neurohypophysis) (Castillo, 2005). The hormones produced by the pituitary include thyroid stimulating hormone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), growth hormone (GH), prolactin (PRL), adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone (MSH), oxytocin, antidiuretic hormone (ADH), etc. (Moore et al., 2002). Among them, GH and PRL belong to the GH/PRL family, the hormones which regulate numerous physiological processes in vertebrates such as growth, metabolism, reproduction, osmoregulation, immune function and behavior (Forsyth and Wallis, 2002).

1.1 Members of the GH/PRL family of hormones

It has become clear that the GH/PRL family contains GH, PRL, placental lactogen (PL), somatolactin (SL), and GH or PRL-related genes as well (Forsyth and Wallis, 2002; Haig, 2008). Both GH and PRL have been described in all vertebrate groups. Although GH and PRL are mainly synthesized from the pituitary and secreted to other tissues, there is evidence showing that they are also produced in other organs such as gonad, kidney, mammary gland and placenta (Anthony et al., 1995; Ho et al., 1985). Researchers have discovered a fragment of human genome in

length of about 47 kilobases as the human growth hormone locus, which contains 5 genes related to GH. Among them, two different *GH* genes are responsible for two GH subtypes, namely GH1 which is mainly expressed in the pituitary and GH2 which is expressed in the placenta (Chen et al., 1989). The main protein encoded by the *GH1* gene is the 22K-GH, which is about 22 kilodalton (kDa) in size. Alternative mRNA splicing of *GH1* further generates different isoforms of the 22K-GH, including 20K-GH and 17.5K-GH (DeNoto et al., 1981). GH-V, the product of *GH2* gene, has 191 amino acids and structurally similar to the 22K-GH. The discovery of the GH-V isoform 20K-GH-V has been reported in the human placenta (Boguszewski et al., 1998). Based on phylogenetic analysis, the GH in lower vertebrates such as in fish can be branched into two clusters, viz. GH1 and GH2. In mammals there appears to be only one single gene for PRL, which comprises five exons split by four introns. The *PRL* gene is about 10 kilobases in length, which is much larger than the *GH* gene due to much longer introns. It is noteworthy that different from the multiple GH-like genes in higher primates, human and other higher primates only have a single PRL gene. However, complex clusters of PRL-like genes occur in ruminants and rodents, giving rise to PRL-like proteins of ill-defined function. The organization of these gene clusters has not yet been reported in details. In lower vertebrates, evidence of complex clusters of PRL-like genes has not been reported. Duplications of PRL genes give rise to different subtypes of PRL in fish, including PRL1 and PRL2 (Bole-Feysot et al., 1998; Huang et al., 2009). PL, which is secreted in the placenta and has significant influence during pregnancy, is so far discovered only in mammals (Forsyth, 1994), while SL is uniquely found in fish. There are two distinctive SL subtypes produced in the pars intermedia of the pituitary, viz. SLα and SLβ. SLα has been proven to exist in all the fish while SLβ is

discovered in freshwater fish (Fukada et al., 2005).

1.2 Evolution of the GH/PRL family of hormones

Vertebrate GH and PRL are believed to arise as a result of gene duplications from a common ancestral gene and subsequent divergent evolution (Cooke et al., 1981; Niall et al., 1971). The divergence of GH and PRL lineages may occur at some 400 million years ago (Cooke et al., 1980; Cooke et al., 1981). SL occurs in fish including lungfish and white sturgeon, and this presumably reflects another duplication event (Amemiya et al., 1999; May et al., 1999). Some scientists hypothesize that the GH/PRL family have a prevertebrate origin, but the nature of this has not yet been discovered. As the matter of fact, proteins related to GH or PRL have not been convincingly described in any invertebrate, including *Caenorhabditis elegans* and *Drosophila melanogaster*, although their complete genome sequences of them are available. Recently, GH but not PRL or other members of the GH/PRL family has been identified in lamprey, which is an extant representative of the most ancient class of vertebrates, indicating that GH is most likely the ancestral hormone in the molecular evolution of this family (Kawauchi et al., 2002). However, the authors can not rule out the possible existence of PRL or PRL-like protein in lamprey. Therefore, more evidence is required to support the notion that GH is the ancestral gene of this family. It is proposed that GH, PRL and SL genes arose during two rounds of genomic duplication during chordate evolution (Sankoff, 2001), while PL is considered to come from GH or PRL during evolution of the mammals (Walker et al., 1991). A fish specific genome duplication was proposed after the discovery of seven *Hox* gene clusters in fish, which was almost twice as many in mammals and this duplication event was hypothesized to happen in the Actinopterygia (Amores et

al., 1998; Postlethwait et al., 1998; Prince et al., 1998). This hypothesis is becoming widely accepted after the sequencing of zebrafish and pufferfish genomes (Van de Peer, 2004). Therefore, subtypes of GH, PRL and SL generally exist in fish. It was also reported that not only GH and PRL, but also SL were discovered in lungfish, whose ancestors evolved to the land vertebrates. However, SL was not found in the land vertebrates after numerous attempts in the screening and cloning of SL from mouse and human genomes. This suggests that SL may be lost during evolution to the land vertebrates (Kawauchi and Sower, 2006). Based on current studies and related evolutionary information, a hypothetical evolutionary model of the GH/PRL family has been generated as shown in Figure 1.1.

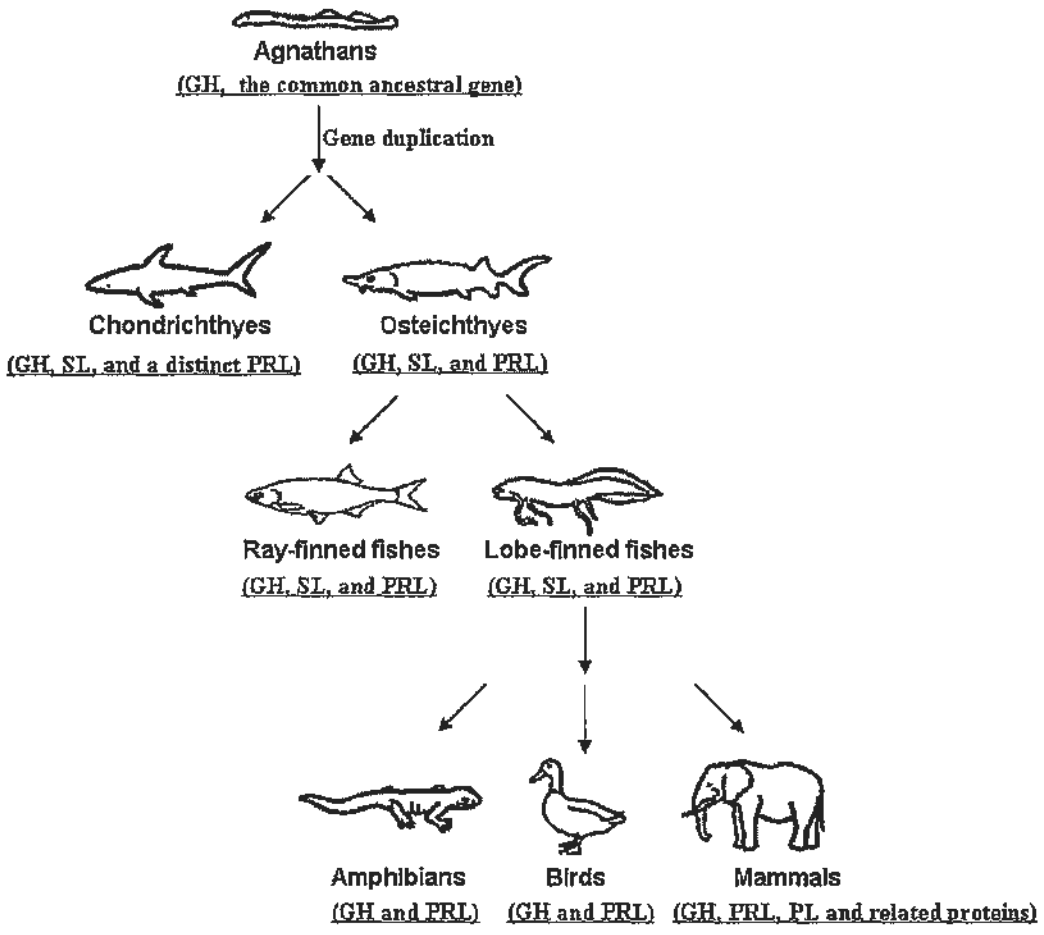


Fig. 1.1 A hypothetical evolutionary model of the GH/PRL family (Amemiya et al., 1999; Kawauchi and Sower, 2006 and references therein).

The molecular evolution of GH and PRL exhibits two particularly interesting features, variable evolutionary rates, which underlie species specificity; and multiple gene duplications, which give rise to the clusters of GH- and PRL-like genes. Phylogenetic analysis illustrates that the sequence of mammal GH is very conserved as compared to that of teleost GH. For example, the sequence of pig GH is almost identical to that of the dog and is also highly similar to that of the horse, elephant and human GH (Forsyth and Wallis, 2002). However, sequences of teleost GHs differ markedly from the pig GH sequences and the primitive fish, the sturgeon. Moreover, the sequences of teleost GHs are also different from each other, suggesting that the evolutionary rate of GH in teleosts is high and variable. The evolutionary pattern of PRL is similar to that of GH. In fish, PRL shows great variation and differences in the sequences. This is not only confined to teleosts, as PRL of sturgeon also shows considerate differences to that of other teleosts (Forsyth and Wallis, 2002; Noso et al., 1993).

1.3 Structure of the GH/PRL family of hormones

As all the GH/PRL family hormones arose from a common ancestral gene, they share very high similarities in genomic organization and protein structure. The classical GH and PRL genes include a 5'-flanking region which contains several gene promoters and regulatory elements, five exons split by four introns and a 3'-untranslated region (Figure 1.2).

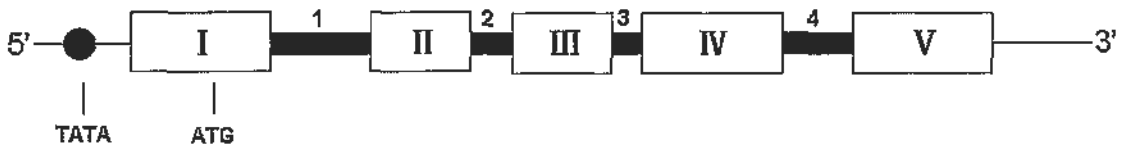


Fig. 1.2 A schematic map of GH genomic organization (not to scale). 5' and 3' flanking regions are indicated as black thin lines. The solid black circle represents the TATA box. The white rectangles with Roman letters indicate the exons and solid black rectangles denote the introns.

Sequences of GH, PRL and SL have been determined using DNA sequencing and direct protein sequencing. All members of the GH/PRL family are single chain polypeptides comprised of about 200 amino acids (aa) with molecular mass of 22-23 kDa in most vertebrate species, ranging from fish to mammals (Kossiakoff, 2004). They have two to three disulfide bonds whose location is conserved among different species. GH of most species contains two disulfide bonds, one existing at the C-terminus and the other one connecting a distant part of the protein. Tetrapod PRLs and fish PRL2, which was recently discovered in our laboratory, harbor six cysteines forming three disulfide bonds, two of those exist in corresponding positions as that of GH, and the additional one forms a loop at the N-terminus. Fish PRL1 is shorter than mammal PRLs and lacks several residues including two cysteines which abolish the formation of the third disulfide bond at the N-terminus (Huang et al., 2009; Keeler et al., 2003). The protein sequence of the N-terminal region of SL and the location of the two cysteines forming a disulfide bond in this region bear some resemblance to tetrapod PRLs and fish PRL2 but not fish PRL1 (Huang et al., 2009; Ono et al., 1990). Post-translational modifications of mature GH and PRL have been reported, including phosphorylation, proteolytic cleavage and glycosylation (Berghman et al., 1987; Ho et al., 1993; Sinha, 1995; Walker, 1994).

The three-dimensional structures of mammalian GH and PRL by X-ray crystallography have already been resolved (Abdel-Meguid et al., 1987; Cunningham et al., 1991; Teilum et al., 2005). These secondary structure studies have demonstrated that GH and PRL have a common characteristic, four α-helix bundles in an up-up-down-down topology. These α-helix bundles in a genetically engineered variant of porcine growth hormone are located in residues 7-34, 75-87, 106-128 and 153-183 (Abdel-Meguid et al., 1987). Small but significant structural differences between GH and PRL can also be recognized. Due to the additional disulfide bond at the N-terminus, the structure of PRL is much more compact than that of GH. Examination of the crystal structure of the complex between human GH and the extracellular domain of its receptor showed that the complex consisted of one molecule of GH per two molecules of receptor. The receptor-binding sites on GH are located on the faces of opposite sides of the four helix bundles. The first binding site on GH (site 1) is formed by the residues on exposed faces of mainly helix 4 and helix 1, together with residues in the connecting region between helix 1 and 2. The majority of second binding site on the GH (site 2) is made up of the exposed faces of helix 1 and 3 (de Vos et al., 1992). The relative extents of the binding areas support a sequential mechanism for receptor dimerization and downstream signaling pathway activation. A schematic map of the structure of GH is shown in Figure 1.3 (Wells and de Vos, 1993). Similarly, the structural studies also showed that one molecule of PRL interacted with two molecules of its receptor (Freeman et al., 2000). To date, attempts to resolve the three-dimensional structure of SL have failed. However, taking advantage of homology modeling approach based on the known crystallographic structure of GH and PRL, the SL structure was predicted, which is

similar to those of GH and PRL (Jiang et al., 2008).

GH and PRL exhibit some degree of species specificities. In many cases, the GH from nonprimates is not active in human, which hinders the treatment of human hypopituitary dwarfism using GH from other sources (Behncken et al., 1997). Studies also show that nonprimate GHs bind poorly to the GHR from human tissues. On the other hand, human GH shows comparable receptor-binding and growth-promoting activities in nonprimates (Souza et al., 1995). PRL also shows notable species variation. Teleost PRLs are not active in human, but human PRL is active in fish (Nicoll et al., 1986).

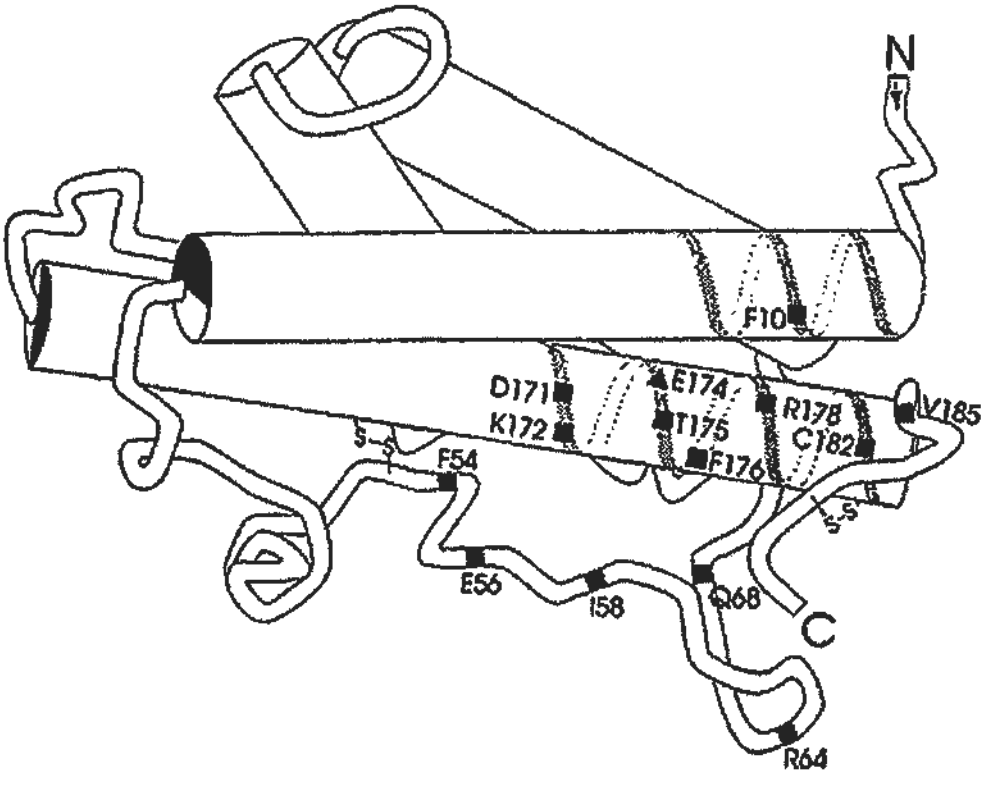


Fig. 1.3 A schematic diagram of human GH (Cunningham et al., 1989). Approximate location of residues in hGH that when mutated cause greater than fourfold weakening (■) or strengthening (▲) of the binding affinity of hormone to

its receptor. The four α-helical bundles are shown in straight columns. The N- and C-terminals are labeled as "N" and "C", respectively.

1.4 Receptors for the GH/PRL family of hormones

Cloning and sequencing of cDNA for the membrane-anchored GH receptor (GHR) and PRL receptor (PRLR) have been accomplished in a number of non-mammalian and mammalian species, including fish, reptiles, birds, and mammals (Goffin and Kelly, 1997; Kelly et al., 1991). These receptors belong to the large superfamily of class I cytokine receptors, which lack the intrinsic tyrosine kinase activity. Although PRLR and GHR show limited overall sequence identity (~30%), their tertiary structures are highly similar. Both GHR and PRLR contain three functional domains, extracellular domain (ECD), transmembrane domain (TMD) and intracellular domain (ICD). Most of the sequence similarities between the two types of receptors are found within their ECD (Wells and de Vos, 1996). Typically, a GHR or PRLR ECD consists of the cytokine receptor homology region, which can be further divided into two subdomains, D1 and D2. Two or three pairs of disulfide bonds in the N-terminal extracellular D1 subdomain are involved in maintaining structural and functional properties in GHR and PRLR, and a tryptophan-serine (WS motif) of the subdomain D2 which is close to the TMD confers high affinity binding of the receptor to the ligand. PRLRs of chickens and pigeons are somehow different, which contain an additional cytokine homology region. However, this additional region shows no significant effect on the ligand-binding affinity and specificity or downstream signal pathway activation (Chen and Horseman, 1994). TMD of the receptor is a single-pass transmembrane domain, which contains about 24 aa (Kelly et al., 1991). Although the ICDs of GHR

and PRLR show relatively lower sequence similarity, two regions of the domain, Box 1 and Box 2, are conserved (Goffin and Kelly, 1997). Box 1, which is enriched in prolines and hydrophobic residues, is crucial for the recognition of the signaling molecules. Box 2, which begins with several hydrophobic residues and ends with positively charged residues, is located approximately 30 aa C-terminal to Box 1 and spans about 15 aa (Kelly et al., 1991). Mutation or deletion of Box 1 and/or Box 2 results in reduced or abolished downstream signaling, suggesting the essential roles of these two boxes in the signaling transduction (Bazan, 1989; Fukunaga et al., 1993). The three-dimensional structure of PRLR ECD has been determined by crystallographic analysis. D1 and D2 domains are linked by a single four-residue segment of polypeptide chain. Each chain contains seven beta-sheets that together form a sandwich of two anti-parallel beta-sheets (de Vos et al., 1992; Somers et al., 1994). A schematic map of rat growth hormone receptor is shown in Figure 1.4 (Wells and de Vos, 1993).

It is widely accepted that the GH/PRL receptor family arose as the result of multiple gene duplications and subsequent divergent evolution, coevolving with the corresponding superfamily of ligands (Kelly et al., 1991). The gene duplication that gave rise to the separate receptors for GH and PRL presumably occurred at about the same time as that giving rise to the cognate ligands, and subsequent divergent evolution has been fairly conserved in nature, with the development of different binding specificities, but retention of general mechanisms of hormone-receptor interaction and possible signal transduction.

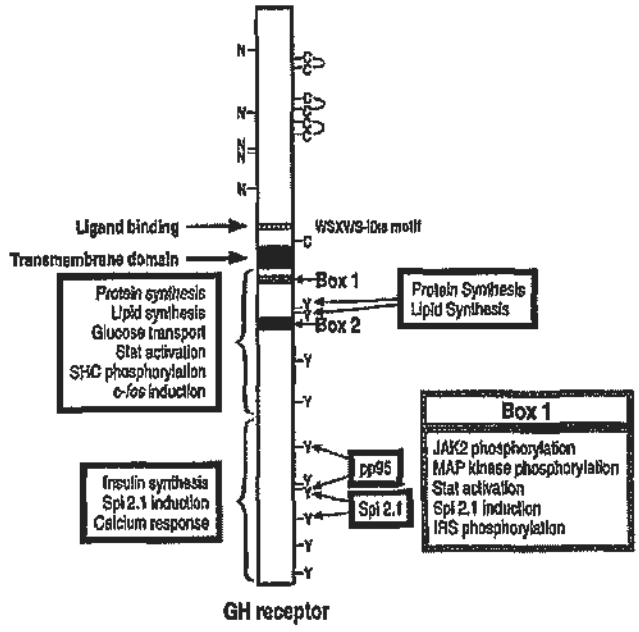


Fig. 1.4 A schematic representative of the growth hormone receptor (Wells and de Vos, 1993). The transmembrane domain is shown in black. The extracellular asparagines (N) that are potential N-linked glycosylation sites are shown on the left. The 10 cytoplasmic tyrosines (Y) present in rat GHR and 7 extracellular cysteines (C) are shown on the right with the 3 pairs of linked cysteines indicated. The position of the WSXWS-like motif (WS motif) is indicated by the striped box. Intracellular Box 1 (proline-rich domain) and Box 2 are shown as gray boxes. Regions of GHR shown to be required for various functions are indicated.

Gene duplications are considered as the main factor responsible for the functional diversification of genes, the creation of gene families and the expansion of genome (Urbain, 1969). Vertebrates evolved by whole genome duplications (WGD), while fish (especially teleost) had undergone an additional WGD, the fish-specific genome duplication, which probably happened after the bichirs (Polyteriformes),

sturgeons (Acipenseriformes), gars and bowfins (Semionotiformes) branched off from the fish stem lineage (Hoegg et al., 2004; Meyer and Van de Peer, 2005). Therefore, more than one copies of both *GHR* and *PRLR* genes were maintained by selection through radiation in fish and the concurrence of two GHRs and PRLRs exist in fish (Fukada et al., 2004; Fukamachi and Meyer, 2007; Huang et al., 2007; Jiao et al., 2006). Based on phylogenetic analysis, these receptors can be clustered into two clades, viz. GHR1/PRLR1 and GHR2/PRLR2. In salmonids, both GHRs identified so far belong to the GHR2 clade. It is proposed that there are even more GHR in salmonids due to the fourth genome duplication particularly happened in salmonids (Jiao et al., 2006). This hypothesis is supported by the fact that salmonids possess about 16 *Hox* family genes which is almost as twice as that of the other teleosts (Moghadam et al., 2005). On the other hand, mammals contain only one copy of GHR and PRLR genes. Different subtypes of mammalian GHR and PRLR proteins are given rise from alternative splicing of the precursor mRNAs or proteolysis, which varies between species and tissues (Edens et al., 1994; Guan et al., 2001). For example, rat PRLR shows multiple forms of the 5' untranslated region. Three PRLR gene promoters have been recognized that are used in a tissue-specific manner. Subtypes of PRLR have been isolated, identical in the ECD, but different in the ICD. These are termed long, intermediate, and short forms. A long form of the receptor (591 aa in the rat) has been described in all species, although in ruminants the cytoplasmic domain is truncated by some 40 aa compared with other known forms (Hu et al., 1998). The rat Nb2 lymphoma cell line, for which PRL is a mitogen, has an intermediate PRLR with a deletion of 198 aa in the cytoplasmic domain. Short forms of PRLR have been described in rodents (291 aa) and ruminants and in man and further variants may be found (Jabbour and Kelly, 1997). Additionally, soluble

isoforms of PRLR have also been identified that circulate in blood as PRL-binding proteins. The generation of binding proteins may involve alternative splicing of mRNA for the receptors or proteolysis of the receptor proteins or both (Kline and Clevenger, 2001).

Despite attempts to search for SLR since the discovery of SL, there is a still much controversy regarding its identity (Fukada et al., 2005). This issue will be further discussed in Chapter 3.

1.5 Signaling pathway activation by the GH/PRL family of hormones

The physiological functions of GH/PRL are mediated through the interaction of the hormones with their receptors in the various target cells followed by a cascade of downstream signaling pathways. The details of these processes will be discussed in the following sections.

1.5.1 Dimerization of the receptors

The earliest event of the GH and PRL signaling appears to be the interaction of the hormones with the receptors and dimerization of the receptors. Binding reactions between GH/PRL and their cognate receptors provide a detailed account of how a polypeptide hormone activates its receptor and more generally how proteins interact with each other. Take the activation of GHR by GH as an example, through high-resolution structural and functional studies, it was found that GH uses two different sites (site 1 and site 2) to bind two identical receptor molecules (Cunningham et al., 1991; Wells, 1996). First a GH molecule interacts via its binding site 1 with a site on the extracellular domain of a receptor, to form an inactive

hormone-receptor complex. The bound hormone then binds to the same site on a second receptor through its binding site 2, which explains why the hormone does not form higher oligomers with the receptor (de Vos et al., 1992). The binding of the receptors by GH shows a bell-shaped dose-dependent curve. At low concentrations, GH can bind to receptors and easily find an empty receptor to form a dimer and affect the signal. However, at high concentrations receptors become occupied to greater extents as 1:1 complexes, and thus fewer empty receptors are available to dimerize (Fuh et al., 1992; Ilondo et al., 1994a). Therefore, very high hormone concentrations have the potential to become inhibitory, by preventing the formation of receptor dimers, as well as by removing receptor from the cell surface by internalization (Goffin and Kelly, 1997). The sequential dimerization reaction activates the receptors, presumably by bringing the intracellular domains into close proximity so they may activate cytosolic components. The finding that a mutated GH (G120R) that fails to induce GHBP dimerization is biologically inactive when added to cells expressing GHR suggests that GH-induced dimerization of GHR is required for GH action (Silva et al., 1994). As a consequence of this mechanism it is possible to build antagonists specifically to the receptor by introducing mutations in GH that block binding at site 2 and to build even more potent antagonists by combining these with mutants which enhance binding at site 1. Alanine-scanning mutagenesis of all contact residues at the site 1 interface shows that only a small and complementary set of side chains clustered near the center of the interface affects binding. The most important contacts are hydrophobic, and these are surrounded by polar and charged interactions of lesser importance (Cunningham et al., 1991). Kinetic analysis shows for the most part that the important side chains function to maintain the complex, not to guide the hormone to the receptor (Cunningham and Wells, 1993). The binding

profile of PRL-PRLR complex is similar to the GH-GHR complex (Fuh et al., 1993). Hormone-induced dimerization reactions are turning out to be general mechanisms for signal transduction. Moreover, the molecular recognition principles seen in the GH/PRL family hormones and receptor complex are likely to generalize other protein-protein complexes.

1.5.2 JAK-Stat signaling pathway activation

It was observed more than ten years ago that hormonal stimulation of PRLR or GHR led to tyrosine phosphorylation of several cellular proteins, including the receptors themselves. Since the cytoplasmic domains of PRLR and GHR (same as the other class I cytokine receptors) are devoid of any intrinsic enzymatic activity, such phosphorylation should be resulted from other associated protein kinase(s) which is (are) also activated upon hormonal stimulation. The first major progress in the understanding of PRLR/GHR signaling is the identification of Janus kinase 2 (JAK2) as one of these protein kinases (Argetsinger et al., 1993; Lebrun et al., 1994). JAK2 belongs to the Janus tyrosine kinase family, which includes four members: JAK1, JAK2, JAK3, and Tyk2 (Finidori and Kelly, 1995). The Janus kinases are involved in the signaling of all cytokine receptors. In the context of PRLR and GHR, JAK2 is the major receptor-associated Janus kinase, although involvement of JAK1 and JAK3 has also been proposed (Forsyth and Wallis, 2002). In 3T3-F442A fibroblasts, GH stimulates the tyrosyl phosphorylation of JAK1 in addition to JAK2 which has a much greater response (Smit et al., 1996). GH also enhances the tyrosyl phosphorylation of JAK3 in the T-cell line, although to a significantly lesser extent than IL-2, a potent activator of JAK3 (Johnston et al., 1994). Therefore, although JAK2 appears to be the primary JAK family member activated by GH,

JAKl and JAK3 may also be activated by GH and therefore mediate at least some effects of GH. It is also shown that Fyn, a member of the Src kinase family, is associated with PRLR and activated by the PRL stimulation in the rat T lymphoma Nb2 cell line (Clevenger and Medaglia, 1994).

The association of JAK2-PRLR complex is in a ligand-independent manner, while GHR associates with the kinase only upon ligand activation suggesting that some ligand-induced conformational changes of GHR might be required prior to JAK2 binding (Argetsinger et al., 1993; Lebrun et al., 1994). Studies using truncated and mutated GHR and PRLR have implicated the Box 1 motif as a necessary component for the JAK2 association with receptors and for the tyrosyl phosphorylation and activation of JAK2, while more distal regions appear to augment the interaction (Dinerstein et al., 1995; Lebrun et al., 1995b; Sotiropoulos et al., 1994). The mechanism by which the kinase is activated remains poorly understood. It is widely accepted that ligand-induced receptor homodimerization brings two JAK molecules close to each other, allowing phosphorylation on tyrosines and subsequent activation of the enzyme (Finidori and Kelly, 1995). In the PRLR/GHR context, the activation of JAK2 occurs very rapidly after hormonal stimulation, strongly suggesting that this event is a common upstream factor of several signaling pathways of both receptors (DaSilva et al., 1994; Lebrun et al., 1995b). Accordingly, receptor mutants in which Box 1 has been deleted or in which proline residues have been mutated, can neither associate with nor activate JAK2 and were reported inactive in all *in vitro* biological responses analyzed so far (Colosi et al., 1993; Goujon et al., 1994).

Tyrosine kinase signaling is often mediated by the binding of Src-homology 2 (SH2) domain-containing signaling proteins to phosphorylated tyrosines in the kinase itself or in other kinase substrates (Pawson and Schlessingert, 1993). In response to GH/PRL, both JAK2 and GHR/PRLR become phosphorylated on tyrosines, presumably as a result of JAK2 activation (Argetsinger et al., 1993; Foster et al., 1988). In the intermediate rat PRLR isoform, which contains only 3 tyrosine residues, it has been identified that the C-terminal Tyrosine 382 undergoes phosphorylation upon receptor/JAK2 activation (Lebrun et al., 1995). It has been also shown that the five C-terminal tryosines of GHR are major phosphorylation sites activated by the JAK2 (Sotiropoulos et al., 1994). Receptor tyrosine phosphorylation seems to be required for the stimulation of proliferation in cells. For example, the short PRLR, as well as the non-phosphorylated Nb2 mutant lacking the C-terminal tyrosine 382, are unable to stimulate the β-casein promoter (Lebrun et al., 1995a; Lebrun et al., 1995b). Similarly, a C-terminal truncated GHR is active in Spi 2.1 transactivation, while point mutations of Tyr 469 and Tyr 516 prevent this signaling activation (Sotiropoulos et al., 1996).

After activation and phosphorylation of JAK2, signal transducers and activators of transcription (Stat) family proteins are further recruited. So far, three members of the Stat family have been identified as the transducer molecules of the GHR/PRLR: Stat1, Stat3 and Stat5 (Takeda and Akira, 2000). In nonstimulated cells, Stats remain in the cytosol in nonphosphorylated status. Upon hormonal activation, GHR and PRLR undergo tyrosine phosphorylation by the associated Janus kinases and these phosphorylated tyrosines then become binding sites for Stat SH2 domains. Once they are associated with the receptors, Stats are phosphorylated by the

receptor-associated Janus kinases, translocate to the nucleus, bind to DNA, and activate transcription of target genes (Darnell et al., 1994). Consistent with their role as transcription factors, Stat proteins are present in GH-induced DNA-binding complexes for several genes. In the c-fos promoter, GH induces the binding of three complexes to the Sis-inducible element (SIE), including Statl and Stat3 homodimers and Statl and Stat3 heterodimers (Campbell et al., 1995; Meyer et al., 1994). GH also stimulates the binding of Stat5 to the IFN-activated sequence (GAS)-like response element (GLE-1) in the Spi 2.1 gene and of binding an unknown Stat-related protein to IFN-response region in IM-9 cells (Wood et al., 1995). It has also been shown that mutation of the C-terminal tyrosine 382 in the Nb2 PRLR, which abolishes tyrosine phosphorylation of the receptor, prevents activation of the β-casein promoter (Lebrun et al., 1995).

1.5.3 MAPK signaling pathway activation

In addition to JAK2 and Stats proteins, a large number of other proteins become tyrosyl phosphorylated in response to GH/PRL, suggesting that JAK2 phosphorylates proteins in addition to JAK2 and Stats and moreover, these proteins that bind to activated JAK2 initiate other signaling pathways involving other tyrosine kinases or phosphatases (Xu and Messina, 2009). The first two GH/PRL-dependent tyrosyl phosphorylated proteins identified are the MAP kinases designated as extracellular signal-regulated kinases (ERKs) 1 and 2. This pathway leading from membrane receptor tyrosine kinases to MAP kinases involves Src homology 2 domain containing transforming protein (SHC), growth factor receptor-bound protein 2 (Grb2), son-of-sevenless (Sos), ras, raf, and ERK kinase (MEK) (Winston et al., 1995). Activation of the mitogen-activated protein kinase (MAPK) pathway has been

reported in several biological systems under PRL and GH stimulation (Blume et al., 2009; Campbell et al., 1992; Das and Vonderhaar, 1995; Piccoletti et al., 1994; Sotiropoulos et al., 1994). Take GH as an example, GH has been demonstrated to promote rapid tyrosyl phosphorylation of the 66-, 52-, and 46-kDa SHC proteins in 3T3-F442A fibroblasts (Campbell et al., 1992). GH also induces the binding of a GHR-JAK2 complex to the SH2 domain of the SHC proteins fused to glutathione S-transferase (GST) and the association of Grb2 with SHC. These results suggest that GH stimulates the association of SHC proteins with JAK2-GHR complexes via the SH2 domain of SHC proteins. SHC is then tyrosyl phosphorylated by JAK2, which permits the binding of Grb2 to SHC. Ras and Raf have also been reported to be required for activation of MAPK by GH (Winston and Hunter, 1995). GH activation of MAPK requires the proline-rich Box 1 of GHR, the same region implicated in the JAK activation, providing further evidence for a role of JAK2 in the activation of MAPK (VanderKuur et al., 1994). MAPK substrates include other protein kinases (e.g. c-Raf-1, the S6 kinases designated as $p70^{s6k}$ and $p90^{s6k}$), phospholipase A_2, cytoskeletal proteins, and transcription factors (e.g. c-Jun and ternary complex factors) (Davis, 1993). Of these, GH has been shown to activate the S6 kinase in 3T3-F442A fibroblasts (Anderson, 1992). Presumably some of the other substrates are also phosphorylated by MAPK in response to GH and are responsible for other actions of GH. Studies also showed that MAPK activity could also be enhanced by PRL signaling. The prominent stimulatory action of PRL on the ERK/MAPK pathway in the hypothalamic, paraventricular and supraoptic nucleus was likely to mediate the neuroplasticity of the neuroendocrine system during lactation (Blume et al., 2009).

1.5.4 PI3K/Akt and other signaling pathway activation

Although the JAK-Stat and MAPK cascades are presumably two of the most important signaling pathways used by the GH/PRL receptors, other signaling pathways are also likely involved in the signaling transduction by these receptors including the Phosphatidylinositol 3-kinase (PI3K)/Akt, insulin-receptor substrate-1 (IRS-1), protein kinase C (PKC), Phospholipase-C (PLC), and calcium ions. These pathways are currently less documented. IRS-1 is phosphorylated presumably by JAK2 upon GHR activation (Argetsinger et al., 1995). This process is achieved by the association of IRS-1's N-terminus and JAK2, which is independent of Stat activation (Liang et al., 2000). Phosphotyrosyl residues of IRS-1 interact with several SHC proteins including the regulatory p85 subunit of PI3K. GH and PRL activation of the PI3K/Akt pathway has been observed both *in vivo* and *in vitro*. It has been shown that the insulin-like effects of GH in adipocytes can be inhibited by the selective PI3K inhibitor wortmannin, suggesting an important role for PI3K in GH action (Ridderstrale and Tornqvist, 1994). Similarly in the primary adipocytes, GH stimulates the tyrosine phosphorylation of the IRS-1 and its association with PI3K (Ridderstrale et al., 1995). Study also reveals that IRS-1 contributes substantially to GH-induced ERK activation in lipid raft membrane in 3T3-F442A fibroblasts (Wang et al., 2009b). Like GH stimulation of phosphorylation of IRS-1 and IRS-2, stimulation by GH of lipid synthesis and glucose transport requires the N-terminal, but not the C-terminal, of GHR (Moller et al., 1994). The treatment of pregnant rats with PRLR antisense oligonucleotide results in the reduction of the phosphorylation level of Akt and $p70^{s6k}$ expression as well as the reduction of glucose-induced insulin secretion by isolated islets, indicating that PRL may have an important role in increasing the islet mass and sensitivity to glucose during pregnancy by activating

downstream proteins of the PI3K cascade (Amaral et al., 2004). Besides, the PI3K/Akt pathway is involved in the process that PRL prevents human spermatozoa cells from defaulting to intrinsic apoptotic pathway associated cell senescence (Pujianto et al., 2010).

PLC generates diacylglycerol (DAG) which in turn activates PKC. Activation of these enzymes by stimulated PRLR and GHR has been suggested, although their role in signaling remains elusive and their substrates are poorly identified. It has been reported that PRL can activate PKC in primary neuronalcultures, and PKC induce the transactivation of the MAPK pathway (DeVito et al., 1991; Pasqualini et al., 1994). Moreover, in the mediobasal hypothalamic neurons culture containing tuberoinfundibular dopamine neurons, PRL can induce ERKs 1/2 phosphorylation in a PKC-dependent manner (Ma et al., 2005). It has been also demonstrated that PKC pathway mediates PRL signals to elevate the levels of Activating Protein-1 (AP-1) complexes which are able to bind DNA in breast cancer cells together with the JAK-stat and MAPK signaling pathways (Gutzman et al., 2004). Finally, PKC-independent increase of intracellular calcium has been reported for both GHR and PRLR. This increase is dependent upon the presence of extracellular Ca^{2+} and is blocked by verapamil and nimodipine (Billestrup et al., 1995). In the GHR context, this phenomenon seems independent of proline-rich Box 1 but requires the ~200 C-terminal residues, indicating that calcium signaling may be independent of JAK2 activation (Billestrup et al., 1995; Braithwaite, 1975; Ilondo et al., 1994b). A schematic map of the major GH/PRL signaling pathways is summarized in Figure 1.5.

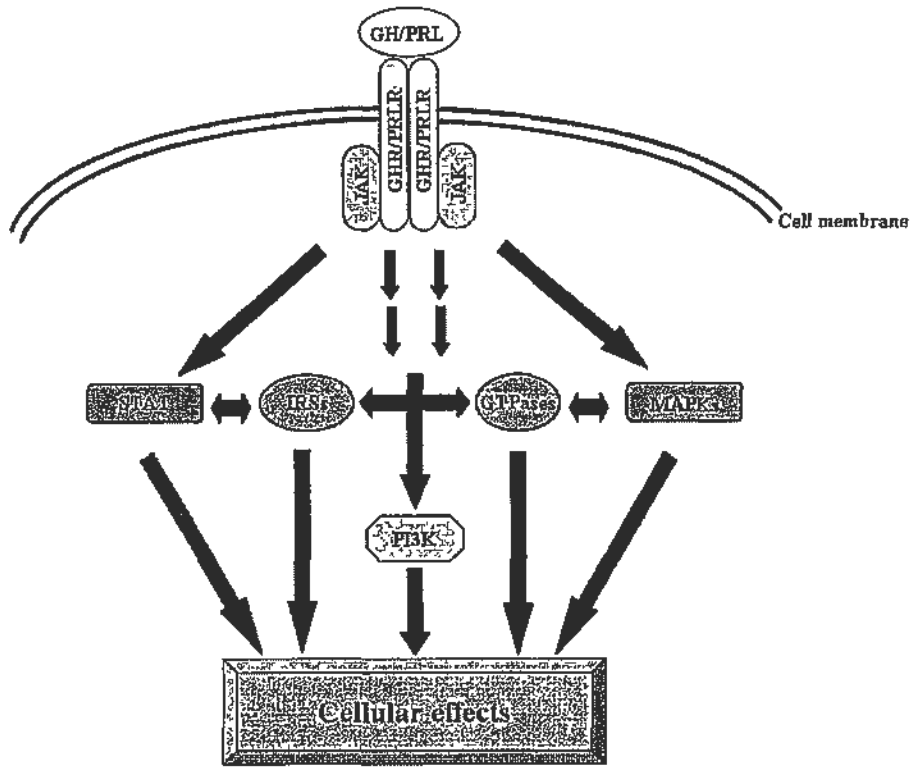


Fig. 1.5 A schematic diagram showing the activation of signaling pathways by GH/PRL hormones.

1.6 Biological functions of the GH/PRL family of hormones

Although GH/PRL family members share many similarities in structures of the hormones, their receptors, and their mechanisms of intracellular signaling, important biological differences can be found among them. The biological functions of GH, PRL and SL are discussed in detail in the following section separately.

1.6.1 Biological functions of GH

The most widely known function of GH throughout the vertebrates is the stimulation of postnatal growth, which is achieved through two major mechanisms. First of all, GH can act on the liver, the major target organ of which, to stimulate the production of insulin-like factors (IGFs), which have growth-stimulating effects on a wide variety of tissues and organs in the autocrine, paracrine and endocrine manners.

Besides, GH also shows direct effects in the postnatal growth through the MAPK pathway (Savage et al., 2010). Growth failure and shorter stature can be found in children due to GH deficiency (Wassenaar et al., 2009).

During the prepubertal period, GH is a major determinant of longitudinal bone growth, skeletal maturation, and acquisition of bone mass via GH-IGF axis, whereas in adults they are important in the maintenance of bone mass (Baroncelli et al., 2003). The skeletal effects of GH and IGF are modulated by the complicated interactions between circulating IGF-1 and IGF binding proteins (IGFBPs) and the locally produced IGF-1 and IGFBPs (Ueland et al., 2002). IGF-1 and IGF-2 are the most abundant growth factors present in the skeletal tissue, and their synthesis and activity are regulated by systemic hormones such as GH and PTH. GH may act by inducing IGF-I in bone or may have direct effects on skeletal cells to participate in the osteoblastogeneis, the proliferation of osteoblasts and the production of osteoprotegerin (Perrini et al., 2010).

Besides bone growth, the role of GH has also implicated in the immune system. Both the pituitary-derived hormones GH and GH-stimulated IGF-1 have been demonstrated to accelerate recovery of the immune system following transplantation of various types of cells and thymopoiesis as well (Murphy et al., 1992a, b). Moreover, the recombinant human GH-treated mice exhibit accelerated the recovery of total thymocytes, B cells, CD4+ and CD8+ T cells (Chen et al., 2003). It seems also very promising to use GH to treat HIV-infected patients. Studies show that GH can improve the immune status of HIV patients by increasing thymic mass and circulating CD4 cells and improved T cell functions (Kelley, 2004). Highly

aggressive anti-retroviral therapy (HAART) has been reported to result in very effective control of HIV infection. It has also been demonstrated that GH can increase the number and function of CD56 NK cells, which are typically depleted in the patients receiving HAART, indicating GH therapy is effective in restoring the immune response to help patient recovery (Pires et al., 2004).

GH also plays an important role in anti-aging as decreased level of GH occurs with increasing age. When treated with GH, men over 60 showed a sharp increase in body mass and bone mineral as compared to the control (Rudman et al., 1990). It is also shown that GH increases muscle and decreases body fat on the healthy elderly (Liu et al., 2007). The mice model studies indicate that treatment with GH agonist, GH, or IGF-1 has positive effects in restoring aging (French et al., 2002; Montecino-Rodriguez et al., 1998).

Apart from the aforementioned functions, GH has been shown to participate in many other biological processes including reproduction, neural development, regulation of cardiovascular development, lipolysis, protein synthesis, gluconeogenesis, calcium retention, etc. (Chandrashekar et al., 2004; Gola et al., 2005; Quik et al., 2010; Scheepens et al., 2005).

GH physiology has also been extensively investigated in many fish such as salmonids, cyprinids and sparides. It participates in almost all major physiological processes of fish, not only including the regulation of ionic balance, lipid, protein, and carbohydrate metabolism, skeletal and soft tissue growth, reproduction and immune function which are the general effects of GH in vertebrates as

aforementioned, but also including appetite, osomoregulation, foraging behavior, aggression, and predator avoidance. For example, besides increasing growth rate, GH increases appetite in juvenile rainbow trout (*Oncorhynchus mykiss*) (Johnsson and Bjornsson, 1994). Again in the studies of salmonids including rainbow trout, it has been shown that peripheral GH can increase feeding and swimming activity, predator avoidance, which can be also observed in the GH transgenic fish (Johnsson et al., 1996; Jonsson et al., 1998; Stevens et al., 1998), consistent with the experiment by Johnsson and Bjornsson (1994).

The role of GH in the osmoregulation of salmonids has also been investigated. It is demonstrated that GH treatment increases the capacity of trout to move from freshwater to seawater (McCormick, 2009). Later studies show that GH has key actions in seawater adaptation of salmonid fishes, the relatively primitive teleosts (Madsen, 1990). Treatment of salmonids with GH stimulates gill Na^+, K^+-ATPase, size and density of chloride cells, and salinity tolerance (Sakamoto et al., 1993). The effect of GH on salinity tolerance and differentiation of salt secretary mechanisms is not restricted to salmonids, as this effect has been found in two other euryhaline species, tilapia and killifish (Mancera and McCormick, 1999; Sakamoto et al., 1997). It is proposed that the osmoregulation effect of GH is achieved through the GH-IGF-1 axis (Sakamoto and McCormick, 2006; Sakamoto et al., 1993). Indeed, circulating levels of GH and IGF-1 increase during smolting and are responsive to photoperiod and temperature (McCormick et al., 2002).

1.6.2 Biological functions of PRL

PRL was originally isolated by its ability to stimulate mammary development

and lactation in rabbits and soon thereafter to stimulate the production of crop milk in pigeons (Chen et al., 1994). Subsequently, a number of additional activities have been associated with this hormone in various vertebrate species. To date, more than 300 actions have been attributed to PRL (Bole-Feysot et al., 1998).

PRL is best known for the multiple effects it exerts on the mammary gland, including growth and development of the mammary gland (mammogenesis), synthesis of milk (lactogenesis), and maintenance of milk secretion (galactopoiesis). Targeted disruption of the *PRL* gene or knockout of the PRLR results in abnormal mammogenesis characterized by complete absence of the lobuloalveolar units in adult homozygous females. Due to impairment of mammogenesis, these knockout mice fail to produce milk. Although PRL knockout heterozygotes appear to have nearly normal mammogenesis that is indistinguishable from wild type, their offspring are unable to lactate (Horseman et al., 1997; Ormandy et al., 1997). Although the hormonal requirements for the induction and maintenance of milk production vary in different species, the common factor is that PRL is the hormone primarily responsible for the synthesis of milk proteins, lactose, and lipids (Cowie et al., 1969; Oppat and Rillema, 1988; Waters and Rillema, 1988). In the process of lactogenesis, prolactin stimulates the uptake of some amino acids, the synthesis of the milk proteins casein and α-lactalbumin, uptake of glucose, and synthesis of the milk sugar lactose as well as the milk fats (Barber et al., 1992; Oladapo and Fawole, 2009). PRL is also involved in the testicular functions. The effects of PRL on the leydig cells, sertoli cells and spermatozoa have been reported (Karthikeyan et al., 2009; Lincoln et al., 1996; Petersen and Soder, 2006; Pujianto et al., 2010b).

Besides its role in the reproduction, PRL shows profound effects on the growth

and development in both lower and higher vertebrates. PRL has been associated with the body growth in both rats and birds (Perez-Villamil et al., 1992; Silverin, 1980). Moreover, PRL stimulates skin melanocyte and keratinocyte growth in mammals (Kanda and Watanabe, 2007). Proliferation of the epithelial cells of pigeon crop sac is linked with the function of PRL, which promotes growth of crop sac (Kansaku et al., 2008). PRL has also been suggested to have function in various developmental processes such as lung maturation, preadipocyte differentiation and germ cell development (Jobe, 1991; McAveney et al., 1996; McFarland-Mancini et al., 2006).

Similar to GH, the role of PRL in immune system has also been investigated. Injection of PRL into the hypophysectomized rats causes an increase in the weight of the spleen and thymus (Bunn, 2007). In addition, PRL activates an immunostimulatory action of the submandibular gland and augments the production of a thymic hormone, thymulin (Savino et al., 1990). It is shown that PRL stimulates mitogenesis in both normal T lymphocytes and the Nb2 lymphoma cell line, which is essential for normal postpartum behavioral responses in the mother during early pregnancy (Larsen and Grattan, 2010; Shiu et al., 1983; Vijayan et al., 1979). It is not surprising to observe that PRL affects lymphocytes since PRLR has been detected on human peripheral lymphocytes and their mRNA expression is regulated by PRL itself (Xu et al., 2010). Moreover, effects of PRL on lymphocytes may involve Interleukin-2 (IL-2) since T-lymphocyte activation by IL-2 requires PRL (Bien and Balcerska, 2008). It appears that immune responses *in vivo* are enhanced by PRL. For example, administration of PRL restores dinitrochlorobenzene-induced contact dermatitis as well as mitochondrion-rich cells expressing Na^+/Cl^- co-transporter impaired by hypophysectomy (Breves et al., 2010; Nagy et al., 1983). In

hemodialysis patients, plasma levels of Th1/Th2 type cytokine are associated with PRL (Liu et al., 2008).

There are increasing data suggesting that PRL influences behaviors. One of the best-characterized PRL-driven behaviors is parental behavior, including the studies in rats, mice, rabbits, hamsters, sheep and human (Angelier et al., 2009; Gubernick and Nelson, 1989; Melo et al., 2009). It is reported that PRL decreases the latency to initiation of the maternal behavior in steroid-primed rats (Anderson et al., 2006; Sakaguchi et al., 1996). Moreover, hypophysectomized rats fail to display a facilitation of the maternal behavior in response to the steroid treatment (Yeh and Moog, 1975). Birds also respond to PRL by an increase in nesting behavior, nest attendance and incubation behavior (Leboucher et al., 1990). Null mutation of the PRLR gene produces a defect in the maternal behavior (Melo et al., 2009). In addition, rapid eye movement sleep and sleep-wake cycle are shown to be related to the function of PRL (Bredow et al., 1994; Roky et al., 1995). PRL also affects other behaviors such as pseudopregnancy, lordosis and libido (Augustine and Grattan, 2008; Dudley et al., 1982; Kleinberg et al., 1999).

Apart from the abovementioned functions, PRL shows profound effects on many aspects of the body such as energy metabolism, lipid metabolism and carbohydrate metabolism (Dyck, 2009; Sangiao-Alvarellos et al., 2006). Indeed, a numbers of diseases and cancers have been associated with the malfunction of PRL. For example, PRL is reported to increase colorectal tumor aggressivity, induce the proliferation of several lines of human breast cancer, activate malignant B lymphocytes and lymphoma cells and induce the proliferation of promyelocytes (Dagvadorj et al.,

2007; Nishiguchi et al., 1993; Tworoger et al., 2007).

PRL also shows significant effects on many aspects of fish physiology (Bole-Feysot et al., 1998). The study of the role of PRL in water and electroplyte balance has been extensively investigated in various fish species such as salmonids, goldfish, tilapia and mudskipper (Manzon, 2002; Sakamoto et al., 2005). Different from GH which is involved in the seawater adaptation, PRL is referred as freshwater-adapting hormone playing an important role in the freshwater adaptation. Indeed, Gene expression, synthesis, secretion, and plasma levels of PRL increase after freshwater exposure (Sangiao-Alvarellos et al., 2006). PRL secretion is also modulated by the plasma factors such as osmolarity and cortisol in tilapia (Borski et al., 1991). PRL treatment primarily reduces ion and water permeability of osmoregulatory surfaces, including kidney, gill and gut which are all lower in freshwater than seawater. In the gastrointestinal tract of euryhaline fish, PRL generally decreases NaCl and water absorption by reducing the permeability of the epithelium, although there is species variability (Nagano et al., 1995). Moreover, a specific prolactin-releasing hormone peptide (Pr-RP) has been described in both mammals and teleosts. This peptide synthesized in hypothalamic neurons can stimulate PRL-releasing cells, increasing synthesis and release of this hormone to systemic blood (Sakamoto et al., 2003). The studies in mudskipper have demonstrated a strong relationship between expression of Pr-RP and environmental salinity, with higher Pr-RP expression in fish acclimated to freshwater and terrestrial environments relative to seawater conditions, suggesting that Pr-RP may affect osmoregulation by PRL (Sakamoto et al., 2005; Sakamoto and McCormick, 2006).

1.6.3 Biological functions of SL

Unlike GH and PRL, physiological significance of SL, which is only found in fish, is less understood, though several studies have revealed its physiological effects on fish. Current data indicate that SL is associated with development, metabolism, reproduction, immune function and background adaptation. The effect of SL on development has been investigated in zebrafish. Zebrafish larvae injected with SLα and SLβ Morpholino lack an inflated gas bladder within 10 days post fertilization (Zhu et al., 2007). The SL-deficient *ci* medaka shows higher accumulations of triglycerides and cholesterol in the liver and muscle than wild type, indicating SL plays a suppressing role in fat accumulation (Fukamachi et al., 2005). Moreover, the treatment of SL in Sea Bass (*Dicentrarchus labrax*) increase carbon dioxide output and oxygen uptake, suggesting the role of SL in the lipid catabolism. Consistently, SL is reported to be able to inhibit the hepatic activity of acetyl-coenzyme A carboxylase (Vega-Rubin de Celis et al., 2003).

Several studies have addressed that SL participates in the fish reproduction. First of all, the mRNA expression level of SL is higher during gonadal maturation and prespawning in salmon (Onuma et al., 2003; Taniyama et al., 1999). During reproductive maturation of coho salmon, the expression of SL increases and reaches its peak at spawning, which is related to the oestrodiol and 11-ketoestosterone levels (Planas et al., 1992; Rand-Weaver et al., 1992). The gradual stimulation of SL synthesis and release during sexual maturation and spawning are observed in striped mullet (*Mugil cephalus*), suggesting that SL may be involved in the control of some steps of reproductive processes such as steroidogenesis, calcium metabolism, and energy mobilization (Mousa and Mousa, 2000). In the study using the recombinant

sole SL to prime gilthead sea bream phagocytes for *in vitro* enhancement of mitochondrial activity, a bell-shape dose-response curve was obtained with a maximum at 50 nM, which is similar to that of GH (Calduch-Giner et al., 1998). In addition, SL shows an important role in the regulation of chromatophores as malfunction of SL have unique defects in proliferation and morphogenesis of certain types of chromatophores on skin (Fukamachi et al., 2004).

1.7 Zebrafish as a model system

To date, several animals have been developed as models to study human diseases and development including *Drosophila melanogaster*, *Caenorhabditis elegans*, zebrafish, rat and mouse (Gold et al., 2006). However, invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* lack many structures and organ systems that are involved in human disease pathogenesis, which therefore hinders their application in the modeling of human diseases. On the other hand, although rats and mice share striking similarities with humans at many aspects from genomic homology to anatomy and structure, they require considerable manpower and infrastructure support, which limits many experiments, especially large-scale genetic screens and drug screens (Carpinelli et al., 2004; Nolan et al., 2000).

The use of zebrafish as an animal model has come to attention as early as 1930s (Laale, 1977). A number of advantages of zebrafish make it an excellent species for the investigation of human diseases and development. The genomes of zebrafish and human share more than 80% similarity with each other (Driever et al., 1994). The zebrafish embryo which is externally fertilized is transparent, facilitating morphological observation during embryonic development. Besides, the period that

the embryos develop from the zygote to a tiny fish is only 24 hours, for a mouse this process requires about 21 days. The sexual maturation of zebrafish from embryo requires only 3 months. Moreover, a pair of adult zebrafish is capable of producing hundreds of eggs per week. Another advantage of zebrafish is that the adult fish are small, about 4 cm in length, and therefore easy to maintain (Bowman and Zon, 2010; Zon and Peterson, 2005). In addition, many *in vivo* assays and manipulation technologies have been well established in zebrafish such as gene knockdown, microinjection, transgenesis and targeted induced local lesions in genomes (Foley et al., 2009; Henikoff et al., 2004; Lieschke and Currie, 2007). Therefore, the zebrafish serves as a good model in both the development and drug screening studies.

1.8 Specific objectives and research plans

As aforementioned, although the physiological functions of SL have been investigated, the receptor for SL remains unknown. It is proposed that GHR1 is in fact the receptor for SL (Fukamachi et al., 2005). However, this hypothesis is contrary to several studies published (Jiao et al., 2006; Reindl et al., 2009). Therefore, the first aim of my study is to examine whether GHR1 is the receptor for SL using physical interaction study and downstream signaling activation study. Meanwhile, a systematic investigation on the interaction and crosstalk of the GH/PRL family of hormones and their receptors is also carried out. A brief research plan of this part is shown in Figure 1.6.

Another part of my study focuses on the role of PRLR1 in the embryonic development of zebrafish. To specify, the aims of this part include the following sections:

(1) To study the temporal and spatial expression patterns of PRLR1 during embryonic development of zebrafish;

(2) To investigate functions of PRLR1 during embryonic development of zebrafish;

(3) To explore the detailed mechanisms of PRLR1 in the embryonic development of zebrafish. The research plan of this part is shown in Figure 1.7.

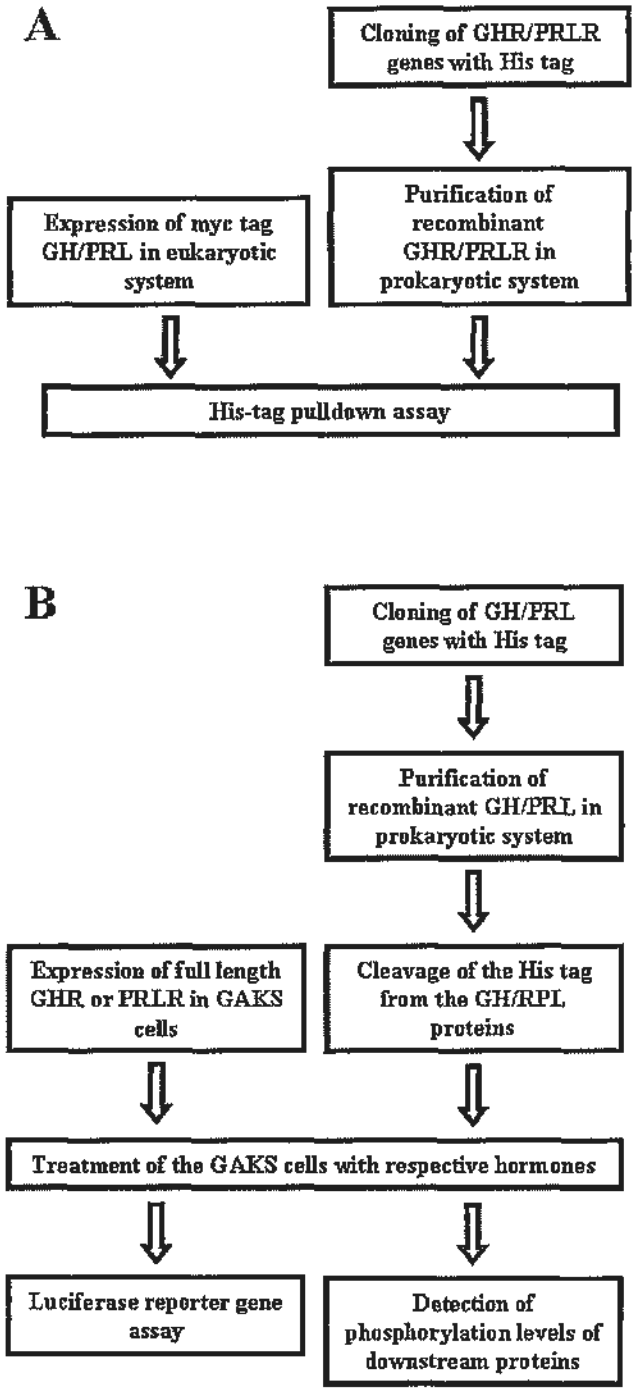


Fig. 1.6 Research plan on the interaction study of the GH/PRL family hormones and their receptors, including His-tag pulldown assay (A), Luciferase reporter assay and detection of phosphorylation levels of downstream proteins (B).

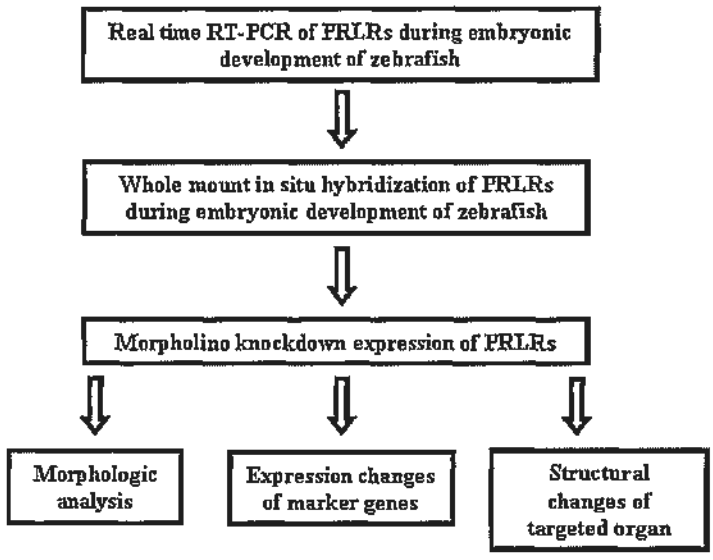


Fig. 1.7 Research plan on the role of PRLRs in the zebrafish embryonic development.

Chapter 2

Cloning, expression and purification of recombinant proteins from the GH/PRL family of hormones and their receptors

2.1 Introduction

Protein purification, which is usually referred to as the process to isolate one single type of protein from a protein mixture, plays a very important role in the characterization of the function, structure and interactions of the protein of interest (Scopes, 2001). Large-scale recombinant protein purification has served widely in the pharmaceutical, industrial and academic applications. For example, insulin has been used to treat type 1 and 2 diabetic patients (Czech et al., 2010; Valla, 2010). Soy protein isolate which has been developed from defatted soy flout is used to improve the texture of meat products in the food industry (Kolb, 1974). Protein purification technique is also frequently applied in academic research, especially in the biological area. The development of techniques and methods for the separation and purification of proteins has been an important prerequisite for many of the advancements made in biological science and biotechnology over the past several decades. Take the determination of protein structure, a cutting-edge field in biological research as an example, X-ray crystallography of a protein is achieved only after the purification and crystallization of the protein of interest (Bassett, 1993).

The use of bacteria to produce recombinant proteins has grown in recent decades. Using recombinant DNA and inserting it into a plasmid of rapidly reproducing bacteria enables the manufacture of recombinant proteins. These recombinant proteins can be of different types such as antibodies, antigens, hormones and

enzymes. Bacteria can produce large quantities of recombinant proteins in rapid, often inexpensive, fermentation processes as bacteria do not possess a fine control of protein production, meaning that ectopic proteins can be continuously produced in large quantities (Kouwen and van Dijl, 2009). The amount of the recombinant proteins can occupy up to 50% of the total proteins generated by the bacterial host. However in most cases, the heterologous recombinant proteins, which are often encoded by eukaryotes, are overexpressed in the bacteria, it is highly possible to form inactive aggregates of proteins known as inclusion bodies due to the lack of protein regulationary mechanisms in bacteria such as carbohydrate modification and protein phosphorylation (Palme et al., 2010). Besides, the microenvironment of the prokaryotic system including chaperones, osmolarity and pH, is rather different from that in the eukaryotic system (Sbarbati et al., 2010). Since the systematic mechanism of protein refolding is still not fully understood so far, many of the ectopic proteins, especially those with many hydrophobic residues, are prone to aggregate with each other by hydrophobic interaction to form inclusion bodies (Muca et al., 2009). Moreover, it is reported that prokaryotic cells lack protein processing systems to clear up the unintended proteins resulting in the accumulation of inclusion bodies in the cells. Indeed, up to 70% to 80% of proteins expressed in *E. coli* system by recombinant method are contained in inclusion bodies (Singh and Panda, 2005). Although inclusion bodies are composed of inactive proteins which do not exhibit biological activity, the inactive proteins in the inclusion bodies possess native-like secondary structure such as amide bond and are resistant to proteolysis (Villaverde and Carrio, 2003). It is generally accepted that maintenance of inclusion bodies is a dynamic process. The formation of aggregates and their soluble proteins is a dynamic equilibrium. The aggregate intermediates of proteins are responsible for the transition

between the inclusion bodies and soluble proteins (Carrio and Villaverde, 2003). There are many factors affecting the formation of inclusion bodies including protein sequence, protein concentration, temperature, pH, ionic stress as well as the cosolutes (such as chaperones and urea) (Ventura, 2005).

The purification of proteins from inclusion bodies is cumbersome. Such purification usually consists of four major steps, including isolation of the inclusion bodies from the protein mixture, solubilisation of the inclusion bodies, recovery of the soluble protein and purification of the soluble protein (Lilie et al., 1998). Among them, solubilisation of the inclusion bodies and recovery of the soluble protein are the limiting steps during the purification. Inclusion bodies can be solubilized using high concentrations (usually 6-8 M) of chaotropic reagents such as urea, guanidine hydrochloride and detergents such as sodium dodecyl sulfate (SDS), N-cetyl trimethyl ammonium chloride and sarkosyl (sodium N-lauroyl sarcosine) (Fahnert et al., 2004). Inclusion bodies can be dissolved in the environment with extreme pH (Long and Wang, 1998). The combination of different solubilizing reagents may give better solubilisation results. Some other reducing reagents such as β-mercaptoethanol or dithiothreitol are added to prevent formation of intermolecular disulfide bonds which may lead to aggregation of the proteins (Fischer et al., 1993).

In order to explore the functions of the GH/PRL family of hormones, recombinant hormones were produced in this study. The cDNAs of the GH/PRL family of hormones and their receptors were cloned in the prokaryotic expression vector pSUMO. Recombinant proteins were produced as inclusion bodies in *E. coli*. After optimization of solubilisation and refolding conditions, the recombinant

proteins were successfully purified using an effective procedure.

2.2 Materials and methods

2.2.1 Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the pituitary of zebrafish by TRIzol. The concentration of the extracted RNA was measured. First-strand cDNA was synthesized using ImProm-II™ Reverse Transcription System (Promega, USA) according to the manufacturer's manual. Synthesis of the first chain cDNA was preformed according to the following condition: 25°C for 5 min, 42°C for 60 min and 70°C for 15 min. The freshly prepared first strand cDNA was used as a template to amplify the target genes using gene specific primers as shown in Table 2.1. PCR amplification was performed according the following cycle conditions: denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, and elongation at 72°C for 60 sec followed by a final elongation reaction at 72°C for 7 min. The cycle was repeated for 25 times to generate target gene fragments. The PCR product was analyzed on a 1.5% TAE agarose gel. After electrophoresis, agarose gel was stained with ethidium bromide (EB) and observed on a UV light transilluminator.

2.2.2 Cloning of the GH/PRL family of hormones and their receptors in zberafish

The amplified cDNA products were separated by agarose gel electrophoresis and were subjected to gene clean using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). The purified target cDNA fragment was ligated into a TOPO pCR2.1 vector (Invitrogen, USA) and then transformed into Top10 competent *E. coli*

cells by heat shock according to the manufacturer's manual of the TOPO TA Cloning Kit (Invitrogen, USA). The positive single clones were identified by PCR. DNA plasmids were produced from Top10 bacteria, purified using Miniprep Kit (Qiagen, Valencia, CA, USA), and finally confirmed by DNA sequencing (TechDragon, Hong Kong). After confirmation of the DNA nucleotide sequences, the target gene fragments were cleaved from the TOPO plasmids with *Age*I and *Kpn*I restriction enzymes by overnight incubation at 37°C. The DNA fragments were purified by gel extraction and respectively subcloned into the pSUMO expression vector (a kind gift from Professor Shannon W. N. Au at The Chinese University of Hong Kong) which harbors a His tag for purification. Detailed information regarding the pSUMO vector is shown in Figure 2.1. Positive clones were screened by PCR and confirmed by DNA sequencing after purified from *E. coli* cultures.

2.2.3 Recombinant protein expression in the *E. coli* bacteria system

The SUMO plasmids harbouring the target cDNAs to be expressed were transformed in *E. coli* strain *BL21*(pLYS) for expression by a general transformation protocol using heat shock at 42°C for 45 sec and recovery on ice for 5 min and grew in Luria-Brertani (LB) medium containing 100 μg/ml ampicillin (Ap). A single BL21 colony transformed with expression plasmid was selected and grown overnight at 37°C with 250 rpm shaking in 20 ml of the LBAp medium. The overnight bacterial culture was then inoculated in 1L LBAp medium. The mass bacterial culture was allowed to grow at 37°C with 250 rpm shaking until the O.D. at 600 nm reached 0.6-0.8. Isopropylthio-β-D-galatoside (IPTG) (Invitrogen, USA) at a final concentration of 0.1 mM was added into the cell culture to induce protein expression in *E. coli*. After addition of IPTG, the bacterial culture was allowed to grow for

several hours at 37°C with 250 rpm shaking. During the optimization of the purification parameters in the small-scale preparation of proteins, different conditions inducing temperatures, times, and concentrations of IPTG were monitored.

Name of primers	Sequences (5' to 3')
GH forward	ACCGGTtccgaaaaccagcggctcttcaa
GH reverse	GGTACCctggattccaactgtaccctgtag
SLα forward	ACCGGTgttcctctggactgtaaggatgac
SLα reverse	GGTACCctcagctgcctgccccaataa
SLβ forward	ACCGGTtctccagtggagtgtccagacca
SLβ reverse	GGTACCactgctccctcttctag
PRL1 forward	ACCGGTgtgggtctgaatgatttgc
PRL1 reverse	GGTACCctagcacatgtcaggcctcttc
PRL2 forward	ACCGGTgcacctatctgtgctcacg
PRL2 reverse	GGTACCttatttactgggatttgatg
GHR1 ECD forward	ACCGGTcaaggatctgagctgttttct
GHR1 ECD reverse	GGTACCagattccaagcaaagaatcaacg
GHR2 ECD forward	ACCGGTacacaaaatgtgcttcctacaa
GHR2 ECD reverse	GGTACCtatacctaacaaagaatcaaga
PRLR1 ECD forward	ACCGGTgtcagtcctccaggcaaa
PRLR1 ECD reverse	GGTACCccaactatattcccagagagcgt
PRLR2 ECD forward	ACCGGTattgtctgggatctggatgaaa
PRLR2 ECD reverse	GGTACCgtatctggttggcagtccaccg

TABLE 2.1 A list of primer sequences used for the cloning of the GH/PRL family of

hormones and their receptors in zebrafish. The upper case letters indicated the *Age*I and *Kpn*I enzyme cutting consensus introduced into the primers.

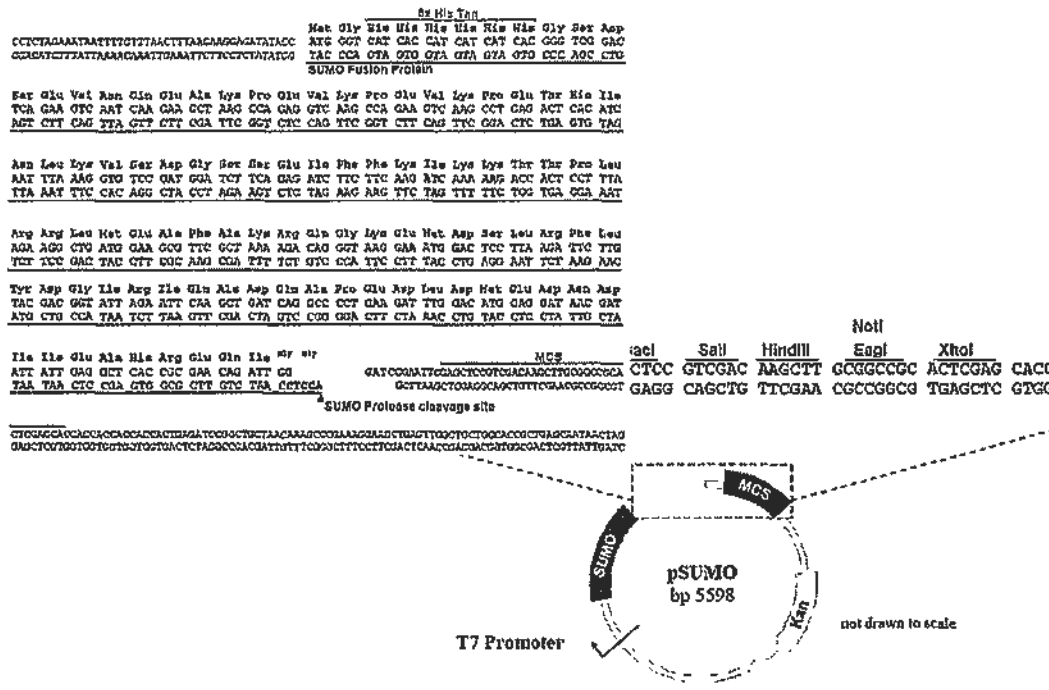


Fig. 2.1 A schematic map of pSUMO vector with multiple cloning sites.

2.2.4 Inclusion bodies isolation and purification

Bacteria culture of *E. coli* containing the expressed recombinant proteins in the form of inclusion bodies was collected by centrifugation at 5,000 rpm at 4°C for 10 min. The cell pellet was then resuspended in the sonication buffer (50 mM Tris-Base and 1 mM EDTA with pH at 8.0). The cell pellet was sonicated in the sonication buffer in a cycle of 30 sec with 30 sec cooling interval for about 30 min. Until the solution became translucent and milky due to release of proteins into the buffer, the inclusion bodies were then isolated from the mixture by centrifugation at 13,000 rpm and 4°C for 20 min. As the inclusion bodies have higher densities, high-speed centrifugation improves the separation of them from the cell debris. The inclusion bodies were washed by the inclusion bodies washing buffer 1 (50 mM Tris-Base, 1

mM EDTA and 1% Triton X-100 with pH at 8.0) and a brief sonication to remove the hydrophobic contaminants, followed by the centrifugation method as mentioned previously. The pellets after centrifugation were solubilized in inclusion bodies washing buffer 2 (50 mM Tris, 2 M urea with pH at 8.0) to separate them from the associated contaminants such as the remaining cellular protein aggregates. The purified inclusion bodies were separated by centrifugation.

2.2.5 Small-scale preparation of solubilised protein from inclusion bodies

100 ml bacteria culture of *E. coli* containing the expressed recombinant GH protein was collected and inclusion bodies of the recombinant protein were isolated and washed according to the methods described in Sections 2.2.3 and 2.2.4. Aliquots of inclusion bodies were solubilised in 50 mM Tris buffer with different pH values or different concentrations of urea, followed by brief sonication. After incubation at room temperature for 30 min, the turbidity of the solutions was measured as the OD at 450 nm. The solutions were then centrifuged 13,000 rpm and 4°C for 20 min and the pellets were discarded. The protein concentrations of the supernatants were measured as the OD at 280 nm.

2.2.6 Solubilisation and refolding of the recombinant proteins

After isolation from other soluble proteins, the inclusion bodies were then dissolved in the solubilisation buffer (2 M urea, 50 mM Tris buffer with pH 12.5) by vigorous shaking using a vortex mix at 4°C for about 1 hour. To optimize the refolding conditions of solubilised inclusion bodies, aliquots of solubilised inclusion bodies solution were dialysed with the following buffers at 4°C for 8 hours respectively: (1) 50 mM Tris buffer at pH 8.0, (2) 50 mM Tris buffer at pH 12.5

followed by 50 mM Tris buffer at pH 8.0, or (3) 50 mM Tris buffer with 2 M urea at pH 8.0 followed by 50 mM Tris buffer at pH 8.0. After dialysis, the concentration of the soluble protein was measured as the OD at 280 nm. In addition, the effects of different original protein concentrations and different temperatures on the dialysis of the solubilised proteins were also investigated by the measurement of the final concentrations of soluble proteins obtained with the OD at 280 nm.

2.2.7 Purification of fusion proteins

The purification process was performed using HisTrap FF column (GE Healthcare, USA). After equilibration with the refolding buffer (50 mM Tris buffer at pH 8.0) of 3× bed volumes, the refolded proteins were loaded onto the column at a flow-rate of around 3 ml/min. The flow-through was collected and the column was washed with the refolding buffer for equilibration. Stepwise elution was used to elute the refolded proteins using different concentrations of imidazole in the refolding buffer, ranging from 50 mM to 500 mM. All the elution fractions were collected followed by SDS-PAGE analysis. The polyacrylamide gel was stained by coomassie brilliant blue R-250 (Sigma, USA) to determine and select the fraction/s with the most abundant pure refolded proteins.

2.2.8 Removal of the His and sumo tags and the purification of non-fused proteins

To further remove the His and the sumo tags from the recombinant hormones to obtain the native proteins, the SUMO protease SENP1C (a gift from Professor K.B. Wong at The Chinese University of Hong Kong) was used. The purified fusion proteins were dialyzed with SENP1C working buffer (20 mM Tris, 200 mM NaCl

and 20 mM β-mercaptoethanol with pH at 7.8) overnight before adding the protease. The ratio of protease to the fusion protein is 1:100 (w/w) and the reaction mixture was incubated at 4°C overnight. The non-fused protein was then purified by metal chelating chromatography performed using the HisTrap FF columns (GE Healthcare, USA) in which the undigested fusion proteins and the cleaved free SUMO tag were adsorbed by the column and therefore removed from the non-fused proteins. Finally, the native non-fused proteins were dialyzed with PBS buffer (1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl with pH at 7.4) overnight. The non-fused protein concentration was determined by the Bradford assay (Bio-Rad, USA). The purity of proteins was determined by SDS-PAGE followed by coomassie blue staining and stored at -80°C.

2.3 Results

2.3.1 The recombinant proteins aggregated as inclusion bodies when expressed in the *E. coli* bacteria system

After successful cloning of recombinant target genes in the pSUMO vector, a small-scale preparation of the recombinant proteins in *BL21* bacterial strain was carried out. This part of study focused on the purification of GH, as it was a representative of these proteins and the purification processes of other proteins were similar to that of the recombinant GH.

The pSUMO-GH plasmid was transformed in *BL21* competent cell and expression of recombinant zebrafish GH was induced by IPTG at 37°C for 8 hours, with the pSUMO vector as a positive control. After cell lysis and subsequent centrifugation, the majority of the sumo protein in the control could be detected in

the supernatant (Lane 3, Figure 2.2). In contrast, the recombinant GH was only found in the pellet, suggesting that GH protein formed inclusion bodies when expressed in the bacteria expression system (Lane 9, Figure 2.2).

The purification of proteins from inclusion bodies could be laborious and time-consuming; therefore I have attempted to optimize the expression conditions in order to obtain soluble recombinant proteins from the bacteria. As aforementioned, the formation of inclusion bodies is affected by many factors such as temperature and protein concentration. A commonly used method is to modify the expression temperature to avoid aggregation of proteins. It is generally believed that recombinant proteins are prone to aggregate at higher temperatures during synthesis. While the temperature is lower than 16°C, protein synthesis in the bacteria is almost suspended (Fahnert et al. 2004). In this study, three different temperatures (18°C, 25°C and 37°C) were selected under 0.1 mM IPTG induction. As compared lanes 2 & 3, 4 & 5 and 6 & 7 in Figure 2.3, it was found that the recombinant proteins produced under all the three temperatures remained in the pellet, indicating that the recombinant protein aggregated as inclusion bodies regardless of the temperature of expression.

Secondly, the induction time was monitored with 0.1 mM IPTG at 25°C. The recombinant GH which was expressed at as early as 2 hours after IPTG induction increased along with the incubation time (Figure 2.4). However, the recombinant protein accumulation was only detected in the pellets, but not in the supernatants, suggesting that IPTG induction time did not affect the formation of inclusion bodies (Lanes 3, 5 and 7, Figure 2.4).

Different concentrations of IPTG used in the induction were also investigated. As shown in Figure 2.5, the GH inclusion bodies were still formed even at a relatively low concentration of IPTG induction at 0.01 mM (Lane 3, Figure 2.5). A similar situation was observed at 0.05 mM IPTG (Lane 5, Figure 2.5), similar to that of 0.1 mM IPTG induction (Lane 9, Figure 2.2).

All strategies used in the present study to improve the solubility of the recombinant proteins during protein expression period turned out to be unsuccessful, therefore I sought to obtain the soluble recombinant proteins from the inclusion bodies.

2.3.2 Optimization of the inclusion bodies solubilisation

Although several methods have been proven effective in the solubilisation of inclusion bodies, including high concentrations of chaotropic agents (such as urea and guanidine-HCl) and detergents (such as SDS and sarkosyl), their treatment always resulted in the complete denaturation of proteins, which might decrease the quantity and quality of the proteins recovered from the inclusion bodies (Fahnert et al., 2004). In the present study, a combination of different methods was used to solubilize the inclusion bodies mildly without disruption of its native-like secondary structure. First of all, we examined the effect of pH on the solubility of the inclusion bodies in the absence of chaotropic agents. Aliquots of inclusion bodies obtained from the *E. coli* were solubilized with Tris buffer at different pH values, ranging from 1 to 14. After 30 min incubation at room temperature, the turbidity of all the solutions was determined as the OD at 450 nm. Greater solubility of inclusion bodies

was observed in the buffers with pH values of 1, 12.5, 13 and 14 (Figure 2.6). After removal of the pellets from the inclusion bodies solution by centrifugation, the OD of the supernatants of all the solutions was measured at 280 nm. Consistently, the concentrations of soluble proteins in the buffers at pH 1, 12.5, 13 and 14 were much higher than all the other buffers at pH 2-12 (Figure 2.7). As extreme pH (pH<2 or pH>13) resulted in the complete destruction of protein secondary structure, the buffer with pH at 12.5 was most appropriate in order to prevent the loss of biological activity after recovery.

However, the Tris buffer with pH at 12.5 alone did not maximize the solubility of inclusion bodies as buffer with 8 M urea was much more effective (compare Column 16 with other columns in Figures 2.6 and 2.7). Therefore, urea was further introduced to the solubilisation buffer. Tris buffers with increasing concentrations of urea at pH 12.5 were used to solubilize the GH inclusion bodies. As shown in Figure 2.8, the turbidity of inclusion bodies solution decreased with addition of urea. The effect of 2 M urea in the buffer at pH 12.5 was comparable to 4-8 M urea at pH 12.5 and to 8 M urea at pH 8.0. Similarly, the concentration of soluble proteins in the inclusion bodies solution was largely increased in the buffers with 2-8 M urea at pH 12.5 (Figure 2.9). In contrast, Tris buffer with 2 M urea at pH 8.0 did not increase the solubilization of inclusion bodies. Therefore, 50 mM Tris buffer with 2 M urea at pH 12.5 was proved to be the best for the solubilization of recombinant proteins from inclusion bodies. This condition was to apply in the subsequent studies.

2.3.3 Optimization of refolding conditions of the recombinant proteins

Protein re-aggregation is one major problem during protein refolding, and this

can be improved by optimization of pH, chaotropic agents, protein concentration, temperature, etc. Two parameters were considered firstly in this study for the refolding of solubilised protein, the existence of urea and alkaline pH in the buffer. Sequential dialysis was carried out to find out an effective method to remove the urea and to adjust the pH from 12.5 to 8.0. One-step dialysis of the solubilized recombinant GH directly from the solubilisation buffer to 50 mM Tris buffer at pH 8.0 resulted in a considerable loss (90%) of soluble recombinant proteins. A two-step dialysis method involving 50 mM Tris buffer at pH 12.5 followed by 50 mM Tris buffer at pH 8.0 resulted in an improvement with a refolding yield of 18%. An alternative two-step dialysis method involving 50 mM Tris buffer with 2 M urea at pH 8.0 followed by 50 mM Tris buffer at pH 8.0 exhibited a refolding yield of about 30% (Figure 2.10).

To test the effect of initial protein concentration on the refolding yield, different concentrations of solubilised proteins were dialysed with the two-step dialysis method (method 3 of Fig. 2.10). Refolding yield was high at protein concentrations ranging from 0.2 to 0.6 mg/ml and decreased at protein concentrations from 0.8 to 1.0 mg/ml, suggesting that lower initial concentrations of protein increased the refolding yields (Figure 2.11). Moreover, lower temperature of dialysis also improved the recovery of proteins. For example, the refolding yield was higher when the dialysis was performed at both 4°C and 10°C than at 25°C (Figure 2.12). This phenomenon was most possibly due to greater protein aggregation at higher temperatures as more amounts of protein pellet were obtained after centrifugation of the refolding protein at 25°C. Therefore, a two-step dialysis method (Method 3 of Fig. 2.10) was chosen to refold the recombinant protein at an initial protein concentration

no more than 0.6 mg/ml at 4°C for 8 hours.

2.3.4 Purification of non-fused soluble recombinant proteins

After optimization of the major reactions, large scale purification of recombinant GH was performed. The refolded recombinant zebrafish GH was purified by metal chelating chromatography using His column followed by elution with increasing concentrations of imidazole. As shown in Figure 2.13, the recombinant GH was eluted by 300 mM imidazole with high purity, which was further processed in the following way. The His and SUMO tags were removed by SENP1C digestion to obtain non-fused GH (Figure 2.14). The yield of non-fused GH determined by the Bradford assay was approximate 1.7 mg/L of cell culture. By using a similar approach, other non-fused zebrafish GH/PRL family of hormones including SLα, SLβ, PRL1 and PRL2 were produced and purified (Figure 2.15). The pictures of the purification of the GH/PRL family receptors were shown in Chapter 3.

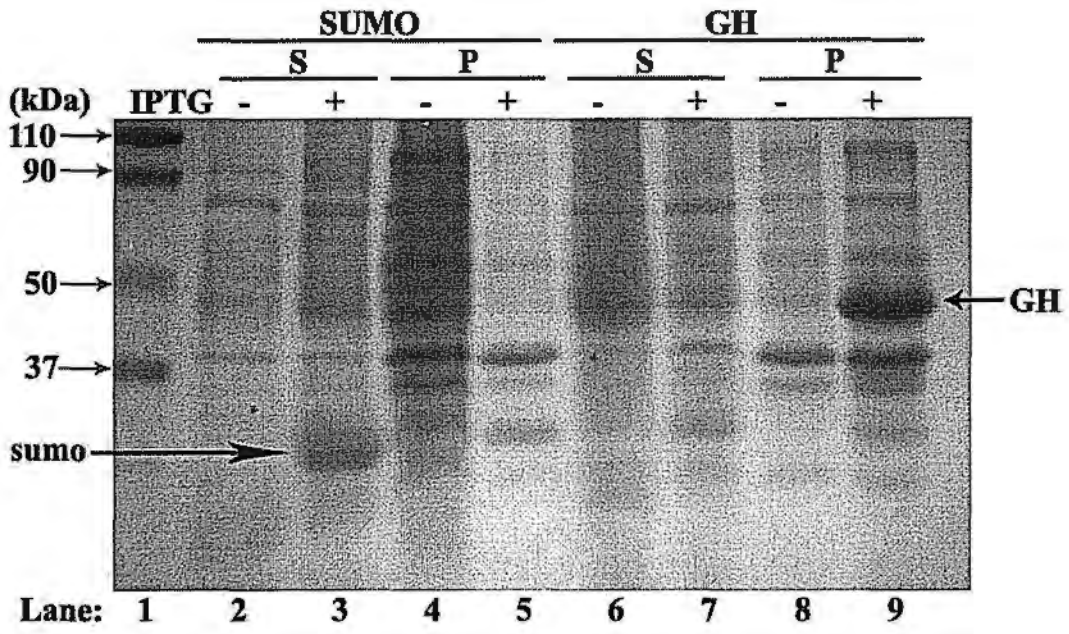


Fig. 2.2 SDS-PAGE analysis of recombinant zebrafish GH expression in *E. coli*. The cells expressing sumo or GH in the presence (+) or absence (-) of 0.1 mM IPTG at 37°C for 8 hours was lyzed and centrifuged to separate the supernatant (S) and pellet (P). The SDS-PAGE gel was stained with coomassie blue. Lane 1: protein marker; Lanes 2 and 3: supernatant of bacteria cell lystate transformed with pSUMO vector with or without 0.1 mM IPTG induction; Lanes 4 and 5: pellet of bacteria cell lystate transformed with pSUMO vector with or without 0.1 mM IPTG induction; Lanes 6 and 7: supernatant of bacteria cell lystate transformed with pSUMO-GH with or without 0.1 mM IPTG induction; Lanes 8 and 9: pellet of bacteria cell lystate transformed with pSUMO-GH with or without 0.1 mM IPTG induction.

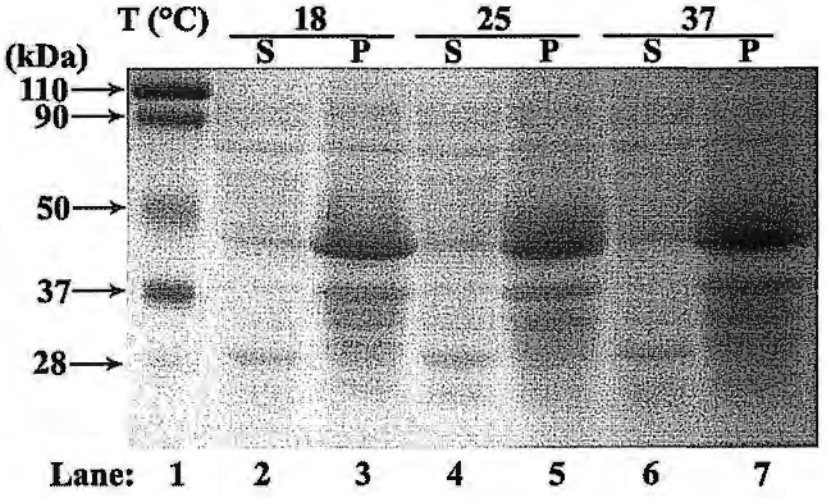


Fig. 2.3 SDS-PAGE analysis of recombinant zebrafish GH expression in *E. coli* under different induction temperatures (T). The SDS-PAGE gel was stained with coomassie blue. Lane 1: protein marker; Lanes 2, 4 and 6: supernatants (S) of bacteria cell lysate under 18, 25 or 37°C incubation temperature; Lanes 3, 5 and 7: pellets (P) of bacteria cell lysate under 18, 25 or 37°C incubation temperature.

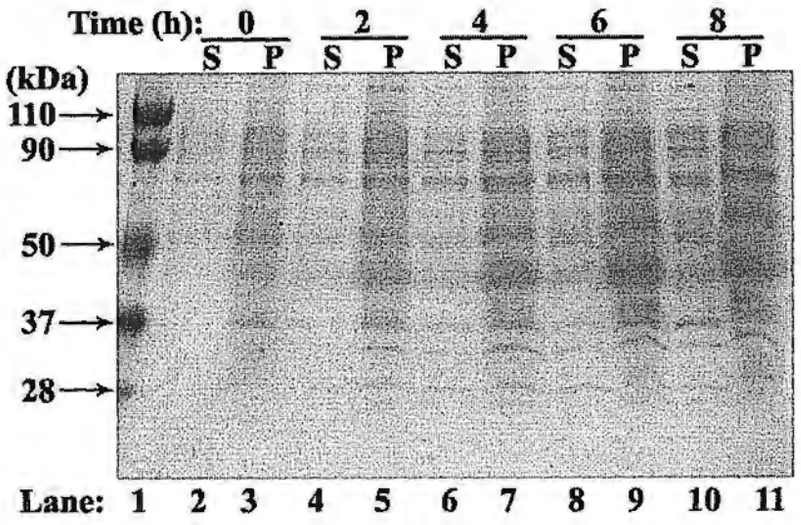


Fig. 2.4 SDS-PAGE analysis of recombinant zebrafish GH expression in *E. coli* at different induction times. The SDS-PAGE gel was stained with coomassie blue. Lane 1: protein marker; Lanes 2, 4, 6, 8 and 10: supernatants (S) of bacteria cell lysate after 0, 2, 4, 6 and 8 hours (h) of 0.1 mM IPTG incubation; Lanes 3, 5, 7, 9 and 11: pellets (P) of bacteria cell lysate after 0, 2, 4, 6 and 8 hours of 0.1 mM IPTG incubation.

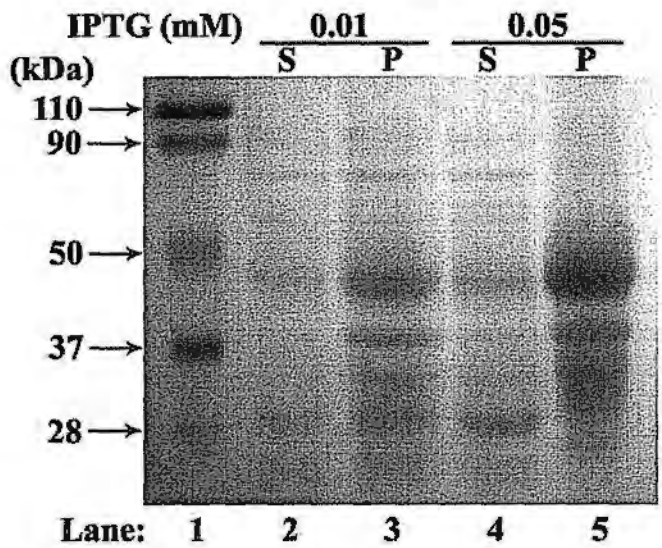


Fig. 2.5 SDS-PAGE analysis of recombinant zebrafish GH expression in *E. coli* under the induction of different IPTG concentrations. The SDS-PAGE gel was stained with coomassie blue. Lane 1: protein marker; Lanes 2 and 4: supernatants (S) of bacteria cell lysate under the induction of 0.01 mM or 0.05 mM IPTG; Lanes 3 and 5: pellets (P) of bacteria cell lysate under the induction of 0.01 mM or 0.05 mM IPTG.

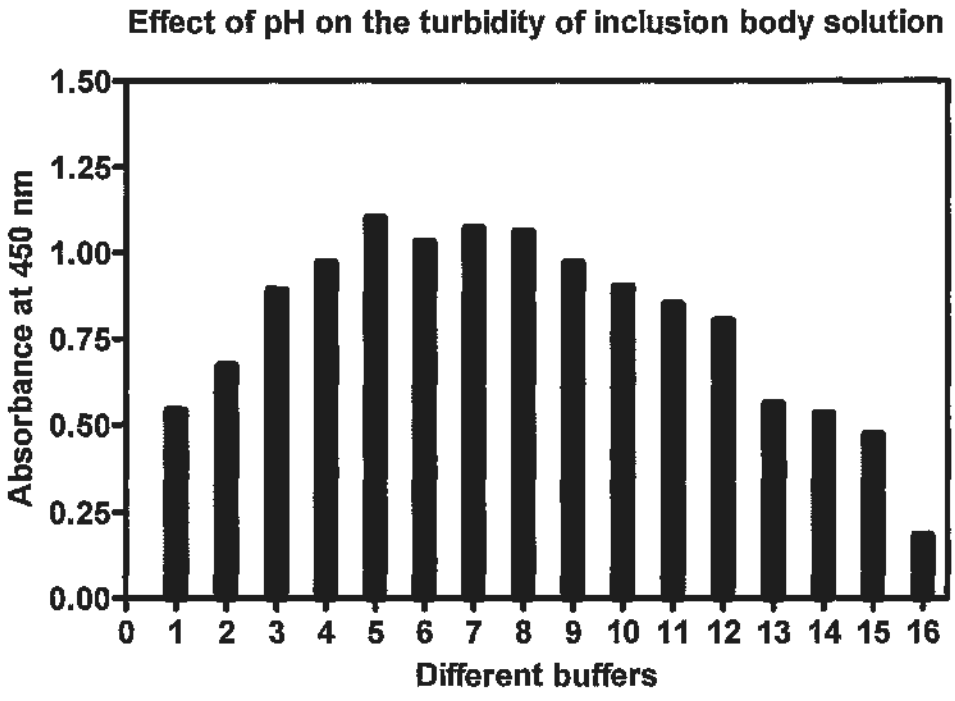


Fig. 2.6 Effect of pH on the turbidity of inclusion bodies solution, using buffer with 8 M urea as the control. Inclusion bodies were solubilized in the buffers with different pH values. The turbidity of the solutions was measured as the OD at 450 nm. Columns 1-15: 50 mM Tris buffer with pH value at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 12.5, 13 or 14 respectively; Column 16: 50 mM Tris buffer with 8 M urea at pH 8.0.

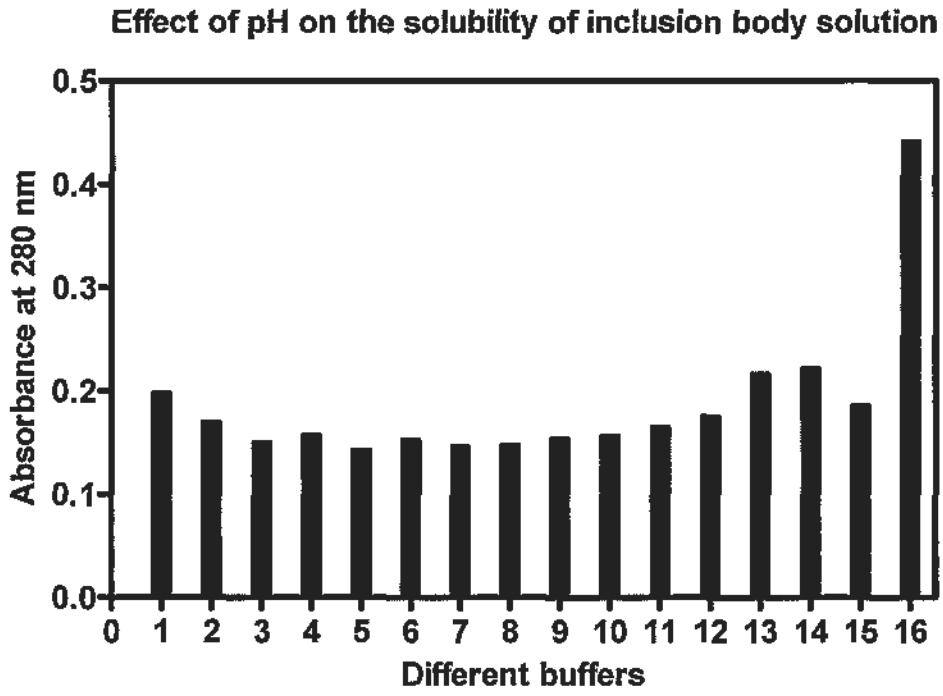


Fig. 2.7 Effect of pH on the solubility of inclusion bodies solution, using buffer with 8 M urea as the control. Inclusion bodies were solubilized in the buffers with different pH values. The protein concentration was measured as the OD at 280 nm after centrifugation. Columns 1-15: 50 mM Tris buffer with pH at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 12.5, 13 or 14 respectively; Column 16: 50 mM Tris buffer with 8 M urea at pH 8.0.

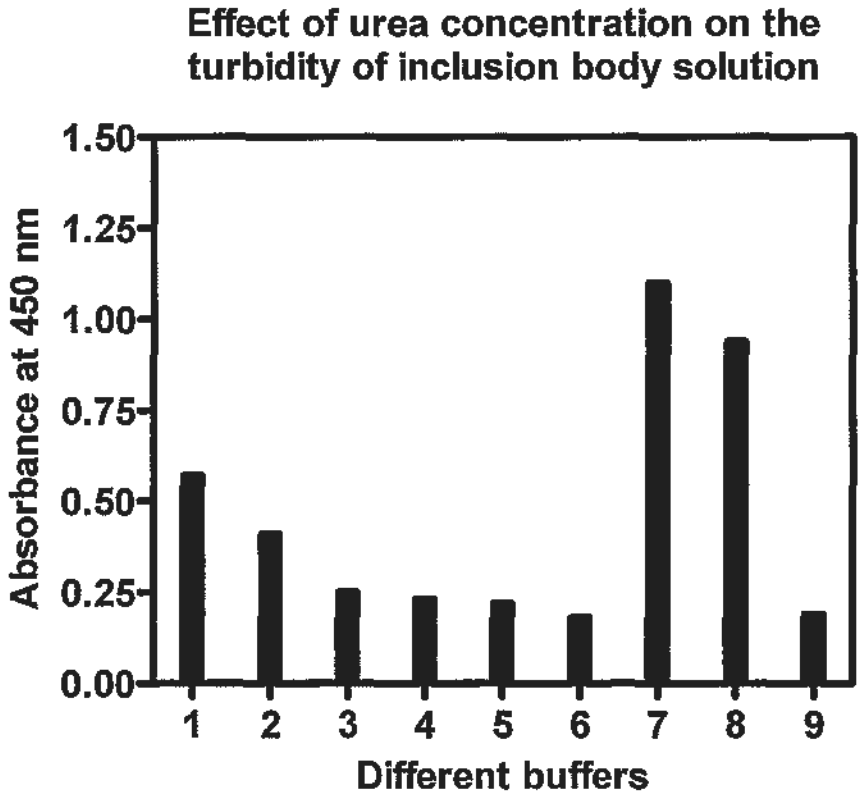


Fig. 2.8 Effect of urea concentration on the turbidity of inclusion body solution. Aliquots of inclusion bodies were solubilized in Tris buffers with different urea concentrations and the turbidity of the solutions was measured as the OD at 450 nm. Columns 1-6: 50 mM Tris buffer with 0, 1, 2, 4, 6, or 8 M of urea at pH 12.5 respectively; Column 7: 50 mM Tris buffer at pH 8.0; Column 8: 50 mM Tris buffer with 2 M urea at pH 8.0; Column 9: 50 mM Tris buffer with 8 M urea at pH 8.0.

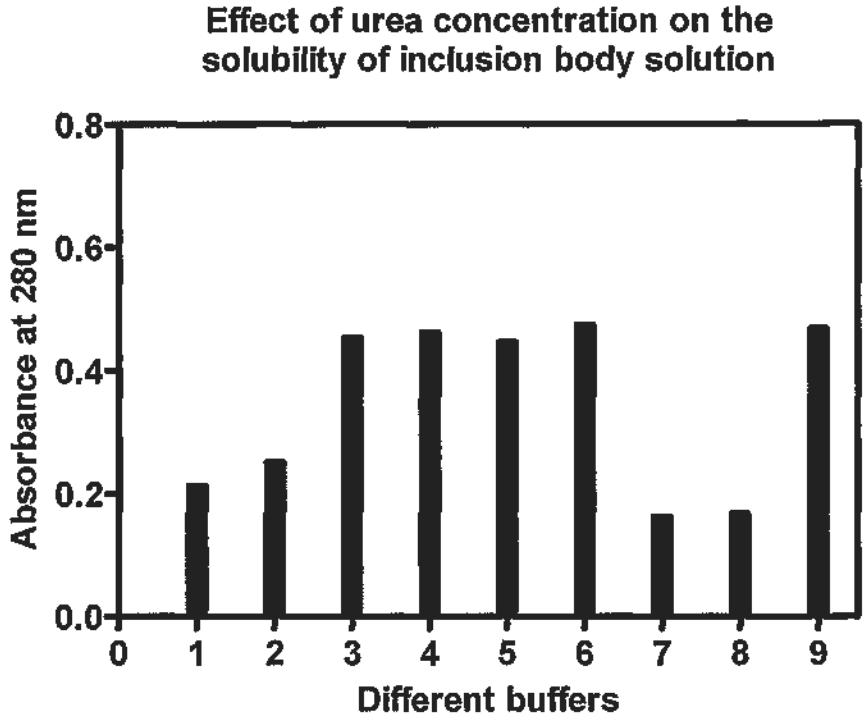


Fig. 2.9 Effect of urea concentration on the solubility of inclusion body solution. Aliquots of inclusion bodies were solubilised in Tris buffers with different urea concentrations and the turbidity of the solutions was measured as the OD at 280 nm. Columns 1-6: 50 mM Tris buffer with 0, 1, 2, 4, 6, or 8 M of urea at pH 12.5 respectively; Column 7: 50 mM Tris buffer at pH 8.0; Column 8: 50 mM Tris buffer with 2 M urea at pH 8; Column 9: 50 mM Tris buffer with 8 M urea at pH 8.0.

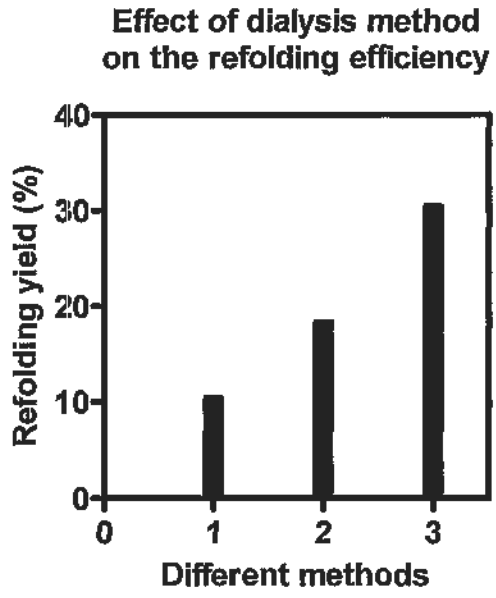


Fig. 2.10 Effect of dialysis method on the refolding efficiency of recombinant zebrafish GH. Solubilised GH (0.6 mg/ml) was dialysed with different methods for 8 hours at 4°C. The refolding yield is the ratio of OD 280 value of dialysed protein solution over OD 280 value of initial protein solution. Method 1: direct dialysis of solubilised GH from 50 mM Tris buffer with 2 M urea at pH 12.5 to 50 mM Tris buffer at pH 8.0; method 2: a two-step dialysis method involving 50 mM Tris buffer at pH 12.5 followed by 50 mM Tris buffer at pH 8.0; method 3: a two-step dialysis method involving 50 mM Tris buffer with 2 M urea at pH 8.0 followed by 50 mM Tris buffer at pH 8.0.

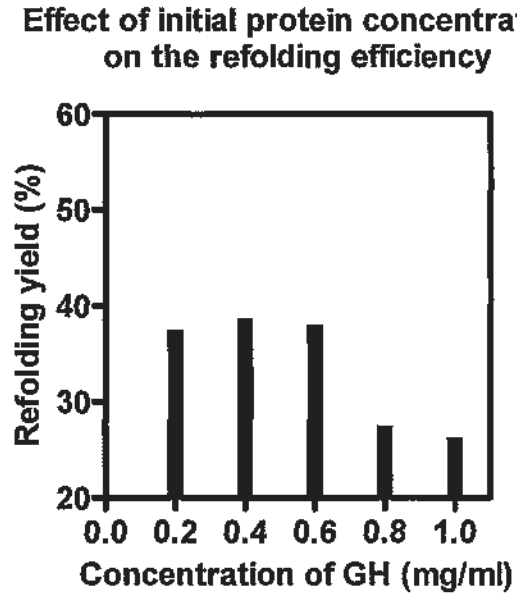


Fig. 2.11 Effect of initial protein concentration on the refolding efficiency of recombinant GH. The refolding yield is the ratio of OD 280 value of dialysed protein solution over OD 280 value of initial protein solution. Different concentrations of solubilised GH were dialysed with a two-step dialysis method (Method 3 in Fig. 2.10) for 8 hours at 4°C.

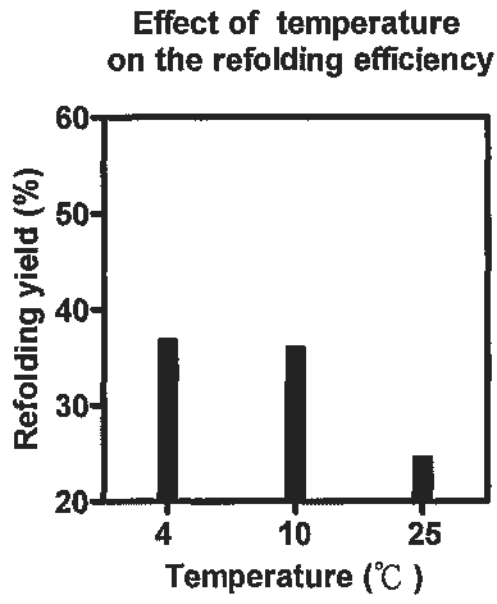


Fig. 2.12 Effect of temperature on the refolding efficiency of recombinant zebrafish GH. Solubilised GH (0.6 mg/ml) was dialysis with a two-step dialysis 2 (Method 3 in Fig. 2.10) for 8 hours at 4°C, 10°C and 25°C respectively. The refolding yield is the ratio of OD 280 value of dialysed protein solution over OD 280 value of initial protein solution.

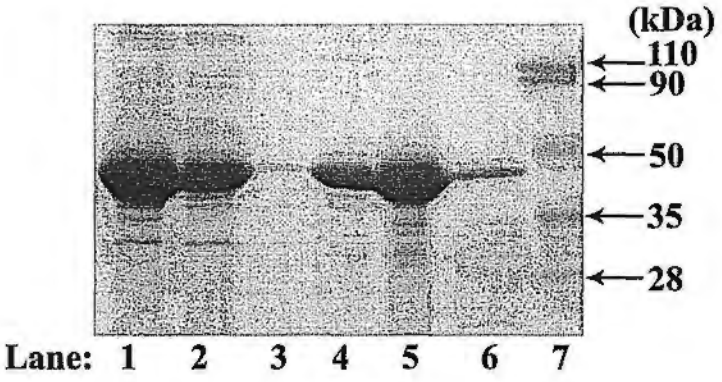


Fig. 2.13 SDS-PAGE analysis of recombinant zebrafish GH protein after recovery from GH inclusion bodies. Lane 1: solubilized GH inclusion bodies; Lane 2: Column flow-through; Lane 3: Wash (50 mM Tris pH 8.0); Lane 4: eluted recombinant GH in buffer involving 100 mM imidazole with 50 mM Tris pH 8.0; Lane 5: eluted recombinant GH in buffer involving 300 mM imidazole with 50 mM Tris, pH 8.0; Lane 6: eluted recombinant GH in buffer involving 500 mM imidazole with 50mM Tris, pH 8.0; Lane 7: protein marker.

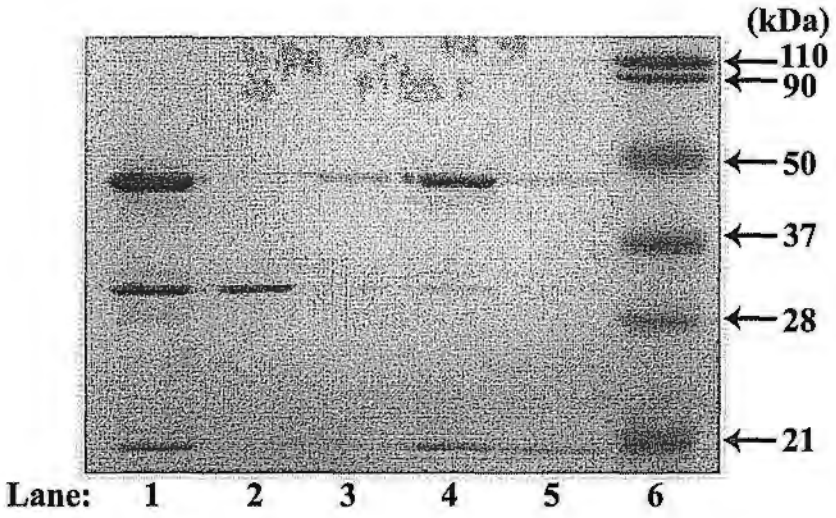


Fig. 2.14 SDS-PAGE analysis of the purification of non-fused GH by metal chelating chromatography after SENP1C digestion. Lane 1: recombinant protein mix after SENP1C digestion; Lane 2: His-column flow-through (non-fused GH); Lane 3: Wash (50 mM Tris, pH 8.0); Lane 4: eluted proteins in buffer involving 100 mM imidazole with 50 mM Tris, pH 8.0; Lane 5: eluted proteins in buffer involving 500 mM imidazole with 50 mM Tris, pH 8.0; Lane 6: protein marker.

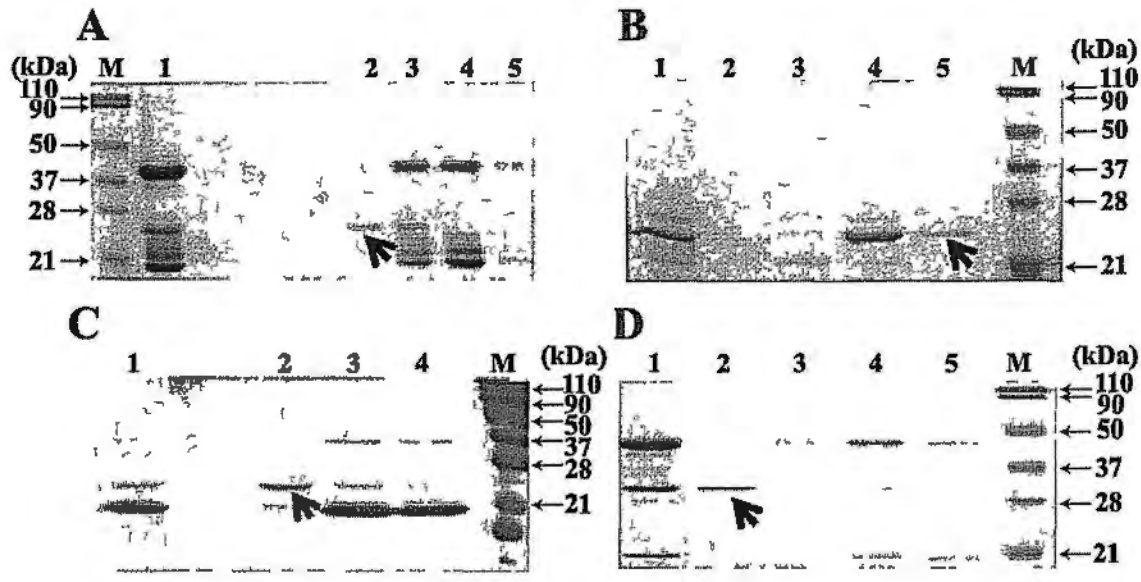


Fig. 2.15 SDS-PAGE analysis of the purification of non-fused SLα (A), SLβ (B), PRL1 (C) and PRL2 (D) by metal chelating chromatography after SENP1C digestion. The arrows indicated the non-fused proteins purified. M: protein marker; Lane 1: recombinant protein mix after SENP1C digestion; Lane 2: His-column flow-through (non-fused recombinant proteins); Lane 3: Wash (50 mM Tris, pH 8.0); Lane 4: eluted proteins in buffer involving 100 mM imidazole with 50 mM Tris, pH 8.0; Lane 5: eluted proteins in buffer involving 500 mM imidazole with 50 mM Tris, pH 8.0.

2.4 Discussion

The aim of the present study was to develop an optimized solubilisation and refolding method suitable for the production and purification of the GH/PRL family of hormones and receptors in zebrafish, as the majority of these proteins were expressed in the form of inclusion bodies in the *E. coli* system (Figure 2.2). The solubility of recombinant GH with retention of native-like secondary structure was increased in the mild buffer combining alkaline pH and low concentrations of urea (2 M). These conditions were demonstrated to enhance the yield and bioactivity of recombinant proteins recovered (Khan et al., 1998). Appropriate concentration of the solubilized GH was refolded by a stepwise dialysis method at 4°C for 8 hours, giving rise to about 3 fold of refolding yield as compared to the one-step dialysis method (Figure 2.10). By combining these methods, the non-fused GH/PRL family of hormones and their receptors were purified with high yield and purity.

2.4.1 Recombinant GH/PRL family hormones formed inclusion bodies

The initial attempt in this study was to purify these proteins directly in soluble form from *E. coli*, and this would be much more convenient and faster. The results of heterologous protein expression, which mainly depend on several complicating factors such as bacteria strain, protein characteristics and induction conditions are always unpredictable. The expression rate and the correct folding rate of the recombinant protein are mainly determined by three factors, including the level of gene induction, the gene codon bias and the mRNA stability. When protein expression rate overwhelms the folding rate, inclusion bodies are most likely produced (Pines and Inouye, 1999). In the actual optimization procedures for soluble and correctly folded proteins, the rate of synthesis is mostly controlled at the

induction level. Lower induction temperature at 18-25°C and lower inducer concentration usually enhance the yield of native proteins (Dracheva et al., 1995). Therefore a low temperature and a long induction time (such as 6-24 hours) are preferred (Fahnert et al., 2004). In the present study, induction of protein expression at lower temperatures (18°C and 25°C) and at lower IPTG concentrations (0.05 mM and 0.01 mM) did not increase the amounts of soluble GH produced (Figures 2.3 and 2.5). Besides, the expression of soluble protein in *E. coli* could not be detected in the early hours post induction, suggesting that the formation of GH inclusion bodies in *E. coli* might be due to the characteristics of the proteins rather than the induction conditions (Figure 2.4). Consistently, GH from other species such as human and ovine also formed inclusion bodies when expressed in *E. coli* (Khan et al., 1998b; Patra et al., 2000; Singh et al., 2008). The formation of inclusion bodies was also frequently observed during the purification of other members of the GH/PRL family including PRL, PL and SL as well as their receptors in bacteria (Fukada et al., 2004; March et al., 1994; Nguyen et al., 2006; Sakal et al., 1997; Sakal et al., 2000), suggesting that it is a tendency for the GH/PRL family of hormones and their receptors to form inclusion bodies when expressed in bacteria.

2.4.2 Purification of recombinant hormones from inclusion bodies

The existence of native-like secondary structure in the inclusion bodies has already been demonstrated by several studies (Oberg et al., 1994; Przybycien et al., 1994) and protection of this structure from disruption during solubilisation of inclusion bodies would be of importance to improve both the yield and bioactivity of recombinant proteins (Khan et al., 1998). Instead of using high concentrations of urea or guanidine-HCl which are commonly employed but always resulted in

complete protein denaturation during the process, alkaline pH combining with a low concentration of urea (2 M) was utilized to protect the native-like secondary structure. The high concentration of OH^- group in the alkaline environment would lead to the gradual unfolding of proteins and induction of inclusion bodies dissociation and solubilisation (Nall et al., 1988). In the context of GH, pH challenge did not unfold its secondary structure or the unfolding was reversible (Aloj and Edelhoch, 1972; Holzman et al., 1990; Zou et al., 1998). On the other hand, the alkaline pH alone did not have the ability to solubilise the inclusion bodies completely (Figures 2.8 and 2.9). Thereofre 2 M urea was added to induce maximum solubilisation of the inclusion bodies. As inclusion bodies were barely solubilised in the 2 M urea in the absence of alkaline pH (Figures 2.8 and 2.9), urea appeared to function to interfere with hydrophobic interactions between proteins and would therefore assist the alkaline pH to solubilise the inclusion bodies. Moreover, the solubilisation effect of this combination was almost comparable to that of 8 M urea. The yield of recovered native protein using this method was much higher than that of 8 M urea (data not shown), indicating that our method of solubilizing the inclusion bodies does not disrupt the native-like secondary structure, and this is the biggest difference with the buffer using 8 M urea. In addition, the success of solubilising the inclusion bodies by an alkaline pH and urea suggests that both the ionic and hydrophobic interactions are present and may play important roles in the inclusion bodies formation.

Protein refolding is often ineffective due to protein re-aggregation after the removal of salts and denaturants. A general refolding method to prevent intermolecular interaction is rapid dilution of the solubilised protein, which however, requires high concentrations of initial protein solution. However, it is more likely for

the proteins to re-aggregate at high concentrations. Different dialysis methods were examined to optimize the refolding yield of soluble proteins from the inclusion body solubilisation buffer. Our data showed that gradual dialysis improved the recovery yield of the denatured proteins as compared to the simple direct one-step dialysis, suggesting that a slow refolding process could reduce intermolecular protein interactions and lead to high recovery yield of protein (Column 1 vs. Columns 2 and 3, Figure 2.10). It was also observed that the longer presence of urea in the refolding buffer was much more effective in preventing re-aggregation of proteins as compared to the alkaline pH, indicating that native GH was stable under the low concentration of chaotropic agents (Column 2 vs. Column 3, Figure 2.10). It is proposed that non-denaturing concentrations of urea bind to the folding intermediates and prevent the aggregation (Misawa and Kumagai, 1999). Indeed, it has been reported that low concentrations of urea or guanidine-HCl can reduce protein aggregation and misfolding, therefore increase the refolding yield of native proteins (Tan et al., 2007). Moreover, decreasing amounts of urea was commonly used to the gradual refolding of recombinant proteins, indicating that urea can inhibit protein aggregation (Swietnicki, 2006). Renaturation with concomitant disulfide bond formation of reduced chymotrypsinogen A was only feasible in the presence of low concentrations of urea or guanidine-HCl (Orsini and Goldberg, 1978).

Protein re-aggregation during the refolding process is a common problem difficult to deal with, especially in the context of the GH/PRL family of hormones due to the existence of several cysteine residues which might form intermolecular disulfide bonds. To avoid the formation of intermolecular disulfide bonds, β-mercaptoethanol was added in the refolding buffer. Several other factors were also

taken into consideration during the refolding process, including protein concentration and temperature. Lower protein concentrations are of importance for efficient refolding because protein aggregation is a process of second order or higher order reaction kinetics (Jaenicke, 1995). At higher concentrations, protein molecules have higher opportunity to interact with each other leading to aggregation. The most efficient protein concentration during refolding was determined to be 0.6 mg/ml, the refolding yield of which was twice as that at 1.0 mg/ml. Although the temperature at which refolding is performed varies, it is generally accepted that 4°C is the best in practice to prevent aggregation (Rudolph and Lilie, 1996; Singh et al., 2008). In the present study, the whole refolding process was performed at 4°C, which was also superior to other temperatures such as 10°C and 25°C (Figure 2.12).

In conclusion, an effective solubilisation and refolding method for the production and purification of recombinant proteins of the zebrafish GH/PRL family of hormones and their receptors was developed in this study. After a comprehensive investigation of the solubilisation conditions and refolding conditions, the non-fused GH/PRL family of hormones and their receptors were successfully purified with high purity. The biological activities of the purified proteins were investigated in the following chapter. My data support that such a mild solubilisation method together with slow gradual refolding of inclusion bodies is a reliable and effective tool for the purification of recombinant proteins from inclusion bodies.

Chapter 3

Studies on the GHR1 in zebrafish: is it a receptor for somatolactin?

3.1 Introduction

As aforementioned, it is generally regarded that the GH/PRL family of hormones has arisen as a result of gene duplications and subsequent divergence from a common ancestor, alongside with their cognate receptors. High similarities among members of the GH/PRL family have been addressed in several aspects, including genomic organization, protein structure and signaling pathway activation (Forsyth and Wallis, 2002; Haig, 2008).

The general existence of both GHR and PRLR is also well documented in all vertebrates, including fish, reptiles, birds and mammals. Due to an additional whole genome duplication event, it is very common that more than one copy of both *GHR* and *PRLR* genes are present in fish (Huang et al., 2007; Jiao et al., 2006; Meyer and Van de Peer, 2005; Saera-Vila et al., 2005). Phylogenetic analysis of GHRs from various vertebrates shows that the fish GHRs can be clustered into two clades, viz. GHR1 (or type I GHR) and GHR2 (or type II GHR). The GHR1 clade contains the sequences previously reported in a variety of fish species including goldfish (*Carassius auratus*), seabream (*Acanthopagrus schlegeli*), tilapia (*Oreochromis mossambicus*), grass carp (*Ctenopharyngodon idella*), zebrafish (*Danio rerio*), etc. The structure of fish GHR1 exhibits higher homology to the non-teleost GHR. On the other hand, the GHR2 clade only exists in teleost (Jiao et al., 2006; Ozaki et al., 2006). Data also indicated that the two GHRs co-existing in the teleosts are both functionally active in terms of signaling pathway activation (Jiao et al., 2006;

Saera-Vila et al., 2005).

SL was first identified in flounder (*Paralichthys olivaceus*) and subsequently in zebrafish, salmon (*Oncorhynchus masou*), seabream, medaka (*Oryzias latipes*) and other fish species as well (Benedet et al., 2008). Two distinct SL subtypes produced by different pituitary cells have been reported in several fish species such as zebrafish and rainbow trout (*Oncorhynchus mykiss*) (Zhu et al., 2004). However, the identity of the receptor for SL (SLR) is controversial. Despite attempts to search for SLR since the discovery of SL, there is a still much controversy regarding its identity. In 2005, the identification of SLR was first reported in masu salmon (Fukada et al., 2005). This SLR was reported to exhibit higher binding affinity to SL than GH and PRL according to the ligand-binding assays. Phylogenetic analysis indicated that the masu salmon SLR clusters within the GHR1 clade. Subsequently, other SLRs were also reported in medaka and other fish species (Benedet et al., 2008; Fukamachi et al., 2005). However, these SLRs were named merely based on phylogenetic analysis without conclusive experimental qualification. Like the masu salmon SLR, all the other aforementioned SLRs cluster within the GHR1 clade, leading to the hypothesis that the GHR1s in fish are in fact SLRs, while the GHR2s are the real GHRs. However, our previous study has demonstrated that the two GHRs in black seabream could only be activated by seabream GH or salmon GH, but not by salmon SL (Jiao et al., 2006). It was also reported that eel SL could not displace the binding of GH from eel GHR1 (Ozaki et al., 2006). Moreover, a recent study showed that in rainbow trout the two GHRs exhibited ligand-binding specificity towards GH, and SL was an extremely weak competitor of GH binding to the two GHRs (Reindl et al., 2009).

To further address this issue, we have employed zebrafish as a model to investigate whether GHR1 is indeed the SLR by various means of studying receptor-ligand interactions. Bioinformatic searching on the zebrafish genome indicates the existence of five members of the GH/PRL family (namely GH, SLα, SLβ, PRL1 and PRL2) and four members of their receptor family (namely GHR1, GHR2, PRLR1 and PRLR2). It should be noted that these GH/PRL family of ligands and their receptors are only named according to their sequence homology with those in other species. There is so far no systematic study to unravel the relationship among the ligands and receptors. The last point is particularly relevant as some of the ligands and receptors are duplicated in the fish genome. A systematic study on the interaction among the ligands and receptors in zebrafish would help to resolve these issues.

Based on a panel of ligand-receptor interaction studies including His-tag pulldown assays, luciferase reporter assays and phosphorylation of signaling molecules, we have provided evidence that both zebrafish GHR1 and GHR2 are specific receptors for GH. GH could specifically and functionally interact with both GHRs in zebrafish. The two zebrafish PRLs also specifically interacted with the PRLRs but not with the GHRs. However, zebrafish SLα and SLβ could not interact with GHR1, GHR2, PRLR1 or PRLR2 in the binding study. Consistently, these two SLs could not activate the receptor-mediated downstream signaling and transcriptional activities of the four receptors in zebrafish. Our finding in zebrafish is in contrast with the report in masu salmon but similar to the report in rainbow trout and eel. Therefore, the true identity of SLR is still an open question.

3.2 Materials and methods

3.2.1 Plasmids and antibodies

The generation of pcDNA-3.1 plasmids harboring the full-length zebrafish GHR1, GHR2, PRLR1 and PRLR2 has already been described (Huang et al., 2009). The sequences encoding the extracellular domains of zebrafish GHR1, GHR2, PRLR1 and PRLR2 as well as the mature peptide coding regions of zebrafish GH, SLα, SLβ, PRL1 and PRL2 were subcloned into the modified pSUMO vector has already been described in Chapter 2. The mature peptide coding regions of zebrafish GH, SLα, SLβ, PRL1 and PRL2 were also inserted into the pCMV-myc vector by taking advantage of the *Eco*R I and *Xho* I restriction enzyme sites. The luciferase reporter plasmids containing the Spi 2.1, β-casein and c-fos promoters were obtained as described previously (Jiao et al., 2006). Mouse anti-myc (9E10) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-Erk, anti-phosphor-Erk (Thr 202/Tyr 204), anti-Akt and anti-phosphor-Akt (Ser 473) antibodies were purchased from Cell Signaling Technology (Cell signaling Technology, MA, USA).

3.2.2 Cell culture and transfections

The monkey kidney fibroblast cell line COS-7 and goldfish scale fibroblast cell line GAKS were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere containing 5% CO_2. The Chinese hamster ovary cell line CHO was cultured in F-12 medium supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml). The

zebrafish liver cell line ZFL was maintained at 28°C as recommended by ATCC. Transient transfection was carried out using Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

3.2.3 Expression and purification of recombinant proteins

The procedures of purifying the recombinant proteins from *E. coli* were described in Chapter 2. Briefly, one liter of transformed *E. coli* harboring the pSUMO plasmids encoding the extracellular domains of zebrafish GHR1, GHR2, PRLR1 or PRLR2 was grown in LB medium at 37°C until the absorbance reached 0.6 at 600 nm. Cells were then incubated with 0.1 mM IPTG at 37°C for an additional 8 hours before centrifugation at 8000 g for 5 m. The bacteria were sonicated in Tris buffer (50 mM Tris, 1 mM EDTA, pH 8.0). The recombinant proteins in the form of inclusion bodies were then washed with washing buffer I (50 mM Tris, 1 mM EDTA, 1% Triton X-100, pH 8.0) and then with wash buffer II (50 mM Tris, 1 mM EDTA, 2M Urea, pH 8.0). The inclusion bodies in the pellet were dissolved in fresh solubilization buffer (50 mM Tris base, 2 M urea, pH at 12.5) by vigorous shaking at 4°C. After dissolution of the inclusion bodies, the denatured proteins were refolded by a two-step dialysis. The refolded proteins were dialyzed against the refolding buffer at 4°C overnight and then purified by Ni-NTA agarose (Qiagen, USA). The concentration and purity of recombinant proteins were determined by the Bradford assay and SDS-PAGE analysis followed by coomassie blue staining.

3.2.4 His-tag pulldown assays

The procedure of the His-tag pulldown assays was adapted from the method

Wang et al (2009). The COS-7 cells were transfected with myc-tag GH, SLα, SLβ, PRL1 and PRL2 plasmids respectively for 24 hours. After pre-blocking with 3% bovine serum albumin (BSA) and washing with washing buffer (20 mM Tris, pH 7.4, 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM imidazole), the Ni-NTA agarose bound with the His-tag extracellular domains of GHR1, GHR2, PRLR1 or PRLR2 was incubated with the total lysates of the ligand-transfected COS-7 cells which were pre-cleared by Ni-NTA agarose for 3 hours at 4°C with gentle rotation. Afterwards, the agarose was washed four times with ice-cold washing buffer and then stored in SDS sample buffer. Detection of myc-tag hormones and His-tag receptors were performed by Western blotting using anti-myc antibody (1:2000) and coomassie blue staining, respectively. Bound antibody was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using horseradish peroxidase-conjugated antibody.

3.2.5 Luciferase reporter assays

The GAKS cells were seeded onto 24-well culture plates at the density of 1.5×10^5 cells per well overnight and then transiently transfected with 50 ng of the pcDNA3.1 vector harboring the zebrafish GHR1, GHR2, PRLR1 or PRLR2 sequences together with 500 ng of luciferase reporter plasmid containing the Spi 2.1, β-casein or c-fos promoters respectively. Six hours after transfection, the recombinant zebrafish hormones (GH, SLα, SLβ, PRL1 or PRL2) were added into the medium respectively and incubated for another 20 hours. The cells were then lysed and measured for luciferase activities, and normalized with the *Renilla*

luciferase activity. Data were presented as mean values ± SD from at least three independent experiments.

3.2.6 Assessment of the phosphorylation of GHR1 signaling factors

CHO cells were seeded onto 6-well plates at the density of 4×10^4 per well 12 hours before transfection. The cells were then transfected with 500 ng of pcDNA3.1-zfGHR1, pcDNA3.1-zfGHR2, pcDNA3.1-zfPRLR1 or pcDNA3.1-zfPRLR2. After 24 hours of transfection, the medium was aspirated and replaced with fresh medium containing 300 ng/ml of recombinant zebrafish hormone (GH, SLα, SLβ, PRL1 or PRL2) for another hour. The cells were lysed in the presence of protease inhibitor cocktail (Sigma, USA) and centrifuged at 15,000 g for 20 m at 4°C. The supernatants were separated on 12.5% SDS-PAGE gels. The separated proteins were transferred onto PVDF membranes and immunoblotted with primary antibodies against total and phosphorylated ERK1/2 and Akt (all antibodies are purchased from Cell Signaling, USA) respectively. The protein bands were visualized by Western blotting after incubation with secondary antibody conjugated with horseradish peroxidase.

3.2.7 Alamar blue assay

The ZFL cells were seeded onto 96-well plates at the density of 1×10^5 and incubated overnight at 28°C. The cells were then incubated in the medium supplemented with 0.1% fetal bovine serum in the presence of the recombinant zebrafish hormones. After 48 hours of incubation, the medium was replaced with 10% alamar blue reagent (Biosource, USA) diluted by the cell culture medium and incubated for another 2-4 hours in the dark. The yielded color was measured on an

automated fluorescent plate reader (Tecan, USA) at the excitation wavelength of 485 nm and the emission wavelength of 595 nm.

3.3 Results

3.3.1 Binding between the zebrafish GH/PRL family of hormones and their receptors

In order to investigate the direct interaction between the GH/PRL family of hormones in zebrafish and their receptors, the His-tag pulldown assay was carried out. The His-tag fused extracellular domains of the zebrafish receptors were expressed in *E. coli* and purified as described in Chapter 2. Western blotting analysis showed that myc-GH could be detected in the GHR1 and GHR2 pulldown precipitates, but not in the PRLR1 and PRLR2 pulldown precipitates (Lane 2, Figure 3.1), indicating that the zebrafish GH can bind to both GHR1 and GHR2. However, neither myc-SLα nor myc-SLβ could bind to GHRs or PRLRs (Lanes 3 and 4, Figure 3.1), suggesting that none of the four receptors in zebrafish could be qualified to be called as SLR. Co-precipitation of both myc-PRL1 and myc-PRL2 with PRLR1 and PRLR2 was also detected. The results demonstrated that both PRL ligands could interact with the homologous PRLR1 and PRLR2 subtypes (Lanes 5 and 6, Figure 3.1).

3.3.2 Both SLs cannot evoke β-casein, Spi 2.1 and c-fos promoter activities

The ability of the zebrafish GH/PRL family of hormones to evoke the receptor-mediated transcriptional activities mediated by the four receptors in zebrafish was evaluated. The β-casein, Spi 2.1 and c-fos promoters, which have been reported to be activated by the JAK-Stat signaling pathway (Campbell et al., 1995;

Wood et al., 1995), were employed in this experiment. Recombinant proteins of the mature zebrfafish hormones were prepared in *E. coli*. Our previous work has proven that the recombinant zebrafish GH, PRL1 and PRL2 are biologically active (Huang et al., 2009). In this study, we have also demonstrated that both recombinant zebrafish SLα and SLβ are biologically active by the alamar blue assays in ZFL cells. Both recombinant SLα and SLβ were able to promote cell proliferation, though to a lesser extent than GH (Figure 3.2). In GAKS cells without transfection of the receptors, the recombinant hormones could not evoke luciferase activities driven by the β-casein, Spi 2.1 or c-fos promoters (Figure 3.3). But in cells transfected with GHR1, GH could bring about the activation of all the β-casein (Figure 3.4A), Spi 2.1 (Figure 3.4B) and c-fos (Figure 3.4C) promoter activities. However, no enhancement of promoter activity was detected in the cells treated with SLα, SLβ, PRL1 or PRL2 (Figure 3.4). Similarly, GHR2-mediated β-casein and Spi 2.1 promoter activities were stimulated only by GH, but not by the other hormones (Figure 3.5). On the other hand when PRLR1 was expressed in the cells, both PRL1 and PRL2 could enhance the activation of β-casein and Spi 2.1 promoter activities (Figure 3.6A and B). PRLR2, however, appears to be specifically activated by PRL1 but not by any of the other hormones (Figure 3.7A and B), indicating the different selectivity of PRLR1 and PRLR2 toward PRLs.

3.3.3 Phosphorylation of Erk and Akt could not be activated by SLα or SLβ

It has been shown that activation of GHR could lead to phosphorylation of a number of downstream factors such as Erk and Akt (Winston and Hunter, 1995). To further ascertain whether GHR1 is indeed the SLR, phosphorylation of Erk and Akt in GHR1 transfected cells were evaluated after activation by different recombinant

hormones. As shown in Figure 3.8, in the GHR1-transfected CHO cells, the presence of GH could increase the phosphorylation of both Erk and Akt proteins as compared with the control, indicating that GH could activate Erk and PI3K/Akt signal pathways through functional interaction with GHR1. In contrast, all the other hormones including SLα and SLβ had no effect on the phosphorylation of Erk and Akt (Figure 3.8), indicating that GHR1 do not seem to be the receptor for SLα and SLβ. Moreover, it could also be concluded that there was no or extremely weak crosstalk between GH/GHR and PRL/PRLR signaling axes in zebrafish based on the present study.

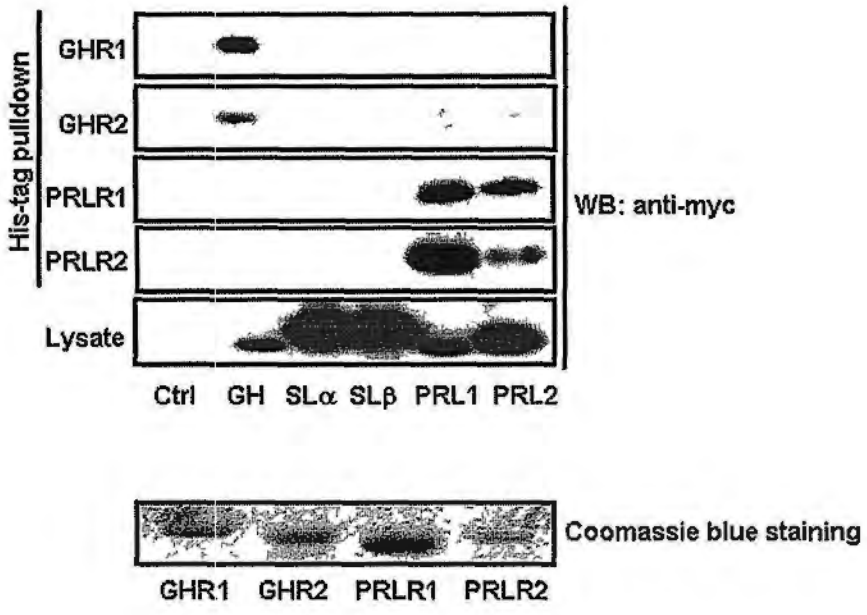


Fig. 3.1 Interaction between the GH/PRL family of hormones and the extracellular domains of their receptors in zebrafish using His-tag pulldown assays. The His-tag fused extracellular domains of zebrafish GHR1, GHR2, PRLR1 and PRLR2 were expressed and purified as described previously (Huang et al., 2009). Myc-tag zebrafish GH, SLα, SLβ, PRL1 and PRL2 overexpressed in COS-7 cells were precipitated separately by the His-tag receptors and loaded on SDS-PAGE. Detection of GH, SLα, SLβ, PRL1 and PRL2 in the precipitates and in the total cell lysates was performed by Western blotting (WB) with anti-myc antibody. The His-tag proteins were detected by coomassie blue staining.

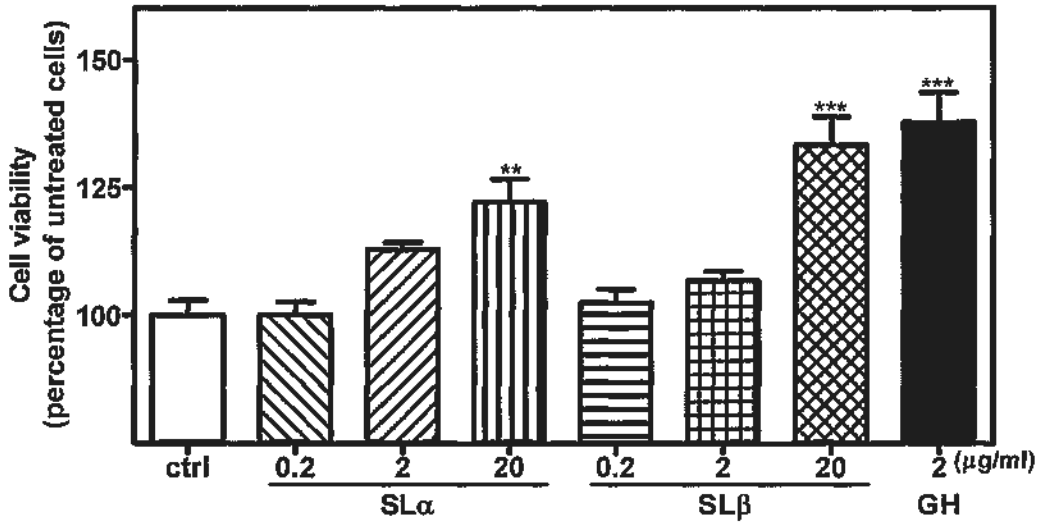


Fig. 3.2 The recombinant zebrafish SLα and SLβ are biologically active. Cultured ZFL cells were treated with different concentrations of the recombinant zebrafish SLα and SLβ for 48 hours. Cell proliferation was examined using the alamar blue assays. Recombinant zebrafish GH was used as a positive control. Results are mean values±SEM (n=3; **P<0.01; ***P<0.001).

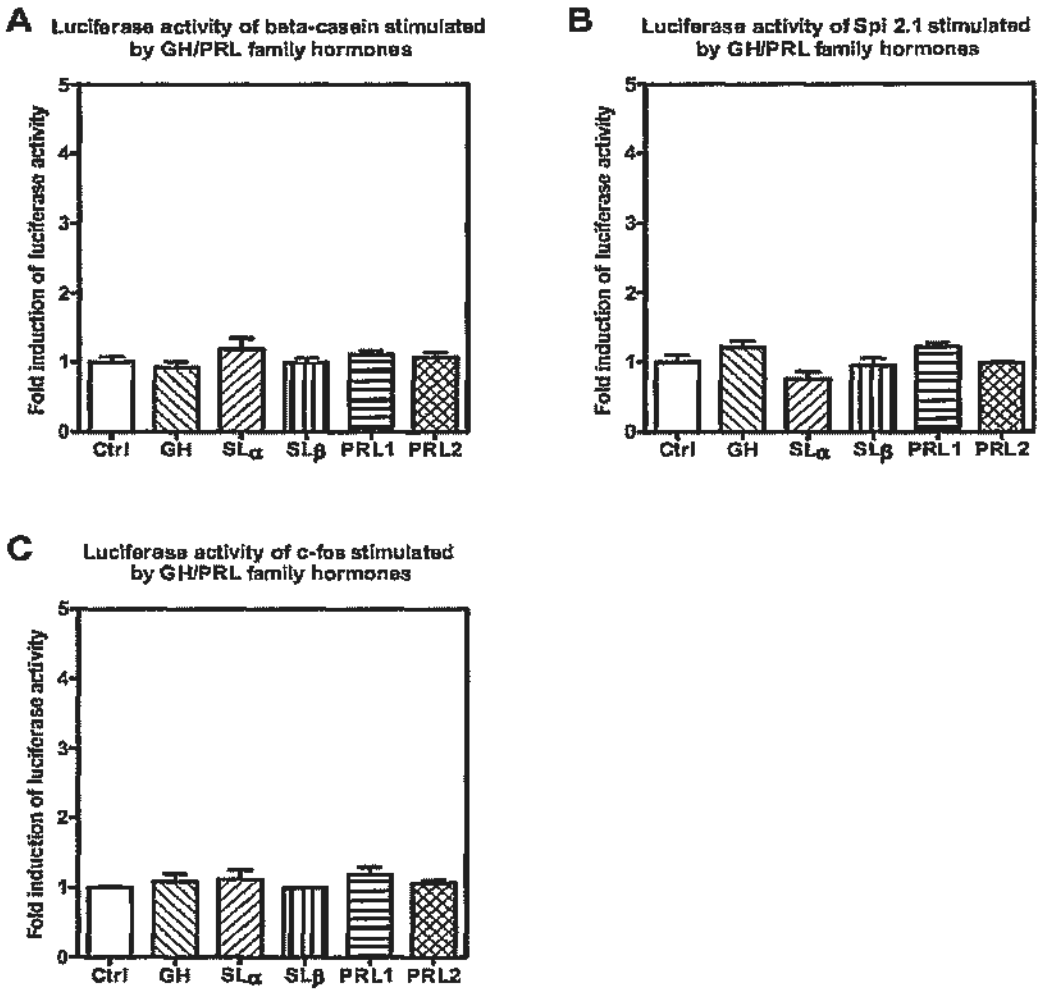


Fig. 3.3 Recombinant hormones did not stimulate promoter activation in GAKS cell. The cells were transfected with luciferase reporter plasmids driven by the Spi 2.1, β-casein and c-fos promoters (A–C), respectively. The transfected cells were subsequently stimulated by adding 300 ng/ml recombinant zebrafish GH, SLα, SLβ, PRL1 and PRL2, respectively. Results are means ± SEM (n = 3).

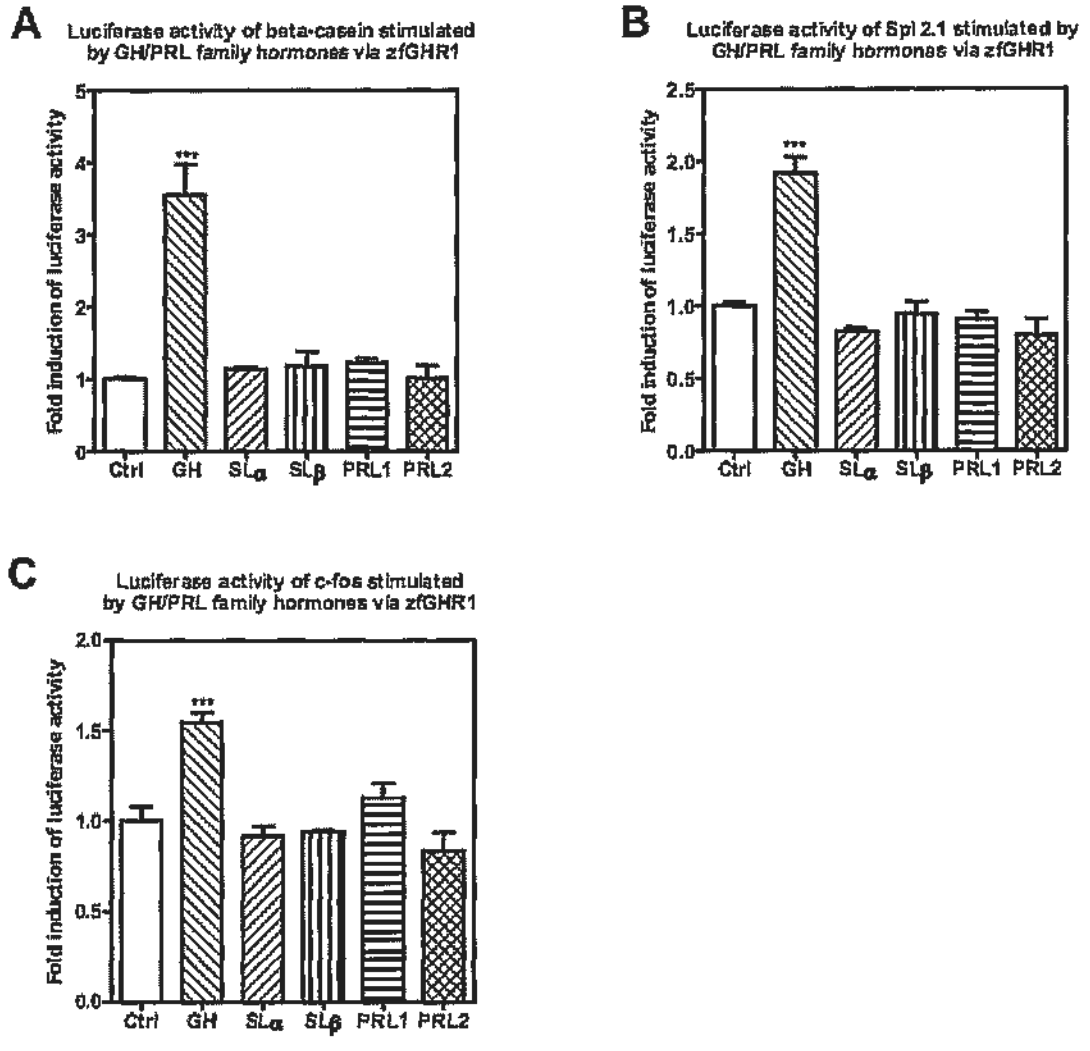


Fig. 3.4 GH activates GHR1-mediated signal transduction. GAKS cells were co-transfected with pcDNA3.1-zfGHR1 and a luciferase reporter plasmid driven by the β-casein (Panel A), Spi 2.1 (Panel B) and c-fos (Panel C) promoters, respectively. The transfected cells were subsequently stimulated by 300 ng/ml of recombinant zebrafish GH, SLα, SLβ, PRL1 and PRL2, respectively. Results are mean values ± SEM (n = 3; ***P<0.001).

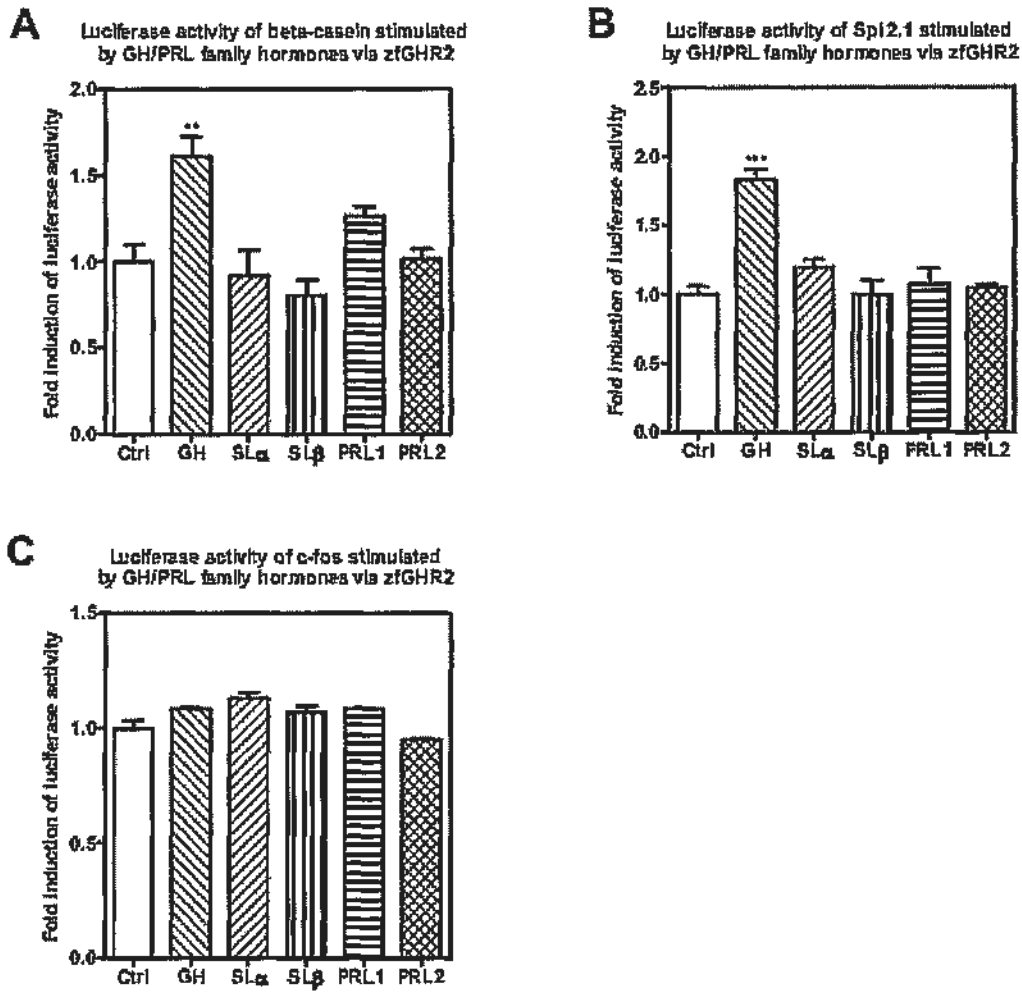


Fig. 3.5 GH activates GHR2-mediated signal transduction. GAKS cells were co-transfected with pcDNA3.1-GHR2 and a luciferase reporter plasmid driven by the β-casein (Panel A), Spi 2.1 (Panel B) and c-fos (Panel C) promoters, respectively. The transfected cells were subsequently stimulated by 300 ng/ml of recombinant zebrafish GH, SLα, SLβ, PRL1 and PRL2, respectively. Results are mean values ± SEM (n = 3; **P<0.01; ***P<0.001).

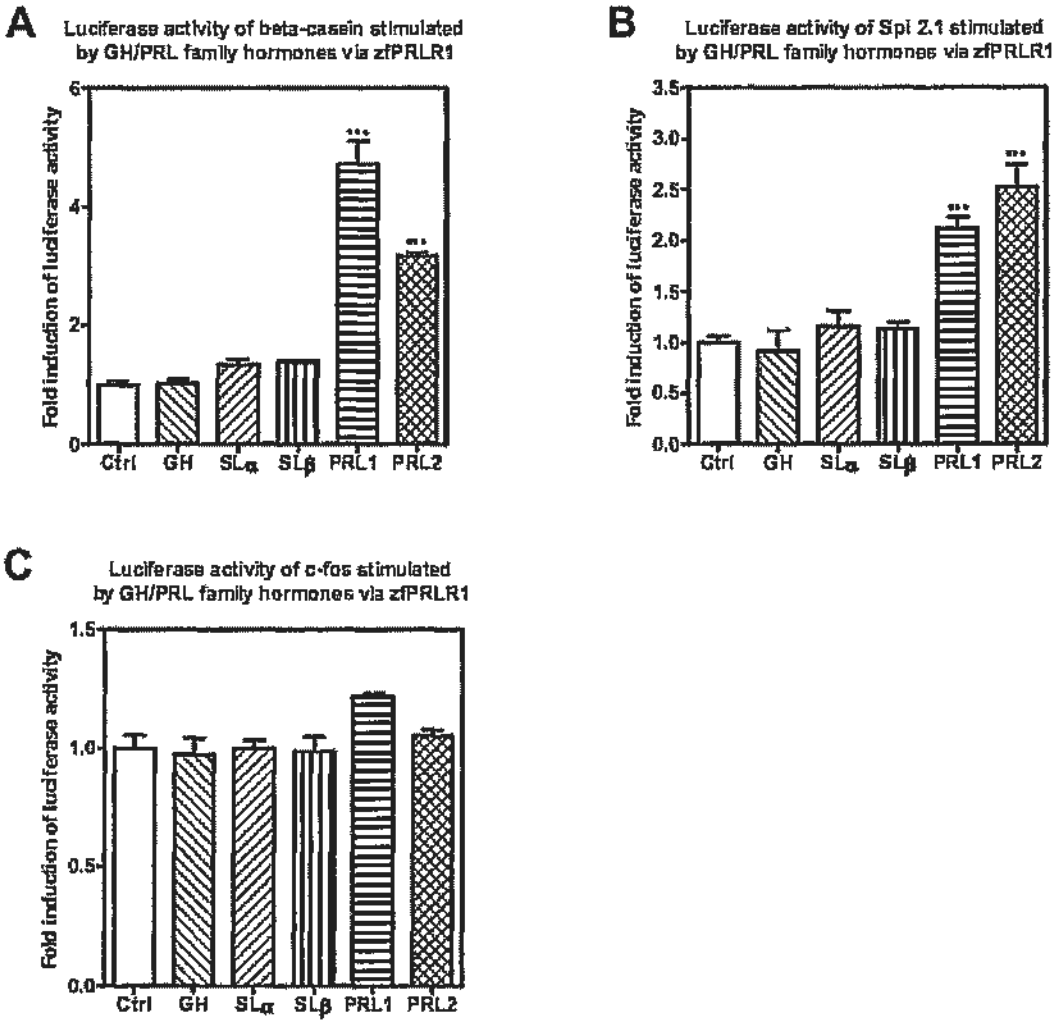


Fig. 3.6 PRL1 and PRL2 activate PRLR1-mediated signal transduction. GAKS cells were co-transfected with pcDNA3.1-zfPRLR1 and a luciferase reporter plasmid driven by the β-casein (Panel A), Spi 2.1 (Panel B) and c-fos (Panel C) promoters, respectively. The transfected cells were subsequently stimulated by 300 ng/ml of recombinant zebrafish GH, SLα, SLβ, PRL1 and PRL2, respectively. Results are mean values ± SEM (n = 3; ***P<0.001).

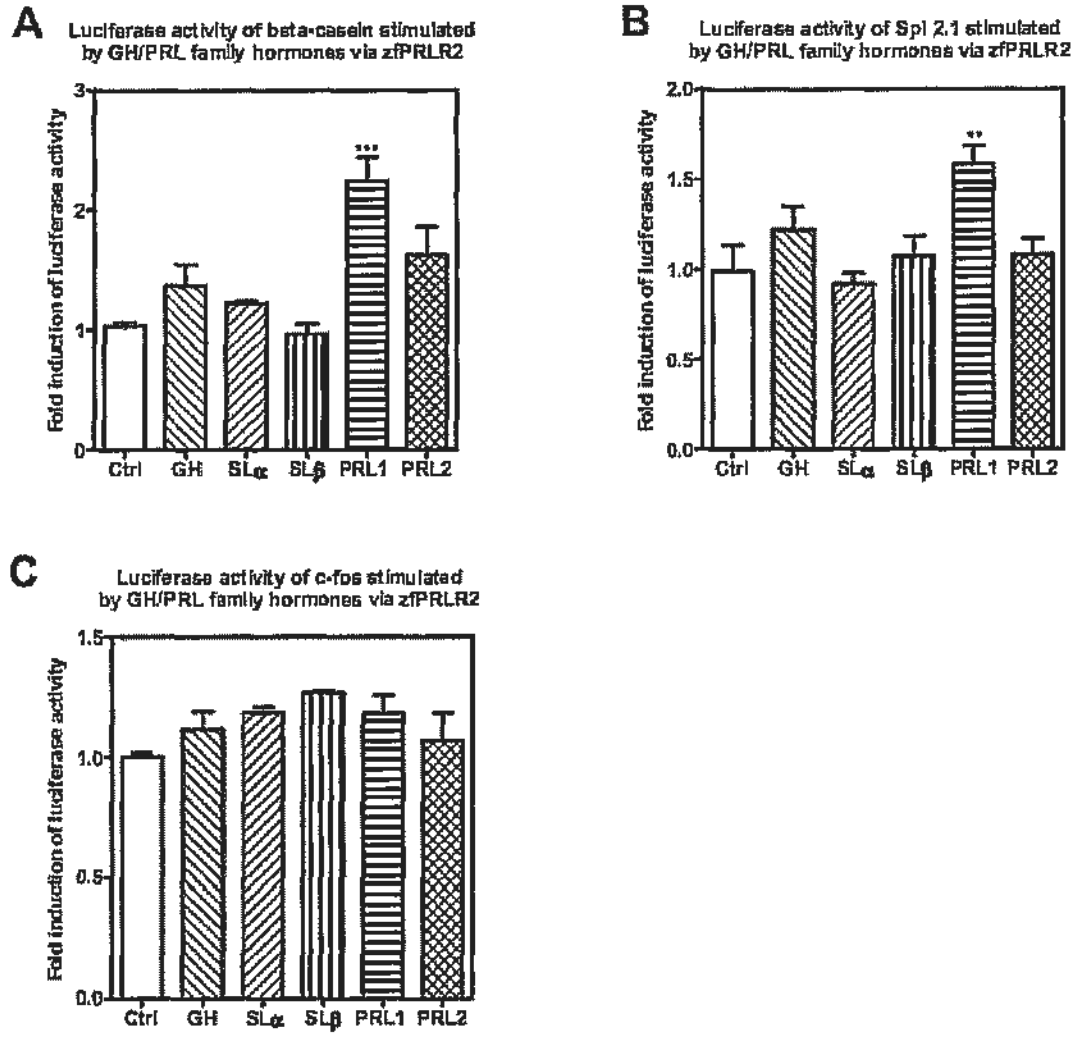


Fig. 3.7 PRL1 activates PRLR2-mediated signal transduction. GAKS cells were co-transfected with pcDNA3.1-zfPRLR2 and a luciferase reporter plasmid driven by the β-casein (Panel A), Spi 2.1 (Panel B) and c-fos (Panel C) promoters, respectively. The transfected cells were subsequently stimulated by 300ng/ml of recombinant zebrafish GH, SLα, SLβ, PRL1 and PRL2 (A-C), respectively. Results are mean values±SEM (n = 3; **P<0.01; ***P<0.001).

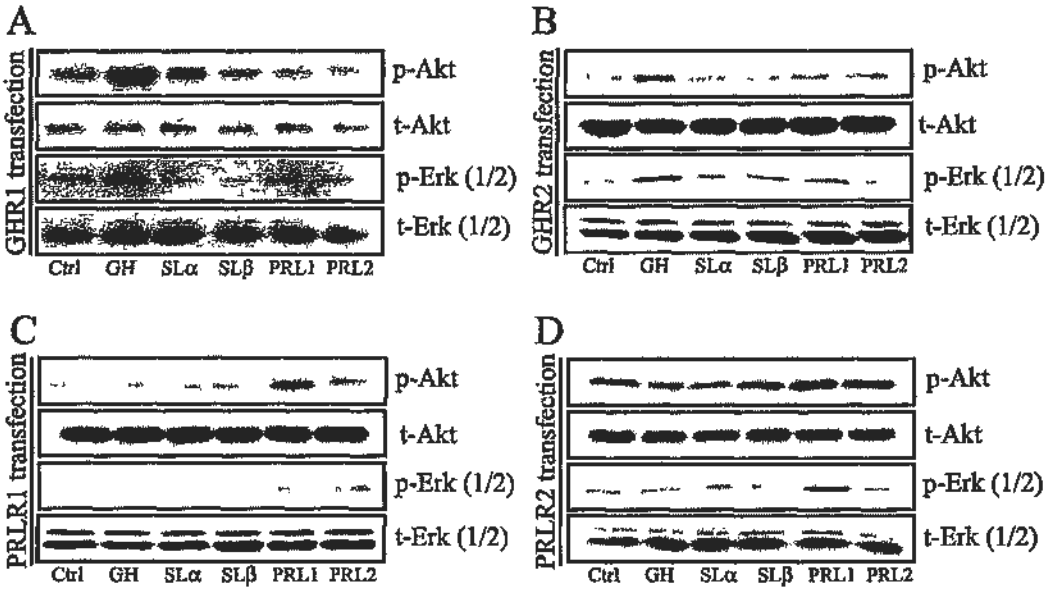


Fig. 3.8 Assessment of the phosphorylation level of Akt and Erk proteins. CHO cells were transfected with pcDNA3.1-GHR1 (A), pcDNA3.1-GHR2 (B), pcDNA3.1-PRLR1 (C) or pcDNA3.1-PRLR2 (D) for 24 hours. Then 300 ng/ml of recombinant zebrafish GH, SLα, SLβ, PRL1 and PRL2 were separately added to the medium for another hour. Western blot analysis was performed to detect both the total and phosphorylated proteins. p-Akt: phosphorylated Akt; t-Akt: total Akt; p-Erk (1/2): phosphorylated Erk (1/2); t-Erk (1/2): total Erk (1/2).

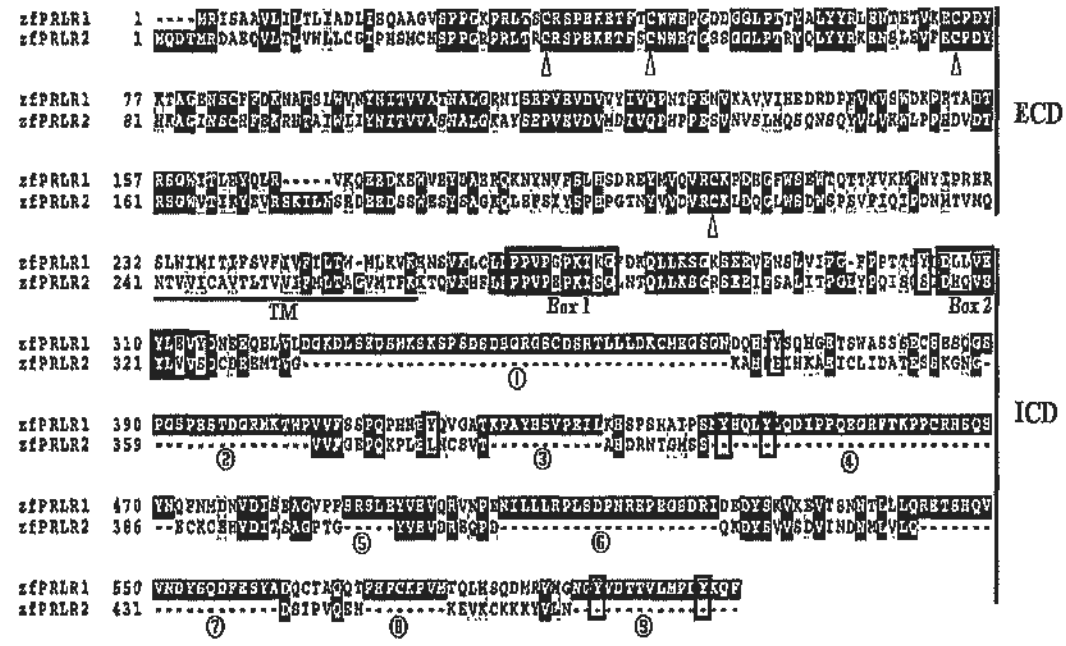


Fig. 3.9 Alignment of zebrafish PRLR1 and PRLR2 protein sequences. The polypeptide sequences were aligned by the Clustal X program. The extracellular domain (ECD) and intracellular domain (ICD) are indicated on the right. The conserved cysteine residues in ECD are marked with triangles at the bottom of the aligned sequences. The Box 1 and Box 2 regions are boxed and indicated at the bottom of the sequences. The conserved tyrosine residues (Y) of ICD are also marked with boxes. The deleted regions in the ICD of PRLR2 are numbered at the bottom of the aligned sequences.

3.4 Discussions

In this study, I have provided evidence that the zebrafish GHR1, GHR2, PRLR1 and PRLR2 could not interact with the two zebrafish SLs. It was shown that neither GHR1 nor GHR2 interacted with SLα or SLβ physically using His-tag pulldown assay. Recombinant SLα and SLβ cannot enhance GHR1-mediated phosphorylation of Erk and Akt. Moreover, the downstream genes of both GHR1 and GHR2 including β-casein, Spi 2.1 and c-fos were not activated by SLα or SLβ in the promoter activity assays.

3.4.1 GHR1 is not the receptor for SL

Current information regarding the identity of SLR is controversial since the report of SLR in masu salmon. To date, one hypothesis proposes that GHR1 is actually SLR. The direct evidence in support of this hypothesis is that the SLR discovered in masu salmon, which is orthologous to the GHR1 of other fish species, exhibits higher binding affinity to SL than GH and PRL (Fukada et al., 2005). Recently, Fukamachi et al. (2007) reported some other orthologs of the salmon SLR in fish, including medaka and fugu (*Takifugu rubripes*). These so-called medaka and fugu 'SLRs' possess one extra exon than GHR, similar to the 'SLRs' of seabream and flounder. However, both eel GHR and 'SLR' contain the same number of exons. Some differences between the GHR and 'SLR' could also be recognized by polypeptide comparisons such as differences of the number of cysteines in the ECDs between GHRs and 'SLRs', which may result in different numbers of disulfide bonds (Fukamachi and Meyer, 2007). For example, three disulfide bonds could be predicted in the extracellular domain of the Atlantic salmon (*Salmo salar*) 'SLR', while both GHRs have only two (Benedet et al., 2008). In view of these phylogenetic studies

and polypeptide comparisons, it was proposed that GHR1 is SLR (Fukamachi et al., 2005). However, more convincing experimental evidence in support of this suggestion is not available to address this issue. On the other hand, results from some physiological studies are not consistent with this hypothesis. For example, according to the aforementioned hypothesis, one of the seabream GHRs should be classified as SLR (Fukamachi and Meyer, 2007). However, it has been demonstrated that GH, but not SL, was capable of activating both of GHRs (Jiao et al., 2006). Recombinant eel GHR1, which was also considered as SLR according to the aforementioned hypothesis, bound to GH and this binding could not be displaced by SL (Ozaki et al., 2006). Similarly in a study on rainbow trout GHR, the salmon ^{125}I-GH binding on the GHR1 and GHR2 could be displaced by salmon GH and SL had an extremely weak interaction with rainbow trout GHRs (Reindl et al., 2009), and this is contrary to the binding result of Fukada et al. (2005). All these data cast doubt on the hypothesis that GHR1 is in fact SLR. In our study, different approaches were adapted to address this issue. All of our results do not support the notion that GHR1 is in fact SLR, at least not in zebrafish. In order to avoid the use of hormones from heterologous organisms, we prepared all the ligands and receptors from zebrafish in our study (see Chapter 2 for details). We did not observe interaction between GHRs and SLs in the His-tag pulldown assay, which is a powerful *in vitro* method and is widely used to determine the physical interaction between two proteins (Figure 3.1). Furthermore, the recombinant SLs did not affect three major GHR downstream signaling pathways, including Akt pathway, Erk pathway (detection of phosphorylation levels of Akt and Erk proteins, Figure 3.8) and JAK-STAT pathway (luciferase reporter assay, Figures 3.3-3.7). Therefore, it can be concluded that zebrafish GHR1 is not the SLR. The real SLR, at least in non-salmonid fish, is still an open question.

3.4.2 No crosstalk between GH and PRL signaling pathways

Crosstalk and overlapping functions among receptors of the same family have already been demonstrated in mammals. For example, insulin receptor mediates both IGF-II and insulin function in prenatal growth (Nakae et al., 2001). It was also reported that besides the placenta lactogen receptor (PLR), GHR can interact with ovine PL through the ECD, leading to the activation of downstream signaling (Helman et al. 2001). In human, GH interacts with recombinant PRLR through its extracellular domain and the interaction is dependent of zinc ion (Cunningham et al., 1990). However, to our knowledge, a systematic investigation of the crosstalk among fish GH/PRL family of hormones and their receptors has not been performed so far. In the present study, no or extremely weak cross-interaction and activation between the GH-GHR and PRL-PRLR signaling cascades was found based on our direct interaction assay (Figure 1) and receptor-mediated promoter assays (Figures 3-6). Although different from the studies in mammals, our observation is consistent to other reports in fish. Phylogenetic analysis illustrates that GH is highly conserved in mammals. However, GHs vary markedly between mammals and teleosts as well as among different teleost species. A similar situation is found in PRL, suggesting that the evolution rates of GH and PRL were high and variable in teleost and the signaling of GH/GHR and PRL/PRLR axes is therefore most likely to be separated from each other (Forsyth and Wallis, 2002). This hypothesis was supported by experimental evidence. For example, GH and PRL elicit downstream signaling activation specifically via their own receptors in seabream (Huang et al., 2007; Jiao et al., 2006). Ligand competition study demonstrated that radioiodine-labeled salmon GH could only be displaced by salmon GH, but not by salmon PRL, indicating high specificity of salmon GHR towards to GH (Fukamachi et al., 2005). Ozaki et al.

(2006) also showed that eel PRL was ineffective in displacing GH from GHR, consistent with the study in rainbow trout (Reindl et al., 2009). Therefore, high ligand-binding specificity may be a distinct characteristic of teleost GH/PRL receptor family.

Interestingly, it was noticed that although direct interaction with both PRL1 and PRL2 was established (Figure 1), PRLR2 did not mediate the downstream signaling events activated by PRL2 (Figure 6). After careful comparison of the protein sequences, several differences can be recognized between the two receptors. PRLR1 and PRLR2 of zebrafish share high similarity in their ECDs but differ markedly in their ICDs. For example, the typical Box 2 sequence is lost in PRLR2, and this region is reported to be important for downstream signaling. Moreover, a number of regions and tyrosine residues conserved in PRLR of other species are also missing in the intracellular domain of PRLR2 (Figure 3.9). All of these depletions and mutations may contribute to the lack of downstream signaling mediated by PRLR2, suggesting the differential functions of PRLR1 and PRLR2.

In conclusion, with different approaches, it was shown that two SLs of zebrafish do not physically interact with or activate GHR1 indicating that GHR1 is unlikely to be the SLR, at least in zebrafish. In addition, the teleost GH-GHR and PRL-PRLR signaling axes were relatively independent and lack of crosstalk to each other. However, as the experiments were performed in the *in vitro* systems which may not reflect the physiological situation, more *in vivo* studies are required to further confirm our finding.

Chapter 4

The role of PRLR1 in embryonic development of zebrafish

4.1 Introduction

The kidney serves as a vital organ which functions in excretion of toxic metabolic wastes as well as in the regulation of body fluids, osmolarity and pH (Reilly and Ellison, 2000). In human, the kidney is a paired bean-shaped organ located at the rear of the abdominal cavity (Ronco and Ricci, 2008). Numerous attempts to unravel the processes and mechanisms that make a functional kidney during embryogenesis have been made for about 100 years (Dressler, 2006). However, a comprehensive understanding on the development of kidney is still far from complete.

Current studies reveal that during vertebrate development, three kidneys develop sequentially from the intermediate mesoderm, viz pronephros, mesonephros and metanephros, which are different from other organs (Wingert and Davidson, 2008b). Kidney development progresses from the pronephros, the first and most primitive kidney, to the more complex mesonephros and metanephros. The pronephros is functional in lower vertebrates, including embryo and larvae of zebrafish. The metanephros is the permanent functional kidney in mammals (Chan and Asashima, 2006; Rumballe et al., 2010). The differences among these three kidneys are listed in Table 4.1. The metanephros is the most complicated kidney among the three and is composed of millions of nephrons, which are the basic structural and functional units of kidney. The metanophros is also different in appearance and morphogenesis from pronephros and metanephros (Michos, 2009).

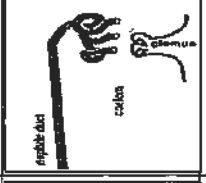
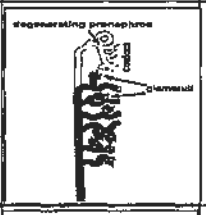
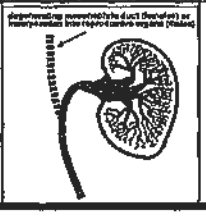
kidney	structure	No. of nephrons	Functions in
Pronephros (first kidney)		**1-2**	**1. Primitive fish** **2. Embryo and larvae of advanced fish** **3. Larvae of amphibians**
Mesonephros (middle kidney)		**~10-50**	**1. Almost all the adult fish** **2. Adult amphibians** **3. Temporary in reptiles, birds and mammals**
Metanephros (final kidney)		**~1,000,000**	**Adult reptiles, birds and mammals**

TABLE 4.1 Comparison of pronephros, mesonephros and metanephros (adapted from Michos, 2009).

Despite different in appearance and complexity, the three kidneys share very high similarity in the process of embryogenesis, which can be conceptually defined in four stages, including (1) specification: the undifferentiated mesodermal cells are committed to a nephrogenic fate and the organ primordium are formed; (2) epitheliazation: the nephric duct is formed, epitheliazed and eventually serve as the kidney's collecting system; (3) patterning: the nephrons are formed and induced to form the glomerulus and the different tubular cell types; and (4) angiogenesis: the glomerular capillary tuft is finally constructed by ingrowing endothelial cells and blood filtration and nephron function get started (Drummond, 2003). Besides, genes which play essential roles in the metanephros development of higher vertebrates have shown similar expression patterns and functions in pronephros development. Table 4.2 contains a selected list of genes which have been reported to express and function

consistently in both metanephros and pronephros. Several signaling pathways such as Wnt, TGF-β, hedgehog and Notch, are also conserved in metanephros and pronephros.

Gene name	Mammal (metanephros)		Zebrafish (pronephros)	
	Expression	Function	Expression	Function
Transcription factors				
pax2a	✓	UB outgrowth, MM induction	✓	PT development, podocyte cell differentiation
wt1	✓	MM development	✓	Formation of GL
Fox	✓	Nephron development	✓	Induction of cilia formation
emx2	✓	$Emx2^{-/-}$ caused absence of kidney	✓	Not determined
Lim-1	✓	Patterning of the kidney	✓	pronephric specification and development
Growth factors				
BMP4	✓	Inhibit ectopic budding, promoting kidney morphogenesis	✓	Formation of pronephros
BMP7	✓	Development of nephrons and collecting ducts	✓	Pronephric differentiation
wnt4	✓	Kidney-tubule formation	✓	Patterning of PT
others				
Cadherin-6	✓	Nephros formation, mesenchyme-to-epithelial conversion	✓	Development of pronephros
Notch	✓	Renal fibrosis	✓	Nephric segmentation

TABLE 4.2 A list of genes conserved during both pronephros and metanephros development. GL: glomerulus; MM: metanephric mesenchyme; PT: pronephric tubule; UB: ureteric bud.

Moreover, a variety of cell types typical of metanephros can also be found in pronephros, including fenestrated endothelial cells in capillary tufts and podocytes with extensive foot processes (Gerth et al., 2005). Zebrafish pronephric tubule cells show brush border with polarized membrane transport proteins which is similar to that of metanephros (Drummond et al., 1998). Therefore, conserved patterns of embryogenesis, gene expression, protein function and cell differentiation between pronephros and metanephros demonstrate that although they are distinct in the final organ morphology, the zebrafish pronephros represents a good model to study kidney

development of higher vertebrates (mesonephros and metanephros) (Vize et al., 1997).

In zebrafish and other teleosts such as killifish, salmon, turbot and herring, the functional embryonic and larval pronephros is composed of only two nephrons with glomeruli fused at the midline ventral to the dorsal aorta (Drummond, 2000; Tytler, 1988; Tytler et al., 1996). The nephron of zebrafish contains three components: glomerulus which functions to filter blood, pronephric duct and pronephric tubule connecting directly to the closed nephrocoele. The pronephric ducts run caudally and fuse at the cloaca, where the metabolic wastes are excreted to the outside. The pronephrons are typically located under somite 3 (Kimmel et al., 1995).

As aforementioned, the development of the three kidneys can be conceptually divided into four stages, the development of zebrafish pronephros was also demonstrated in four stages as shown in Figure 4.1 (Drummond, 2000). In zebrafish, the first morphological sign of kidney organogenesis is the formation of pronephric primordium as a mass of intermediate mesoderm lying ventral and lateral to the anterior somites (Vize et al., 1997). At about 13-14 hour post fertilization (hpf), progenitor cells of the posterior intermediate mesoderm are committed to the pronephric fate and the nephrogenic field is established. The epithelialization of the pronephric duct as well as the formation of nephron primorida is completed by 24 hpf. The pronephric tubules are formed a little later, at about 30 to 40 hpf (Kimmel et al., 1995). Vascularization of the glomerulus in zebrafish by ingrowth of capillaries from the dorsal aorta happens after the completion of both pronephric duct and tubule development, at about 40 hpf. In zebrafish as well as in *Xenopus*, the

endothelial cells of dorsal aorta branch out as they invade the glomerular epithelium, suggesting that the glomerular capillaries form by sprouting (Drummond et al., 1998; Tytler et al., 1996; Vize et al., 1997). The onset of glomerulus function as a blood filter occurs at about 48 hpf (Drummond et al., 1998). Formation of the cardinal vein and vascular sinus that will surround the pronephric tubules and eventually receive small molecules from glomerular filtrate occurs concurrently with pronephric duct growth and epithelialization (Brand et al., 1996; Cleaver et al., 1997). The epithelialization of pronephric duct is performed by a mesenchyme-to-epithelium transformation in the layer of intermediate mesoderm owing to its position between the paraxial, somitic mesoderm and the lateral plate mesoderm (Schier, 2001). The pronephric duct forms in an anterior-to-posterior progression adjacent to the somites. When complete, the epithelial cells of the duct are polarized with an apical brush border, well-defined cell-cell junctions and a basolateral membrane domain containing ion-transport proteins that are essential for the osmoregulatory function of the duct (Drummond et al., 1998).

In the present study, we focused on the role of PRLRs in the development of zebrafish pronephric duct. Whole mount in situ hybridization (WISH) study showed that PRLR1 was mainly expressed in the pancreas and pronephric duct, while PRLR2 was only expressed in the pronephric duct. In the PRLR1 morpholino (MO) knockdown embryos, the yolk extension (YE), the formation of which is reported to be associated with pronephric duct development, disappeared at 24 hpf. This phenotype could not be observed in the PRLR2 MO knockdown and control embryos. Real time RT-PCR and WISH data revealed that several genes expressed in the pronephric duct were up- or down-regulated. The protein expression pattern of

pronephric duct marker atp1a1 was also affected in the embryos injected with PRLR1 MO. In addition, histological studies showed malformation of the pronephric duct in the PRLR1 MO embryos. In conclusion, these results suggest that PRLR1 plays an important role in the development of the pronephric duct in zebrafish embryos.

4.2 Materials and methods

4.2.1 Maintenance of zebrafish and embryos

Wild-type zebrafish were purchased from a local pet shop. The zebafish were maintained at 28°C with a 14:10 h light/dark cycle in a filtering system and fed brine shrimp twice per day. About half of the water was replaced once a week. Embryos were collected by natural spawning after beginning of the light cycle in the morning. They were staged according to the hour post-fertilization (hpf) and morphological features. The embryos were kept in incubator at 28°C. In whole mount in situ hybridization experiment, the embryos were treated with 0.2 mM 1-phenyl-2-thiourea (PTU) from 12 hpf to prevent pigment formation. All experiments were conducted in accordance with guidelines established by the University Committee on the use and care of laboratory animals at The Chinese University of Hong Kong.

4.2.2 RNA extraction, RT-PCR and real time RT-PCR

Total RNAs of zebrafish embryo from different stages were isolated by TRIzol standard protocol. The concentration of the extracted RNA was measured. First-strand cDNA was synthesized using the ImProm-II Reverse Transcription System (Promega, USA) according to the manufacturer's manual. The first strand

cDNA was synthesized according to the following conditions: 25°C for 5 min, 42°C for 60 min and 70°C for 15 min. The examination of gene expression by RT-PCR was preformed according the following cycle conditions: denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, and elongation at 72°C for 60 sec. The cycle was repeated 30 times. The sequences of the gene-specific primers are shown as follows:

RT-PRLR1 FW: 5'-GTCAGTCCTCCAGGCAAA-3'

RT-PRLR1 RV: 5'-CCAACTATATTCCCAGAGAGCGT-3'

RT-PRLR2 FW: 5'-ATTGTCTGGGATCTGGATGAAA-3'

RT-PRLR2 RV: 5'-GTATCTGGTTGGCAGTCCACCG-3'

Real-time PCR was performed in thin-wall 8-tube strips with the Chromo 4 Four-Color Real-Time System (MJ Research®, USA). The 18s ribosomal RNA was used as the internal control for normalization. SYBR Green PCR Master Mix (Applied Biosystems, USA) was used for the real-time PCR analysis. The sequences of the forward and reverse primers for the target genes are shown in Table 4.3. The PCR primer sets were designed using the online real-time PCR Primer Design tool (https://www.genscript.com/ssl-bin/app/primer), and the primers were synthesized by TechDragon, HK. The amplification conditions of the real-time PCR for the target genes were validated before hand. The analysis of real time PCR data was based on the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The relative expression of each gene was calculated by the equation: relative fluorescence = $2^{\Delta\Delta Ct}$.

Primer name	Sequence (5' to 3')
Realtime PRLR1 FW	CTTCTCAAGAGCGGGAAATC
Realtime PRLR1 RV	TCCTCGTTGTCGTACACCTC
Realtime PRLR2 FW	CTAAAGAGCGGGAGGTCTGA

Realtime PRLR2 RV	CTTCATCACAGTCGGACACC
zfWT1A FW	AGCCAACCAAGGATGTTCAG
zfWT1A RV	CCTCGTGTTTGAAGGAGTGG
zfWT1B FW	TGCTGATCCTCCTTCTAGCC
zfWT1B RV	GAACGGAGGAGTGTGTTGTG
zfCDH17-RT FW	CGCTGGGCTTGGCATCACAC
zfCDH17-RT RV	TGCCAGGATGCCGATGGTGC
zfNBC1 FW	GGCTGCCACTGCACACCTCC
zfNBC1 RV	ACGCCCACGTCGCATTCAGG
zfJAK2A FW	CGTCATTGACATCAGCATCA
zfJAK2A RV	TAGCCGTCGATCAGAGACAC
zfJAK2B FW	CGACGAAACGGTACATTCAC
zfJAK2B RV	CTCCAGGCTCCTTAACCTTG
zfRET1 FW	GTGCCCTGATGGTTACTGTG
zfRET1 RV	ACAGGTCCCTTTCATAACCG
zfSLC4A2 FW	GGCACCTGGTTAAGAAGAGC
zfSLC4A2 RV	ATCCAGCTTGGTCCGAATAC
zfGATA3 FW	CGTGTTGTGTGTATCGGTGA
zfGATA3 RV	GGTGATGTGTCTCAGGATGC
zfPAX2A-RT FW	TGAATGGTCAGAGGGATTGA
zfPAX2A-RT RV	GTTCAGTGATGGTGCCAAAG
zfATP1A1 FW	TGGATCCGTGAAAGAAATGA
zfATP1A1 RV	GGTGGATGGAGAGCTGGTAT

TABLE 4.3 Nucleotide sequences of primers used in the real-time PCR assays.

4.2.3 Microinjection of morpholino oligonucleotides

Two morpholino antisense oligonucleotides (MOs) targeting PRLR1 were purchased from Gene-Tools (Philomath, OR, USA). These two MOs were designed either to block translation initiation of PRLR1 (prlr1MO-1, 5'-TCAGCACAGCGGCGGAAATCCTCAT-3') or to block pre-mRNA splicing (prlr1MO-2: 5'- AGAATAGAAACTTACTTCTCCAGGC-3'). They were dissolved in sterile Danieau's buffer (8 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES, pH 7.6). MO of PRLR2 (prlr2MO-1: 5'-CTGCATCTCTCATTGTGTCCTGCAT-3') and the negative MO control (control: 5'-CCTCTTACCTCAGTTACAATTTATA-3') were also synthesized. Different amounts of MOs dissolved in 0.25% Phenol Red, ranging from 1 ng to 12 ng, were injected into the embryos at 1-cell stage to 2-cell stage by PV820 picoPump (World Precision Instruments, USA). The microinjection needles were produced from Narishige GD-1 glass capillaries using MODEL P-97 micro-electrode puller (Sutter Instrument Co., USA). For statistics of morphology analysis, the number of embryos defective in yolk extension formation was counted after 24 hpf after injection.

4.2.4 Construction of pEGFP-PRLR1 plasmid

The PRLR1 ECD fragment was amplified by PCR using pSUMO-PRLR1 as a template with two specific primers prlr1-fw (5'-GAATTCATGGTCAGTCCTCCAG GCAAA-3') and prlr1-rv (5'-GGATCCCCAACTATATTCCCAGAGAGCGT-3'). The purified target PRLR1 ECD fragment was ligated into a TOPO pCR2.1 vector and then transformed into Top10 competent *E. coli* cells by heat shock using the TOPO TA Cloning Kit (Invitrogen, USA). The positive single clones were identified

by PCR. DNA plasmids were produced from Top10 bacteria, purified using Miniprep Kit (Qiagen, Valencia, CA, USA), and finally confirmed by DNA sequencing (TechDragon, HK). After confirmation of the DNA nucleotide sequences, the target gene fragments were cleaved from the TOPO plasmids with *Eco*RI and *Bam*HI restriction enzymes by overnight incubation at 37°C. The DNA fragments were purified by gel extraction and subcloned into pEGFP-N1 eukaryotic expression vector (Clontech, USA). Positive clones were screened by PCR and confirmed by DNA sequencing after purified from *E. coli* cultures.

4.2.5 Examination of MO efficiency

To test prlr1MO-1 (translation blocking form) knockdown efficiency, 50 ng of pEGFP-PRLR1 was injected into 1-cell stage to 2-cell stage with or without the co-injection of 6 ng prlr1MO-1. The injected embryos were incubated at 28°C. GFP expression was analyzed after 24 hpf on a Leica DM1L fluorescence microscope with the Leica filter set (excitation wavelength= 485 nm; emission wavelength = 520 nm) and the pictures were captured on a Leica camera DFC420. To test the efficiency of prlrMO-2 knockdown (splicing blocking form), the mRNAs of embryos injected with prlrMO-2 or control MO were exacted using TRIzol reagent as described in Chapter 2. The synthesis of first-strand cDNA was performed as described in Section 4.2.2. The freshly prepared first strand cDNA was used as a template to amplify the PRLR fragment using gene specific primers, prlr-fw1 (5'-ATGGTCAGTCCTCCAG GCAAA-3') and prlr-rv1 (5'-CATCATCTCCAGGCTCCCACC-3'). The PCR products were analyzed by gel electrophoresis.

4.2.6 Synthesis of DIG-labeled RNA probes by *in vitro* transcription

The PRLR1 and PRLR2 were subcloned to pGEMT vector using pSUMO-PRLR1 and pSUMO-PRLR2 as templates respectively. The sequences of the primers used are shown as follows:

Insitu-PRLR1-F1: 5'-TATTCCCAGAGAGCGTTCGT-3'

Insitu-PRLR1-R1: 5'-GCTTTCAAAGTCCTGGCTGT-3'

Insitu-PRLR2-F1: 5'-GGATTACCACAAGGCTGGAA-3'

Insitu-PRLR2-R1: 5'-GGTTGCTCTCTGTCGACCTC-3'

The pGEMT-pax2a and pGEMT-cdh17 were generated by amplifying cDNA reverse-transcribed from mRNA of zebrafish embryos and ligated to TOPO pCR2.1 vector (Invitrogen, USA). The genes of interests were further cleaved from the pCR2.1 vector and ligated to pGEMT-easy vector (Promega, USA) according to method as described in Section 2.2.2. The primers used in the PCR are shown as follows:

Insitu-zfPax2a FW: 5'-AGCTTGGCGGTTCAGCAACC-3'

Insitu-zfPax2a RV: 5'-GATTGTGTATCACAGAAAATG-3'

Insitu-zfCDH17 FW: 5'-ATTGATGCCCGTAATCCCGAG-3'

Insitu-zfCDH17 RV: 5'-CCGATTATGCAAATCTTGAAC-3'

The positive single clones were identified by PCR and confirmed by DNA sequencing. The pGEMT-wt1a plasmid was a kind gift of Professor Alan J. Davidson at the Center for Regenerative Medicine of the Massachusetts General Hospital. The DIG-labeled otg and tal1 RNA probes were generously provided by Professor Anskar Y. H. Leung at the department of Medicine in the University of Hong Kong. The plasmids were linearized by digesting with the appropriate restriction enzymes at 37°C overnight. The linearized fragments were recovered by gene clean using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). 1 μg of linearized

plasmid template, 2 μl of 10X transcription buffer, 2 μl of DIG-RNA mix, 2 μl of T7 RNA polymerase, 1 μg of RNase inhibitor (40 unit per μg) and RNase-free H_20 were mixed in a total reaction volume of 20 μl. The mixture was incubated at 37°C. After 2 hours, DNase I (1 μl) was added and the incubation continued for another 30 min. After stopping the reaction by the addition of EDTA (1 μl, 500 mM), the probe solution was purified using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The quality of the RNA probes was examined by gel electrophoresis.

4.2.7 Whole mount *in situ* hybridization (WISH)

The chorions of zebrafish embryos before 48 hpf were removed by 10 % pronase E treatment for 10 min or manually with Dumont Watchmaker's Forceps no. 5. The embryos were then fixed in 4% paraformaldehyde (PFA) in 1×PBS buffer (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4, pH at 7.3) overnight at 4°C. The embryos were washed with PBS several times and transferred gradually into 100% methanol (MeOH) and stored at -20°C until use. In WISH, the embryos were rehydrated in graded concentrations of MeOH (75%, 50% and 25%) to 1×PBS buffer. After pre-hybridization with Hyb buffer (50% formamide, 5×SSC (sodium chloride sodium citrate), 50 μg/ml Heparin, 5 mM EDTA, 0.5 mg/ml ribosomal RNA (Sigma R7125), 0.46 ml 1 M citric acid (pH 6.0) and 0.1% Tween-20 to a final volume of 50 ml), the embryos were mixed with the RNA probes dissolved in the Hyb buffer at 65 °C overnight. Following hybridization with the RNA probes, embryos were washed sequentially in Hyb buffer, 75% Hyb buffer, 50% Hyb buffer, 25% Hyb buffer (in 2×SSC buffer), 2×SSC (0.3 M NaCl and 0.03 M Na citrate, pH at 7.0) at 65°C for 15 min each and then 0.2×SSC for 30 min twice at room temperature. The embryos were further washed sequentially by 75%

0.2×SSC, 50% 0.2×SSC, 25% 0.2×SSC (in PBT buffer) and PBT (1×PBS with 1% Tween-20) for 5 min at room temperature. After washing, the embryos were incubated with anti-DIG antibody (1:5000) in PBT buffer containing 2% lamb serum at 4 °C overnight. The embryos were then washed by PBT for 30 min three times and AP buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5 and 0.1% Tween-20) for 5 min three times. The embryos were then incubated with staining solution (1% NBT in AP buffer) at room temperature in the dark. The staining reaction was monitored by color examination using stereo microscope. The embryos were then destained in 100% EtOH, 75% EtOH (in H_2O), 50% EtOH (in PBT), 25% EtOH (in PBT) and PBT for 5 min at room temperature. The embryos were stored in PBT buffer at 4°C and pictured on a Leica camera DFC420.

4.2.8 Whole mount *in situ* immunostaining

The embryos were dechorioned as described in Section 4.2.7 and fixed in the Dent's fixative (80% MeOH and 20% DMSO) at 4°C overnight. The fixed embryos were washed by PBS for 5 min three times and then blocked in the blocking buffer (0.5% Triton X-100, 4% normal horse serum in PBS) for 4 hours followed by PBST (1×PBS with 0.5% Triton X-100) wash three times at room temperature. The embryos were incubated with a6f antibody (1:5) in blocking buffer at 4°C overnight. After incubation, the embryos were washed by PBST for 60 min and blocking buffer for 60 min for 4 hours. The embryos were further incubated with 488-alexa donkey anti-mouse secondary antibody (1:300) at 4°C for 2-4 hours followed by blocking buffer washing for 10 min three times at room temperature in the dark. Fluorescence was detected on a Leica DM1L fluorescence microscope with the Leica filter set (excitation wavelength= 485 nm; emission wavelength = 520 nm) and the pictures

were captured on a Leica camera DFC420.

4.2.9 Embryo sectioning and H&E staining

The embryos injected with MO were fixed in 4% PFA at 4°C overnight and dehydrated sequentially as described in Section 4.2.7. Samples were embedded in molten paraffin wax and the transverse sectioning of zebrafish embryo was performed at 6 μm using a Leica microtome. The samples were deparaffinized with xylene and rehydrated with graded EtOH (100% EtOH, 95% EtOH, 70% EtOH, 50% EtOH and tape water sequentially). The specimen were stained with 5 mg/ml hematoxylin for 3-5 min and washed with tape water. After decolorization by acid alcohol and immersation of Scott's buffer (2 mg/ml sodium hydrogen carbonate and 20 mg/ml magnesium sulphate), the samples were stained with 1% eosin for 10 min followed by graded dehydratation (70% EtOH, 90% EtOH and 100% EtOH sequentially). The samples were then cleared by xylene and mounted with Glycergel Mounting Medium. Finally, the samples were examined and pictured using a Leica camera DFC420.

4.3 Results

4.3.1 PRLR1 is expressed in the zebrafish pronephric duct during embryonic development

The temporal expression patterns of PRLR1 and PRLR2 were detected by RT-PCR and real-time PCR using 18s ribosomal RNA as the internal control. As shown in Figure 4.2, PRLR1 was expressed throughout embryonic development in zebrafish. Within the 72 hpf that we have showed, the peak level was observed at 24 hpf. A remarkable increase of PRLR1 expression was observed from 12 hpf to 24 hpf.

In fact during this period, the cells from the intermediate mesoderm are committed to the pronephric fate (Drummond et al., 1998), suggesting the role of PRLR1 in the development of pronephric system (Figure 4.2). Similarly, the expression of PRLR2 was found to increase from 0 hpf to 24 hpf and decrease subsequently (Figure 4.3). The peak of expression was again found to be at 24 hpf.

The WISH data revealed the spatial expression patterns of PRLR1 and PRLR2 in zebrafish embryos. At the early stages such as 4 and 8 hpf, PRLR1 and PRLR2 were ubiquitously expressed in the embryo (Figure 4.4 A-D and Figure 4.5 A-D). From 16 hpf to 48 hpf, PRLR1 expression was observed as bilateral stripes in the anterior pronephric duct, running caudally to the middle of the duct (Figure 4.4 E-H and Figure 4.5 E-J). A small amount of PRLR2 was also found at the posterior region of the pronephric duct (Figure 4.5 E-J). It was also found that PRLR1 mRNA was expressed in the pancreas, while PRLR2 was only expressed in the pronephric duct (Figure 4.4 G and H).

4.3.2 PRLR1 knockdown caused malformation of yolk extension

To determine the roles of PRLR1 and PRLR2 in the embryonic development of zebrafish, especially on kidney development, antisense MOs were used to knock down PRLR1 and PRLR2 respectively. At 24 hpf, yolk extension was totally destroyed in prlr1MO-1 injected zebrafish embryos, which died about 4 to 5 days post fertilization (dpf) (Figure 4.6 C and D). This phenomenon was not observed in either the control MO or prlr2MO-1 injected embryos, even at very high doses (Figure 4.6A and E and also Table 4.4), suggesting that only PRLR1 but not PRLR2 interferes with the development of yolk extension.

The efficiency of prlr1MO-1 knockdown was evaluated by co-injection of prlr1MO-1 and the pEGFP-PRLR1 plasmid. In the embryo injected with pEGFP-PRLR1 only, green fluorescence could be readily detected (Figure 4.7A). The fluorescence was largely decreased when the embryo was co-injected with pEGFP-PRLR1 and prlr1MO-1, suggesting that the expression of PRLR1 was blocked and prlr1MO-1 was effective in down-regulating PRLR1 expression (as compared Panels A and B in Figure 4.7). At the dose of 6 ng, the ratio of prlr1MO-1-injected embryos with abnormal yolk extension was up to 80%. This dose of prlr1MO-1 was used in the subsequent studies. However, a dose of 9 ng of prlr1MO-1 would cause non-specific effects resulting in the death of embryos (Table 4.4).

To further confirm the results, a pre-mRNA splicing blocking MO of PRLR1, prlr1MO-2, was synthesized and injected into the embryos. Disruption of yolk extension was detected at 24 hpf, which was the same as the inhibition of prlr1MO-1, suggesting that malformation of yolk extension was a robust effect of PRLR1 specific knockdown (Figure 4.8A and B). The efficiency of prlr1MO-2 was evaluated by RT-PCR, which showed that prlr1MO-2 induced defective splicing of PRLR1, and thus depleted the function of PRLR1 (Figure 4.8C).

4.3.3 PRLR1 MO induced developmental defect of zebrafish pronephric duct

The yolk extension, which is the posterior elongated region of the yolk, is developed adjacent to pronephric duct at about 15 hpf (Kimmel et al., 1995). A number of studies have shown that the formation of yolk extension is associated with

the development of pronephric duct (Horsfield et al., 2002; Kubota et al., 2007). Considering the fact that PRLR1 was expressed in the pronephric duct, it was further investigated whether development of pronephric duct was affected by PRLR1 knockdown using WISH of pronephric duct marker genes. The expression patterns of these marker genes, including pax2a and slc12a3 (Raciti et al., 2008; Wingert et al., 2007), were examined in this study. In the control embryo, pax2a was expressed along the pronephric duct at 24 hpf (Figure 4.9A and B). In contrast, expression of pax2a in prlr1MO-1 embryos almost disappeared completely (Figure 4.9 C and D).

Consistently, expression of slc12a3 in prlr1MO-1 injected embryo decreased as compared to the control (Figure 4.10). On the other hand, expression of wt1a which is a marker gene for the glomerulus (Perner et al., 2007), and also of tal1 and otg, which are marker genes for the hematopoietic system (Gering et al., 2003), remained unchanged as comparing the control and the prlr1MO-1 injected embryos, indicating that PRLR1 was not required for the development of glomerulus and hematopoietic system (Figures 4.11 and 4.12).

Whole mount in situ immunostaining was also carried out to detect the expression level of atp1a1 protein, which is another marker of pronephric duct (Rajarao et al., 2001). In the control, the atp1a1 protein was expressed in the whole pronephric duct (Figure 4.13A and B). In the prlr1MO-1 injected embryo, the expression of atp1a1 was only found in the posterior pronephric duct, indicating that development of pronephric duct was affected by knockdown of PRLR1 (Figure 4.13C and D).

To further provide structural information of the zebrafish pronephric duct after PRLR1 MO injection, H&E staining on transverse sections of zebrafish embryo was performed at 48 hpf. The pronephric duct is a lumen which is surrounded by epithelial cells regularly (Haugan et al., 2010). However, in the prlr1MO-1 injected embryos, the structure of the pronephric duct was disrupted, including disorder of epithelial cells and destruction of pronephric duct lumen, indicating that the pronephric duct was destroyed. This may disrupt waste excretion of the fish and may be responsible for the death of the embryos several days post fertilization (Figure 4.14B).

To further investigate the molecular basis of the developmental defect of pronephric duct induced by PRLR1 MO, the expression of 12 genes which are located in the pronephric duct, including wt1a, wt1b, atp1a1, NBC1, CDH17, slc4a2, GATA3, JAK2a, JAK2b, PRLR2, pax2a and ret1, were investigated by real-time PCR (Vainio and Lin, 2002; Wingert et al., 2007). Among them, the expression of wt1b and atp1a1 was suppressed, while CDH17, NBC1 and slc4a2 mRNA levels were up-regulated by PRLR1 MO injection. The expression of the other genes remained unchanged (Figure 4.15).

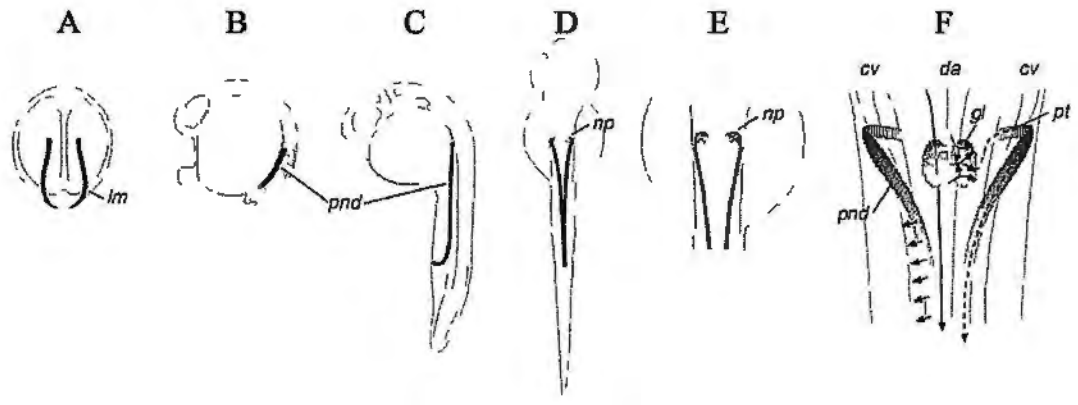


Fig. 4.1 Developmental stages in the formation of zebrafish pronephros. (A): Commitment of intermediate mesoderm to the pronephric fate at about 12 hpf. (B) and (C): Pronephric duct (pnd) growth and formation of nephron primordial at about 24 hpf. (D) and (E): Patterning of the nephron primordial (np) giving rise to the pronephric glomerulus (gl) and pronephric tubules (pt) at about 40 hpf. (F): Onset of glomerular filtration at about 48 hpf. Angiogenic sprouts from the dorsal aorta (da) invade the glomerulus and form the capillary loop. The cardinal vein (cv) is apposed to the tubules and duct and receives recovered solutes (Drummond et al., 1998).

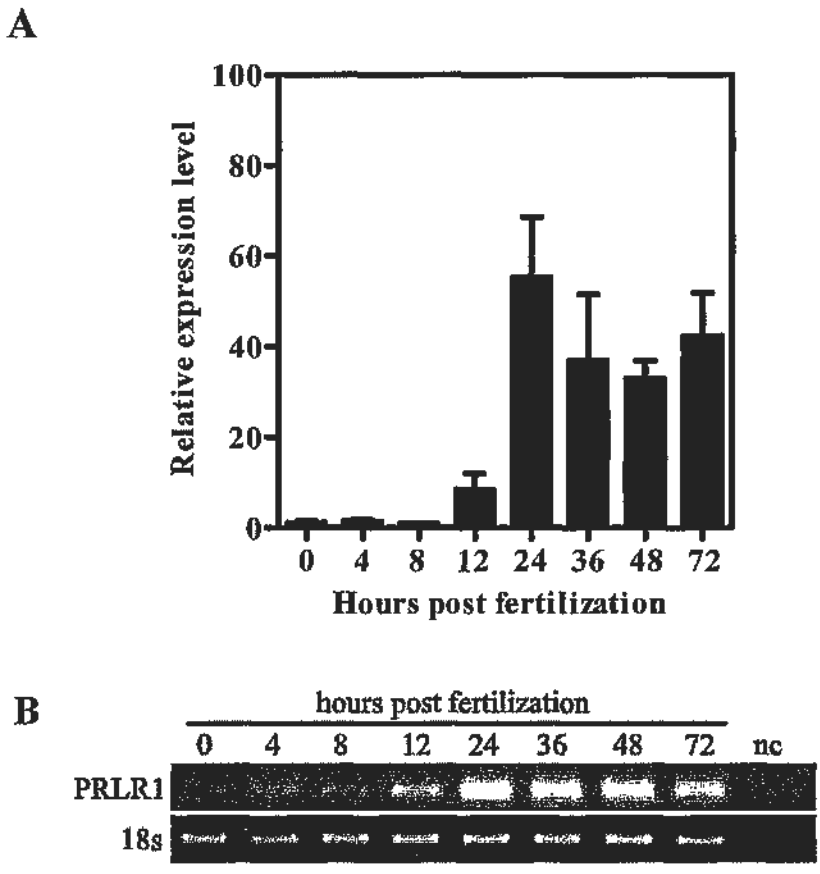


Fig. 4.2 Temporal expression of PRLR1 during zebrafish embryonic development was detected using real-time PCR (A) and RT-PCR (B). The mRNAs of zebrafish embryos at different developmental stages were extracted and used as templates. In the real time PCR, the 18s ribosomal RNA was used as the internal control to quantified the expression level of PRLR1. nc: water negative control.

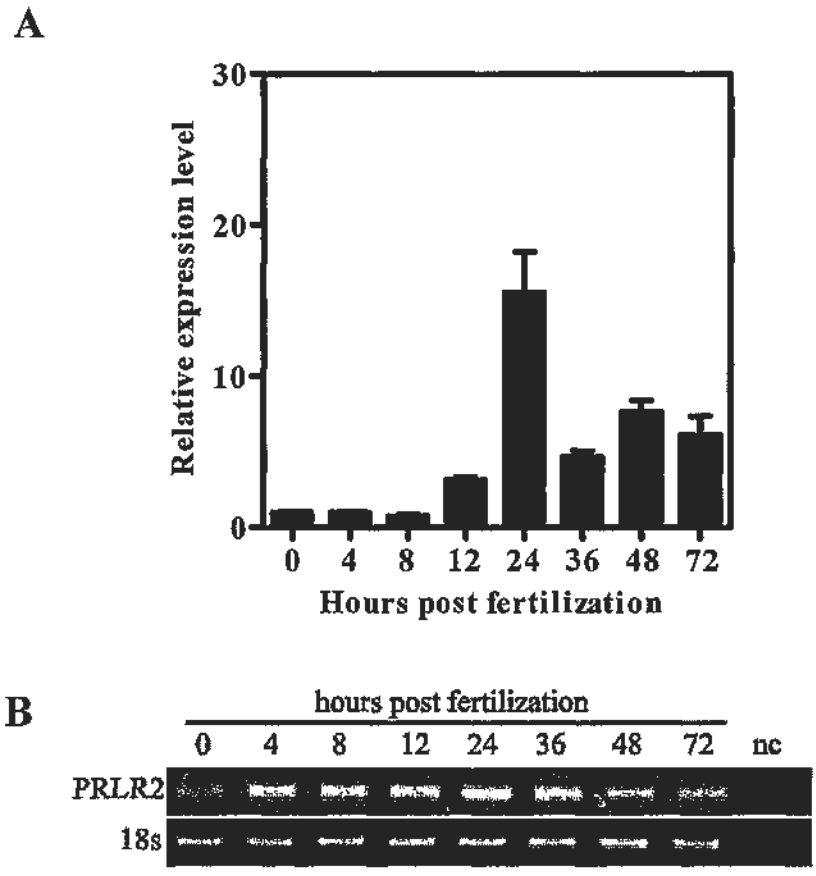


Fig. 4.3 Temporal expression of PRLR2 during zebrafish embryonic development was detected using real-time PCR (A) and RT-PCR (B). The mRNAs of zebrafish embryos at different developmental stages were extracted and used as templates. In the real time PCR, the 18s ribosomal RNA was used as the internal control to quantified the expression level of PRLR1. nc: water negative control.

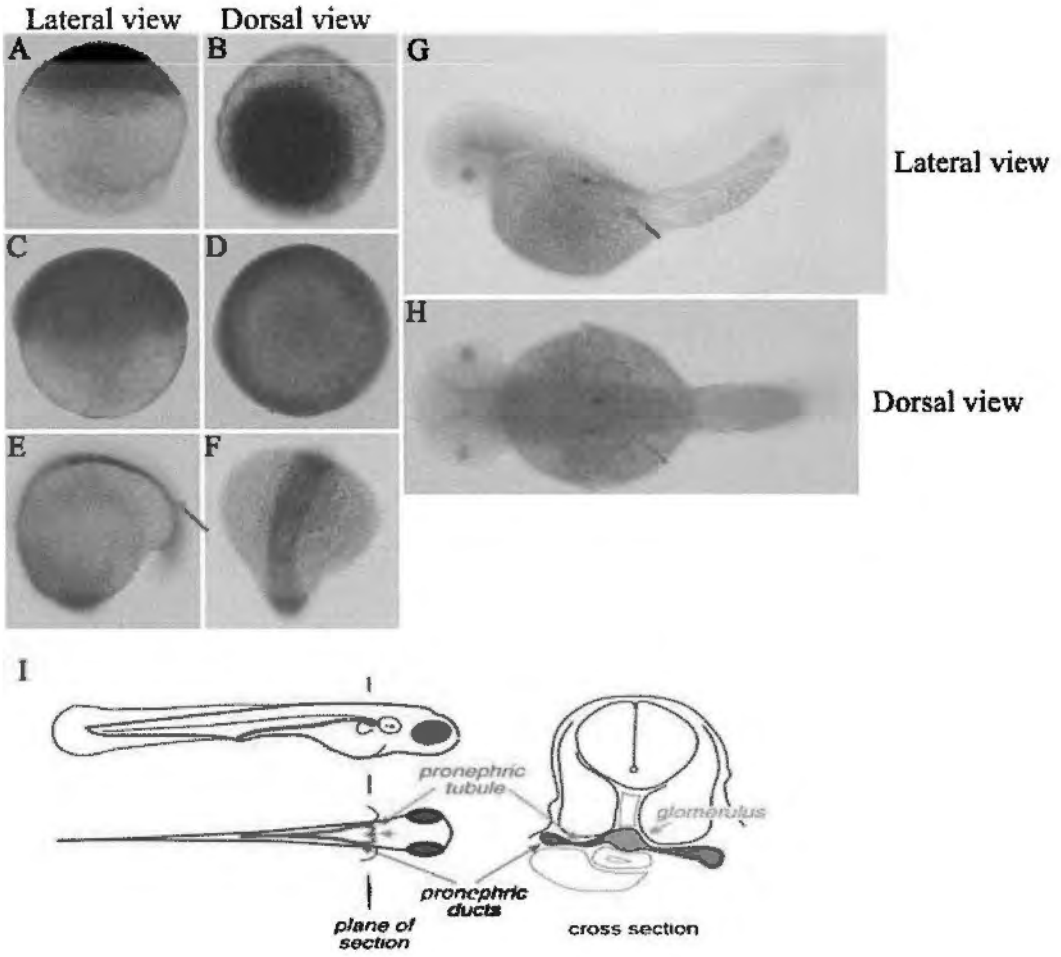


Fig 4.4 Detection of PRLR1 expression in the zebrafish embryos at 4 hpf (A and B), 8 hpf (C and D), 16 hpf (E and F) and 48 hpf (G and H) using WISH. Panel I shows a schematic map of the pronephric duct of zebrafish at 48 hpf (Drummond, 2003). The red arrows indicate the WISH signal of PRLR1.

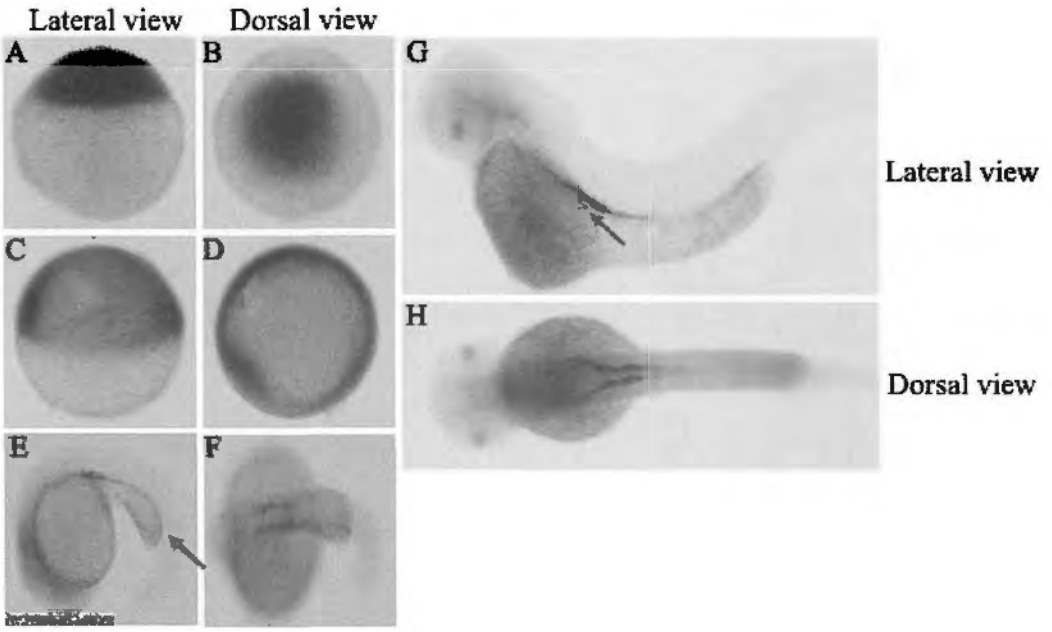


Fig 4.5 Detection of PRLR2 expression in the zebrafish embryos at 4 hpf (A and B), 8 hpf (C and D), 16 hpf (E and F) and 48 hpf (G and H) using WISH. The red arrows indicate the WISH signal of PRLR2.

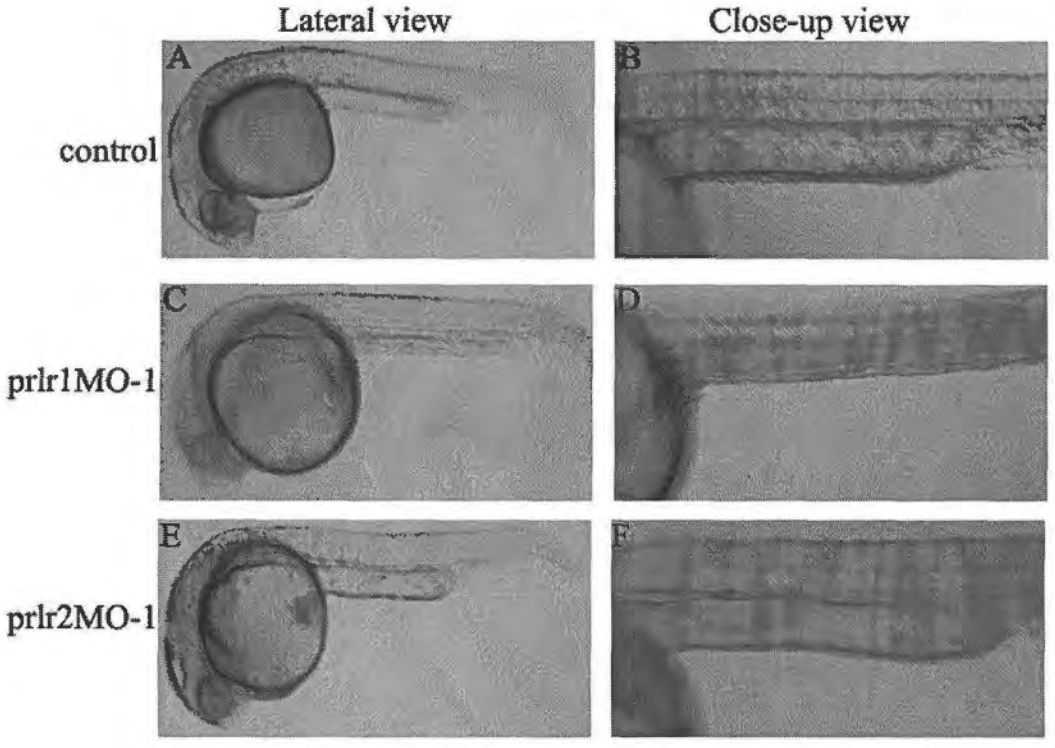


Fig. 4.6 Morphological analysis of zebrafish embryos after PRLR MO injection. The zebrafish embryos were injected with 6 ng control (A), prlr1MO-1 (C), or prlr2MO-1 (E) at 1-cell stage respectively. The morphology of the MO-injected zebrafish embryos was analyzed under the Leica microscope. Close-up views of the pictures are shown on the right panels (B, D and F).

Morpholino	Ctrl	prlr1MO-1			prlr2MO-1		
Dose (ng)	9	3	6	9	3	6	9
Numbers of embryos injected[a]	245	461	486	239	198	155	169
Abnormal YE	5	309	396	98	2	4	8
Mortality	3	17	25	108	2	6	59
Ratio of abnormal YE (%)	2	67	81	41	1	2	4

Table 4.4 Statistics regarding the malformation of yolk extension (YE) induced by MO injection. Different doses of control, prlr1MO-1 and prlr2MO-1 were injected to the zebrafish embryos. The numbers of embryos with abnormal yolk extension or dead embyros were counted at 24 hpf. [a]: The non-fertilized embryos were removed at 12 hpf.

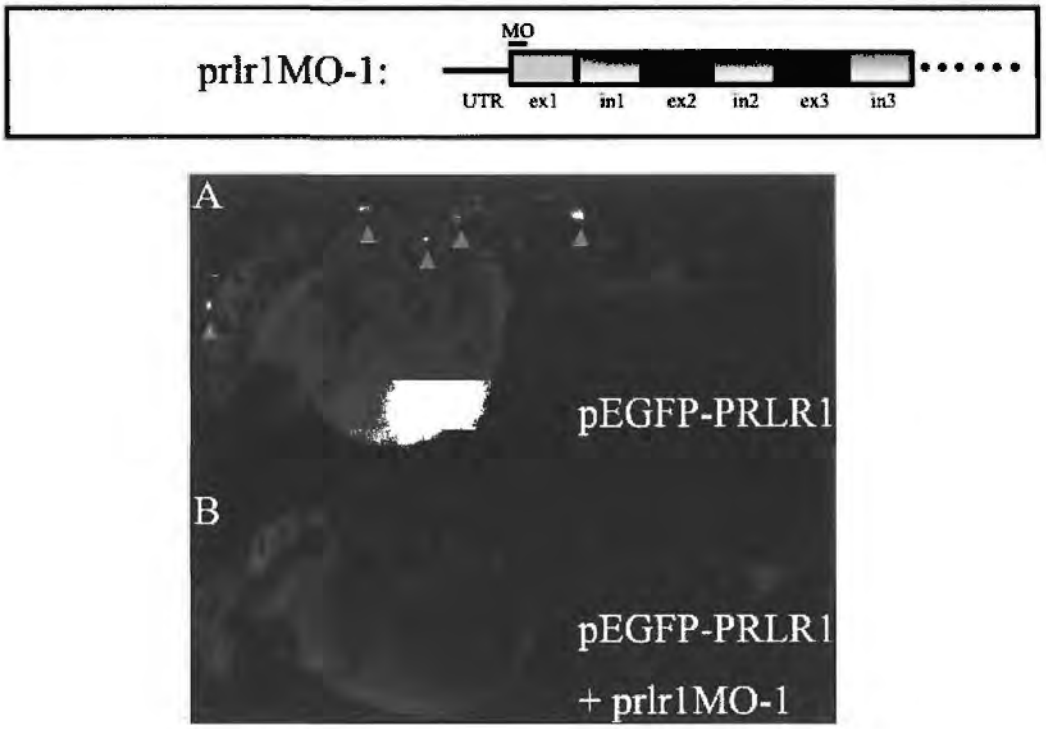


Fig. 4.7 Efficiency test of PRLR1 MO. The pEGFP-PRLR1 plasmid was injected in the zebrafish embryos in the absence (A) or presence (B) of translation blocking prlr1MO-1. Green fluorescence as indicated by red triangles was detected at 24 hpf.

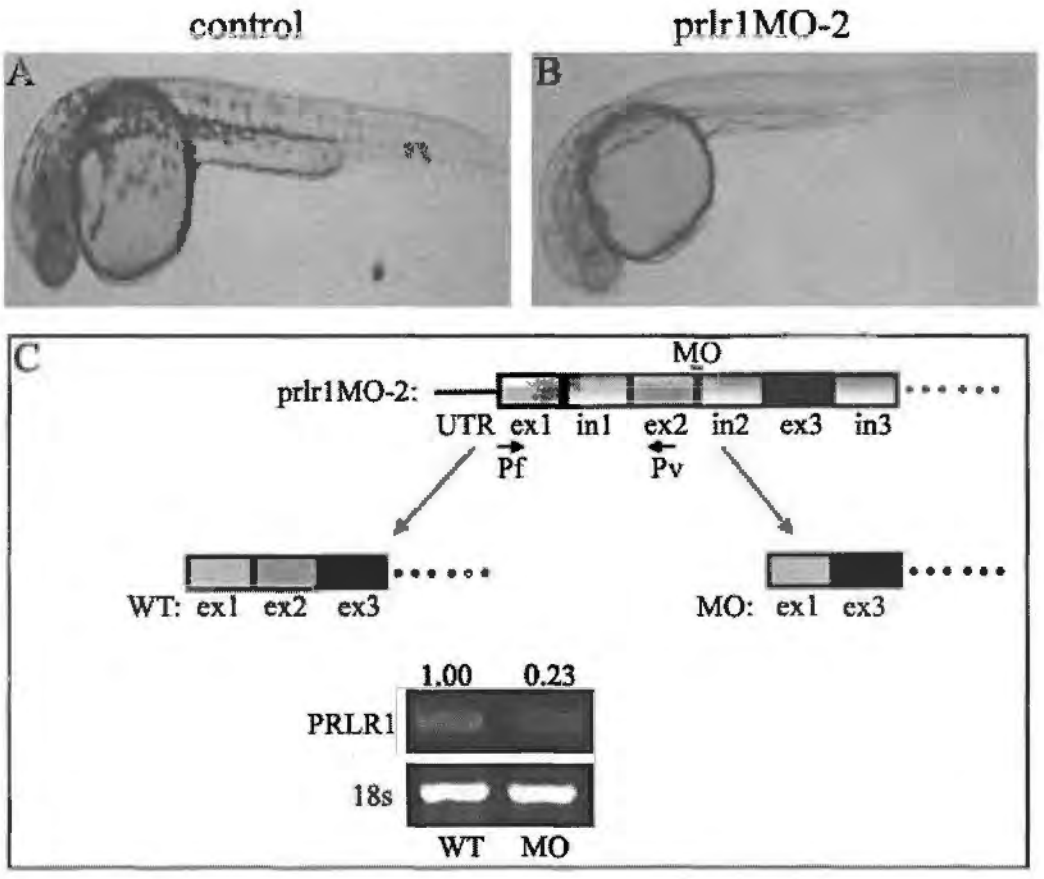


Fig. 4.8 Morphological analysis of zebrafish embryos after prlr1MO-2 injection. 9 ng of control MO (A) or prlr1MO-2 (B) was injected to 1-cell stage zebrafish embryos. The morphology of MO-injected zebrafish embryos was analyzed under the Leica microscope. The efficiency of prlr1MO-2 was evaluated by RT-PCR (C). The 18s ribosomal RNA was used as the internal control. UTR: untranslated region; WT: wild type; ex: exon; in: intron; Pf: forward primer; Pr: reverse primer.

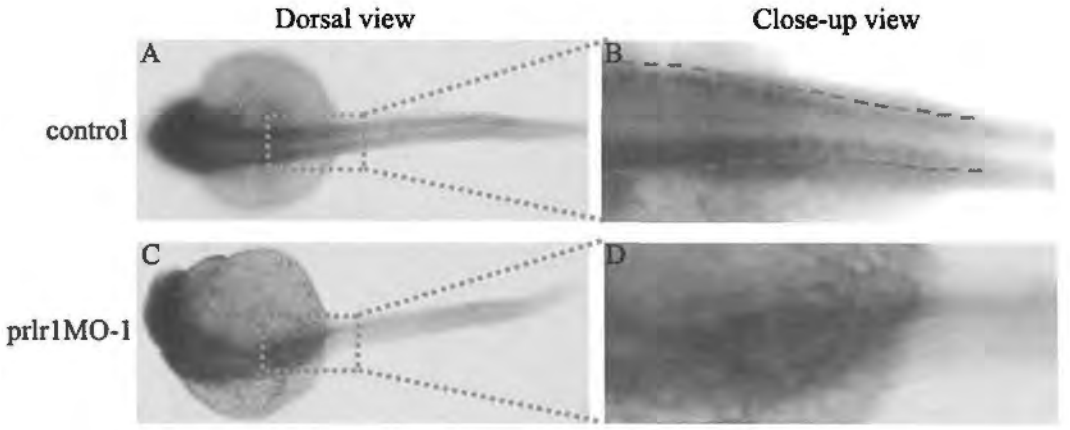


Fig. 4.9 Expression pattern of pax2a in the zebrafish pronephric duct after MO injection. The control MO (A and B) or prlr1MO-1 (C and D) were microinjected into 1-cell stage embryos respectively. The expression pattern of pax2a was determined using WISH by at hpf. The pictures were taken on the dorsal view (mag 5X) and close-up view (20X).

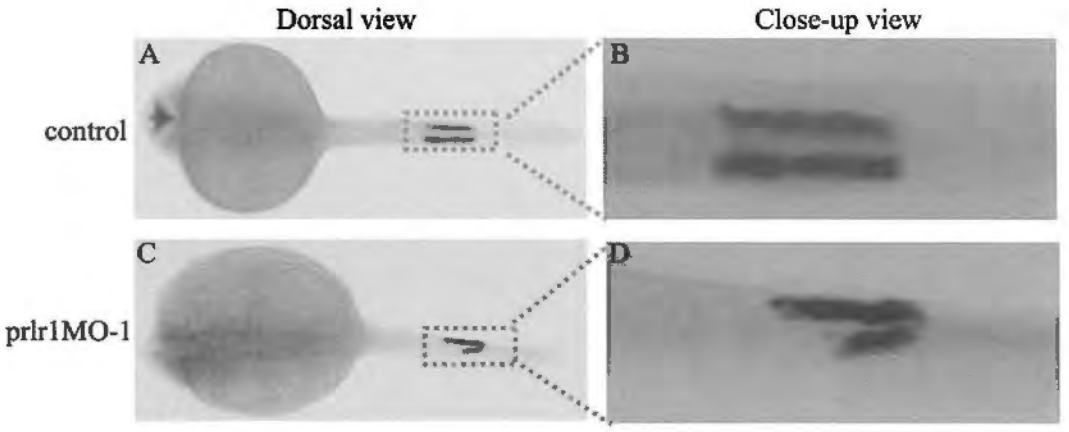


Fig. 4.10 Expression pattern of slc12a3 in the zebrafish pronephric duct after MO injection. The control MO (A and B) or prlr1MO-1 (C and D) were microinjected into 1-cell stage embryos respectively. The expression pattern of slc12a3 was determined using WISH at 24 hpf. The pictures were taken on the dorsal view (mag 5X) and close-up view (20X).

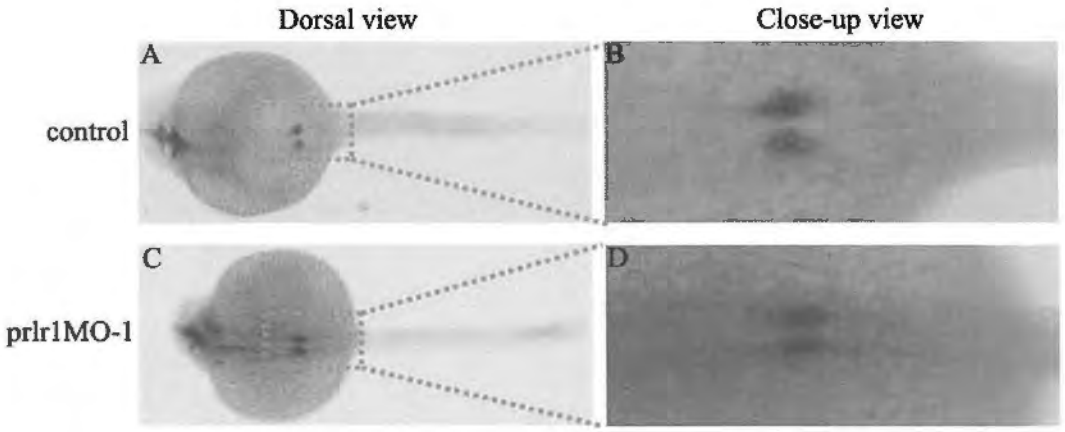


Fig. 4.11 Expression pattern of wt1a in the zebrafish glomerulus after MO injection. The control MO (A and B) or prlr1MO-1 (C and D) were microinjected into 1-cell stage embryos respectively. The expression pattern of wt1a was determined using WISH at 24 hpf. The pictures were taken on the dorsal view (mag 5X) and close-up view (20X).

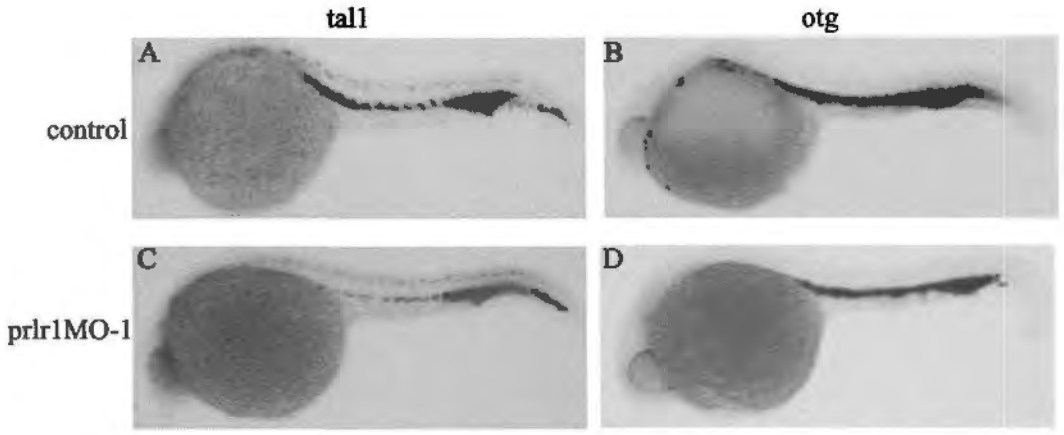


Fig. 4.12 Expression patterns of tal1 and otg in the zebrafish hematopoietic system after MO injection. The control MO (A and C) or prlr1MO-1 (B and D) were microinjected into 1-cell stage embryos respectively. The expression patterns of tal1 (A and B) and otg (C and D) were determined using WISH at 24 hpf. The pictures were taken on the dorsal view (mag 5X).

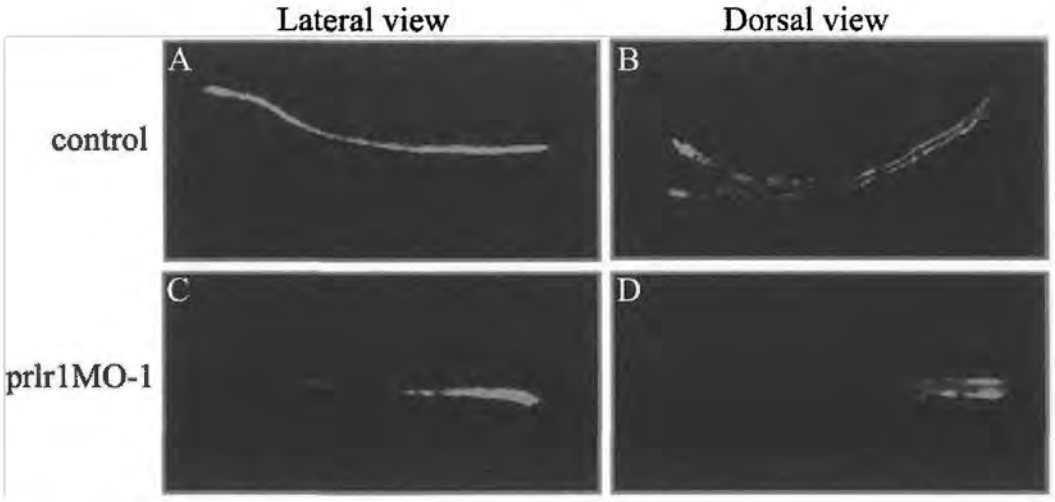


Fig. 4.13 Expression pattern of the atp1a1 protein in the zebrafish pronephric duct after MO injection. The control MO (A and B) or prlr1MO-1 (C and D) were microinjected into 1-cell stage embryos respectively. The expression pattern of the atp1a1 was determined using whole mount in situ immunostaining at 24 hpf. Green fluorescence indicates the atp1a1 protein. The pictures were taken on the dorsal view (mag 5X) and close-up view (20X).

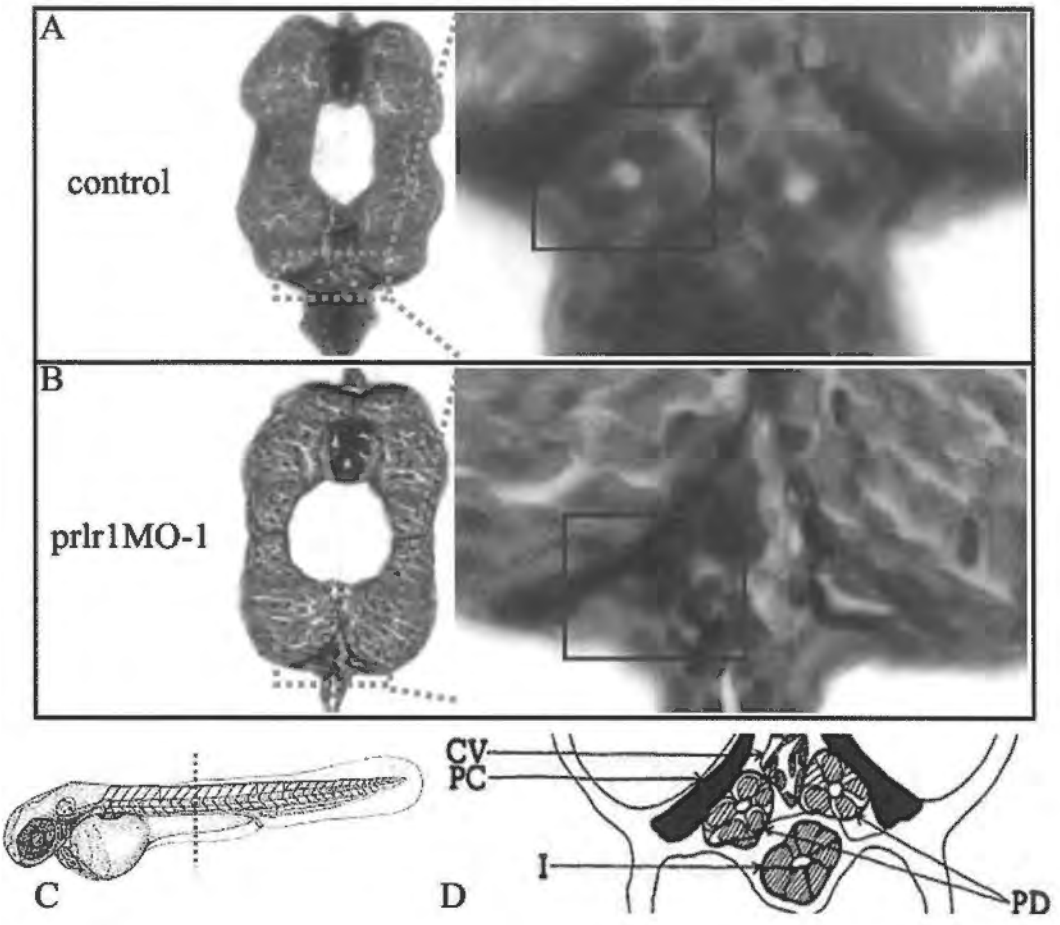
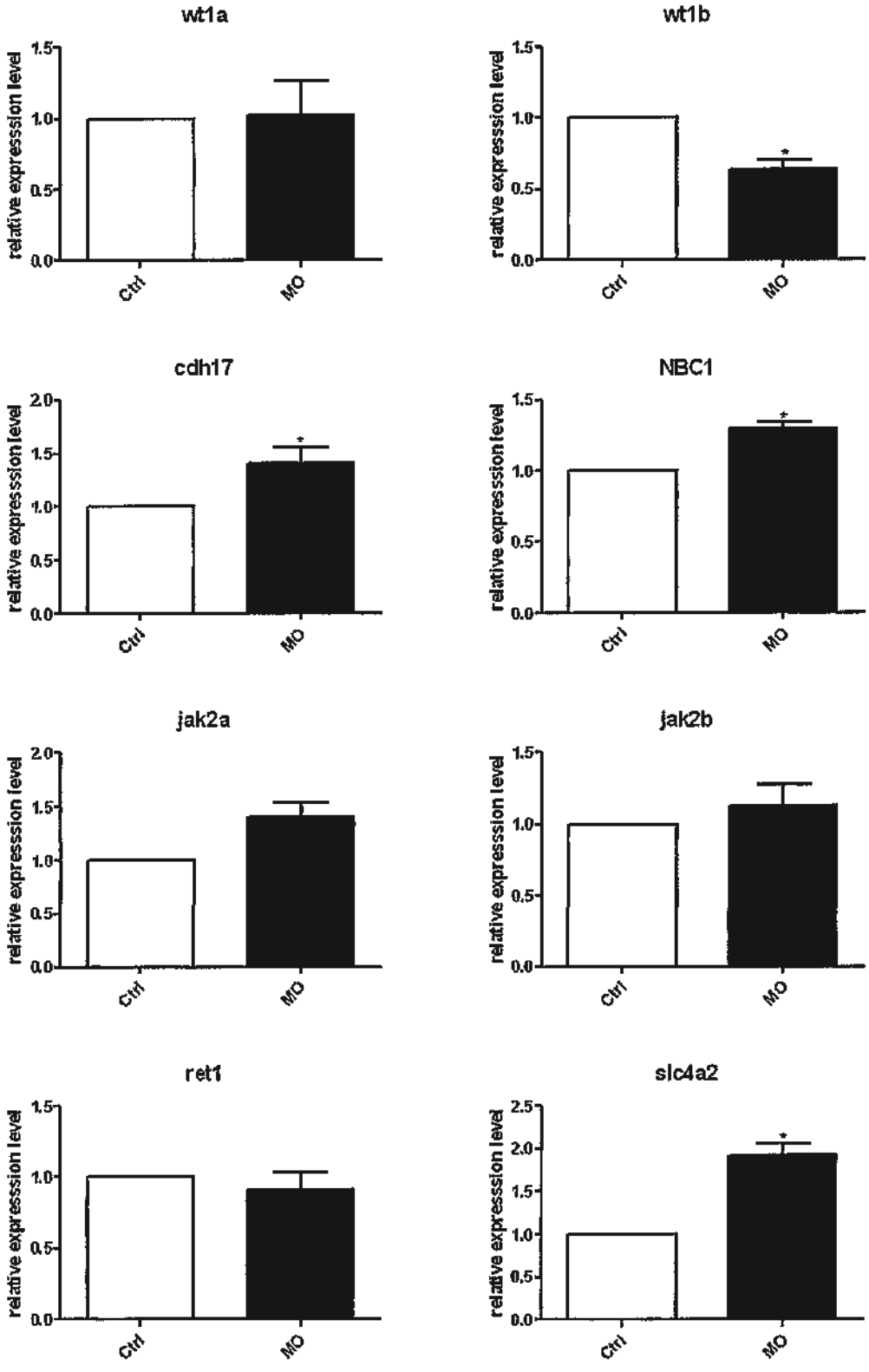


Fig. 4.14 Histological analysis of the zebrafish pronephric duct after MO injection. The control MO (A) or prlr1MO-1 (B) was injected into 1-cell stage zebrafish embryos respectively. The transverse sectioning of zebrafish was performed at 6 μm at 48 hpf (C) and stained with hematoxylin and eosin. A schematic map of the zebrafish pronephric duct is indicated in Panel D. The blue box indicates the pronephric duct. CV: caudal vein; I: intestine; PC: pigment cell; PD: pronephric duct.


wt1a
wt1b
cdh17
NBC1
jak2a
jak2b
ret1
slc4a2
relative expression level
Ctrl
MO
*

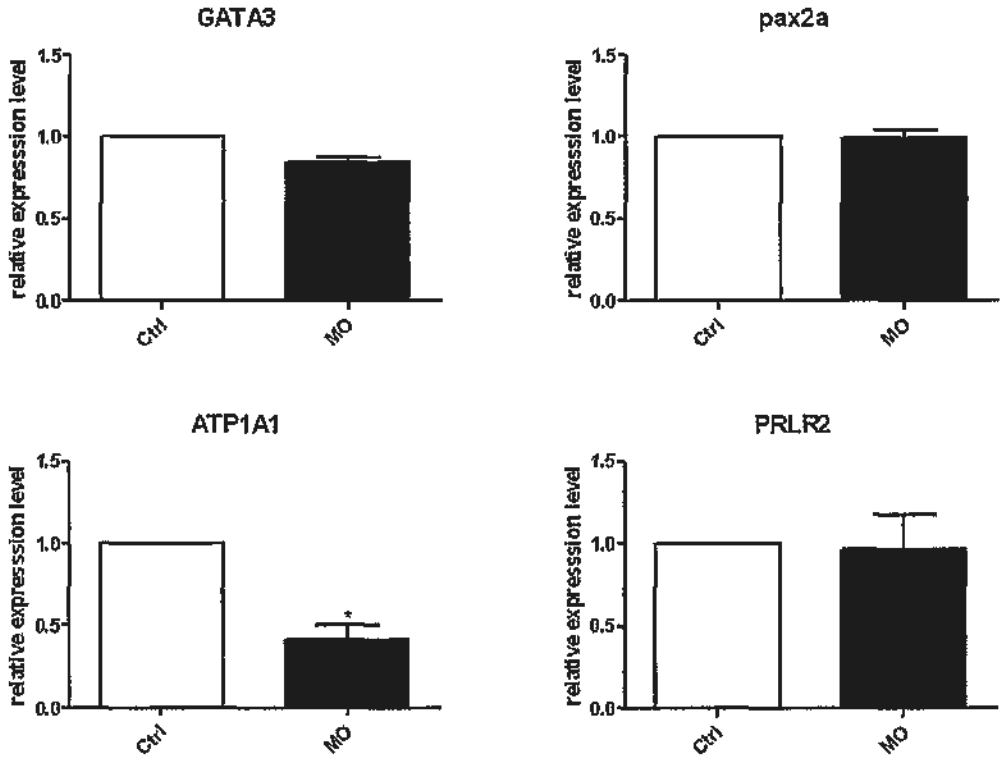


Fig. 4.15 Real-time PCR analysis of genes located in the pronephric duct. The mRNAs of zebrafish embryos at 24 hpf were extracted and used as templates. The 18s ribosomal RNA ribosomal RNA was used as the internal control. (n = 4; *P<0.05)

4.4 Discussion

In the present study, we have shown that both PRLR1 and PRLR2 are expressed in the pronephric duct during embryonic development of zebrafish. PRLR1 exhibited its significance on the pronephric duct formation. Expression levels of pronephric duct markers, including slc12a3, pax2a and atp1a1, showed remarkable decrease after prlr1MO-1 injection using WISH or whole mount *in situ* immunostaining. The typical structure of pronephric duct was also disrupted due to the knockdown of PRLR1. All the evidence demonstrated that PRLR1 played an important role in the development of zebrafish pronephric duct.

4.4.1 PRLR1 was expressed in the pronephric duct and pancreas

It is found that zebrafish PRLR1 is expressed in the pronephric duct and pancreas during early development in the present study. This observation is consistent with previous report, in which PRLR1 could also be detected in the pronephric duct at 24 hpf, although no detailed functional studies was performed (Liu et al., 2006). In the present study, zebrafish PRLR1 is found to express in the pronephric duct during the whole process of pronephric duct development (Figure 4.4 E-H). In zebrafish, the stages from 14 hpf to 24 hpf are critical for pronephric duct development from the intermediate mesoderm (Drummond, 2003). The real-time PCR data showed that PRLR1 expression increased remarkably from 12 hpf to 24 hpf, and remained relatively high afterwards. This trend is correlative to the development of pronephric duct, strongly suggesting the role of PRLR1 in the development of zebrafish pronephric duct (Figures 4.2 and 4.4).

Moreover, PRLR1 was also detected in the pancreas during the embryonic

development of zebrafish using WISH (Figure 4.4). It would be of great interest to investigate the role of PRLR1 in zebrafish pancreas development. As a matter of fact, PRL signaling has been demonstrated in the metabolism of pancreas such as promotion of β-cell growth and enhancement of insulin production (Sorenson and Brelje, 1997; Sorenson et al., 1987). The PI3K and MAPK pathways are involved in these processes (Amaral et al., 2004). Targeted deletion of PRLR resulted in the reduction of islet density, β-cell mass and pancreatic insulin mRNA expression (Freemark et al., 2002). The expression of PRLR in the pancreas during embryonic development has also been reported in several species including turkeys, chickens, rats and mice (Bole-Feysot et al., 1998; Leclerc et al., 2007; Royster et al., 1995). All the evidence strongly indicated the role of PRLR in the embryonic development of pancreas. However, very few detailed studies have been performed regarding the function of PRLR on pancreas organogenesis. As morphogenesis, patterning, differentiation and gene regulation mechanisms of pancreas organogenesis are similar in zebrafish and mammals, zebrafish may serve as a good model to explore the role of PRLR in the embryonic development of pancreas (Kinkel and Prince, 2009; Tiso et al., 2009).

4.4.2 Knockdown of PRLR1 caused abnormal development of pronephric duct

MOs were used to specifically knock down PRLR1 expression during embryogenesis. The PRLR1 MO disrupted the formation of pronephric duct as shown by the measurement of marker gene expression patterns as well as the structure of the pronephric duct (Figures 4.9-14). The onset of the pronephric duct function to excrete metabolic wastes to the outside is at about 40 to 48 hpf

(Drummond, 2003). Disruption of pronephric duct by PRLR1 MO therefore disrupted waste excretion, which may lead to the death of zebrafish. Consistent with our study, Cadherin-6 is important for proper formation of the glomerulus, the pronephric tubule and anterior region of pronephric duct during embryonic development. MO knockdown of Cadherin-6, which caused abnormal pronephric duct, is lethal to the zebrafish embryos (Kubota et al., 2007). Cadherin-17, another member of cadherin family, is also expressed in the pronephric duct and the injection of MOs directly against Cadherin-17 caused defect of pronephric duct resulting in the death of embryos between 5 to 7 dpf (Horsfield et al., 2002). Therefore, maintenance of pronephric system integrity is critical for the survival of zebrafish embryos.

Interestingly, only PRLR1 MO but not PRLR2 MO caused abnormal formation of yolk extension in zebrafish. The analysis of PRLR2 protein sequence may explain this phenomenon. Despite high sequence similarity in the ECDs of these two receptors, remarkable differences can be recognized in their ICDs, including atypical Box 2 region, a number of missing regions and tyrosine residues in PRLR2 (Huang et al., 2007). These may abolish the activation of the downstream signaling pathways and therefore the physiological functions of PRLR2 (Chapter 3 and Figure 3.9). However, we do not role out the possibility that PRLR2 plays a role in the embryonic development of pronephric duct and its role may be compensated by PRLR1. Therefore, further investigation on the PRLR2 is required to demonstrate this issue.

The Wilms' tumor suppressor wt1a which encodes a zinc finger transcriptional

regulator, and Pax2a is a member of the paired-domain containing homeobox transcriptional regulators. They are both involved in the development of pronephros, mesonephros and metanephros, indicating the conserved development pattern of the the three kidneys (Carroll and Vize, 1996; Heller and Brandli, 1997). Malfunction of wt1a in human results in the Wilms' tumor (Gessler et al., 1990). In zebrafish embryo, wt1a is dominantly expressed in the glomerulus and inactivation of wt1a leads to the failure of glomerular differentiation and morphogenesis resulting in a rapidly expanding general body edema (Perner et al., 2007). The expression of pax2a in zebrafish is found in the early intermediate mesoderm and later in the pronephric tubule and anterior region of pronephric duct (Krauss et al., 1991). The zebrafish mutant *no isthmus* which is lacking in the pax2a expression shows a depletion of pronephric tubule (Majumdar et al., 2000). In the present study, the expression pattern of pax2a, but not wt1a, was affected by PRLR1 MO microinjection, indicating that the glomerulus remained intact by the knockdown of PRLR1 (Figures 7, 8). Moreover, both the hematopoietic system and the pronephric system are developed from the mesoderm and they are adjacent to each other during embryonic development (Drummond, 2003). In the present study, no difference of the hematopoietic system marker genes (tal1 and otg) between the control and PRLR1 MO injected embryos was found, further confirming that PRLR1 specifically acts on the pronephric duct (Figure 4.12).

4.4.3 Possible signaling pathways involved in the abnormal development of pronephric duct caused by knockdown of PRLR1

Physiological effects of PRL signaling are mediated by a series of signaling pathways, mainly including the JAK-Stat, PI3K and MAPK pathways (Bole-Feysot

et al., 1998). To date, the expression of several genes in these pathways has been found in the development of pronephric system. WISH analysis revealed that Jak2b is expressed in the pronephric duct at 24 hpf, together with its downstream protein STAT5.1, STAT5.2 and STAT6 (Lewis and Ward, 2004; Low et al., 2006; Oates et al., 1999). In MAPK signaling, erk5 and p38α are distinctly expressed in the pronephric duct at 24 hpf (Krens et al., 2006). Some genes involved in the PI3K signaling are also found in the pronephros development of zebrafish (Costagli et al., 2006; Deepa and Dong, 2009; Wingert and Davidson, 2008). It would be of interest to investigate which signaling pathway is involved in the PRLR1 knockdown induced developmental defect of the pronephric duct.

Organogenesis is a complicated process which requires perfect cooperation and regulation of a variety of genes and proteins (Yokoo et al., 2008). To date, a number of genes and proteins are involved in kidney development of both zebrafish and mammals. These genes can be categorized into several main signaling pathways including TGF-β, Wnt, BMP, Notch, and the Hedgehog pathways (Davies and Fisher, 2002; Wingert et al., 2007). In the present study, it was demonstrated that PRLR1 is also important for the development of zebrafish kidney, especially the pronephric duct. It would be interesting to dissect how the PRLR signaling pathway cooperates with the other pathways in order to ensure the normal development of kidney.

The present study has focused on the role of PRLR1 in the embryonic development of zebrafish kidney. The existence of PRLR1 in the adult kidney has been reported in a variety of vertebrates (Freemark et al., 1996; Leclerc et al., 2007; Sandra et al., 1995). The best demonstrated function of PRLR in the kidney is

regulation of water and electrolyte balance (Bole-Feysot et al., 1998; Hirano et al., 1971). It is worthy to note that PRLR is also expressed in the kidney during embryonic development of mammals. In mouse, the kidney expresses high level of PRLR from the day 18 fetus (Tzeng and Linzer, 1997). In the human fetal kidney at 7.5 to 14 weeks of gestation, PRLR is expressed in the kidney, especially in collecting ducts (equivalent to pronephric duct in zebrafish) and tubules, which is perfectly consistent with our data (Freemark et al., 1997). However, very few reports have demonstrated the role of PRLR in kidney development. Given the fact that the high similarities between the pronephric and metanephric development, it would be expected that PRLR functions similarly in the kidney development of mammals and zebrafish (Drummond, 2003). Further studies are required to testify this hypothesis.

In conclusion, the present study has demonstrated that PRLR1 was expressed in the pronephric duct during embryonic development of zebrafish. The knockdown of PRLR1 expression caused severe defect of pronephric duct by examination of the marker gene expression patterns and histological analysis. These results suggested that PRLR1 plays an important role in the embryonic development of the pronephric duct in zebrafish.

Chapter 5

General discussion and future perspectives

5.1 General discussion

GH, PRL and SL constitute a family of polypeptide hormones which are related in structure and function. These three hormones have a similar size (~200 aa), and share similar protein sequence and protein structure (Goffin and Kelly, 1997). It is widely accepted that these three hormones have arisen by duplication of an ancestral hormone gene, co-evolving with their cognate receptors (Niall et al., 1971). The GH/PRL family of hormones participate in many diversified biological processes of life (Forsyth and Wallis, 2002).

Despite numerous attempts to screen and clone the SL gene in a variety of vertebrates, SL is only found in fish and it is postulated that SL was lost during the evolution to land vertebrates (Chen and Horseman, 1994). While GHR and PRLR have been discovered in all the vertebrate species, the identity of the receptor for SL is still controversial. The first paper regarding the discovery of SLR was reported in masu salmon in 2005, in which the so-called 'SLR' exhibited higher affinity to SL than to GH and PRL using *in vitro* binding assays. The masu salmon SLR clusters within the GHR1 clade according to phylogenetic analysis (Fukada et al., 2005). Subsequently, other 'SLRs' found in medaka and other fish also cluster within the GHR1 clade, leading to the hypothesis that all the GHR1s in fish are in fact 'SLRs', while the GHR2s are the real GHRs (Benedet et al., 2008; Fukamachi et al., 2005). However, studies in black seabream, eel and rainbow trout performed by our lab and others demonstrated that the two GHRs could only be activated by GH, but not by

SL (Jiao et al., 2006; Reindl et al., 2009). To better unravel this controversy, a series of experiments have been performed in the present study including direct binding assays, luciferase promoter reporter assays and phosphorylation detection of the post-receptor signaling factors. All our results support the view that GHR1 is a real receptor for GH but not SL (see Chapter 3 for details). This gives rise to another question: what is the receptor for SL? One possibility is that after several rounds of evolution, one member of the class I cytokine receptor superfamily was endowed with the ability to bind to SL and therefore became the receptor for SL. Although members of class I cytokine receptor superfamily share limited primary protein sequence homology, they all contain a conserved domain of about 200 aa called the cytokine receptor homology domain (CHD) in the ECD (Boulay et al., 2003). The CHD of Class I cytokine receptors possess two pairs of conserved disulfide bonds and a 'WS' motif which are important for the specific binding of ligands (Bazan, 1990; Bravo and Heath, 2000). Moreover, the majority of diversification of this receptor superfamily is suspected to occur after the divergence of urochordates and vertebrates approximately 794 million years ago (MYA), but before the divergence of ray-finned from lobe-finned fishes around 476 MYA. Since then, only relatively limited lineage-specific diversification within the different Class I receptor structural groups has occurred (Liongue and Ward, 2007). In addition, the fish specific genome duplication has given rise to more than one copy of each receptor and thus the possibility exists that one of these receptors adopted a novel function as the receptor for SL. Another possibility is that the gene encoding the receptor for SL is still not found. Although the zebrafish genome has been sequenced for a long period, around 1/4 of the genome sequence remains unfinished (http://www.sanger.ac.uk/Projects/D_rerio/), making it possible that the gene for

zebrafish SL receptor is located in the unknown region of the genome. Further studies are required to unveil the mystery of the receptor for SL.

Interestingly, crosstalk between GH-GHR and PRL-PRLR signaling axes in zebrafish was not observed in the present study, though crosstalk in the mammalian receptors was reported, suggesting that the GH/PRL family of hormones and their receptors in zebrafish diverse to a larger extent than mammals during evolution (Cunningham et al., 1990). Indeed, GH and PRL are highly conserved in mammals but vary markedly between mammals and teleosts as well as among different teleost species, suggesting that the evolution rates of GH and PRL are high and variable in teleost. This might result in the absence of or the extremely weak crosstalk between the two signaling pathways in teleost (Forsyth and Wallis, 2002). Studies in seabream, salmon, eel and rainbow trout support this hypothesis: the cross binding happens only at the pharmacological concentrations (Huang et al., 2007; Jiao et al., 2006; Reindl et al., 2009). Therefore, it is very likely that in teleost, the GH/PRL family of hormones act on their respective receptors to exert biological effects at the physiological level. However, more extensive experimental evidence such as three-dimensional analysis of the protein structures and *in vivo* characterization are required to address this issue.

Nephrogenesis involves many cellular processes such as cell proliferation and differentiation, cell adhesion and apoptosis, all of which require molecules from various classes and families to ensure the appropriate formation of the kidney. In recent years, many essential developmental control genes that coordinate the assembly of the kidney have been discovered such as pax2a, wt1, sim1 and CDH17

(Bracken et al., 2008; Horsfield et al., 2002; Majumdar et al., 2000b). Besides, several signaling pathways are also involved in the regulation of kidney development in both zebrafish and mammals including Wnt, TGF-β, hedgehog and the Notch pathways (Dressler, 2006). In the present study, it was demonstrated for the first time that PRLR1, which is expressed in the pronephric duct during embryonic development, plays an important role in the formation of the zebrafish pronephric duct. PRLR1 MO induced abnormal formation of yolk extension at 24 hpf and embryo death at 5 dpf. The morphological defect on the yolk extension was likely resulted from the disruption of the pronephric duct. Further studies using WISH and whole mount in situ immunostaining showed that expression of pronephric duct markers including pax2a, slc12a3 and atp1a1 were all decreased in the PRRL1 MO injected embryos as compared to the control. Histological analysis also indicated that the structure of pronephric duct was disrupted by the injection of PRLR1 MO, confirming the above observation. Real-time PCR data showed that several genes expressed in the pronephric duct including CDH17, NBC1, slc4a2, wt1b and atp1a1 were up or down-regulated in the PRLR1 MO-injected embryo. Therefore it could be concluded that PRLR1 plays an important role in the development of zebrafish pronephric duct.

5.2 Future perspectives

As aforementioned, nephrogenesis is a complicated process which requires an intact regulatory network composed of a number of signaling pathways and molecules. Although several studies have focused on the development of the kidney in zebrafish, none has so far been designed to explore the whole regulatory network and the coordination of the signaling pathways (Majumdar et al., 2000a; Wingert et

al., 2007). Future studies on the developmental mechanism of kidney in zebrafish should focus on the systematic regulation of proteins and pathways. Large-scale screenings such as two dimensional electrophoresis analysis and RNA microarray may help to construct the regulatory pyramid of kidney development in zebrafish.

Another interesting issue to discuss is whether the zebrafish kidney can serve as a model to study human kidney diseases. Therapy of kidney diseases has made little progress over these years due to the lack of suitable model systems to study such diseases (McTaggart and Atkinson, 2007). It would be of great value to explore the possibility of zebrafish as the animal model to study human kidney diseases. Acute renal failure is a serious defect which causes high mortality (Pannu et al., 2008). A study showed that when treated with the antibiotic gentamicin and the cancer therapeutic cisplatin compounds, which are well known to cause kidney damage in human, the zebrafish embryos suffered from severe swelling in a time and dose dependent manner. This phenotype reflected that the embryos were unable to regulate water and electrolyte balance, which was due to malfunction of the glomeruli in filtration. Moreover, this phenotype could be rescued by treatment of taurine and Ucf-101, both of which were used in the treatment of renal failure (Hentschel et al., 2005). The zebrafish was also used to study the ciliopathies, which have the common denominator of renal disease (Tobin and Beales, 2008). These findings are highly suggesting that the zebrafish could act as a good model to study human kidney diseases and to explore new therapies and drugs in the treatment of such diseases.

Zebrafish has emerged as a powerful model for development, human disease and

environmental toxicology. The transparency of zebrafish embryos and larvae enables real-time observation of internal organs. Recently, a zebrafish line named *casper* lacking in melanocytes and iridophores has been generated. This line is thus almost transparent during the whole adult life, and this has greatly facilitated the imaging of the organs in adult zebrafish (White et al., 2008). The use of zebrafish in embryonic development study has been reported extensively such as in neurogenesis, angiogenesis and nephrogenesis (Childs et al., 2002; Drummond, 2003; Jiang et al., 1996). The zebrafish has also proven to be powerful in serving as a good model for human diseases including cancers, hematopoietic disorders and cardiovascular diseases (Bendig et al., 2006; de Jong and Zon, 2005; Matthews, 2004). In recent years, drug screening using zebrafish guided by phenotype analysis arises. In these screenings, thousands of zebrafish embryos are treated with a large number of compounds or chemicals and the desired phenotype changes are scored. This phenotype-based drug screening overcomes several obstacles in the conventional target-based drug screening such as uptake efficiency, side effects and *in vivo* bioactivity of drugs (North et al., 2007). Several compounds have been successfully identified to be effective in the restoration of blood circulation or suppression of cancer from such screening exercises (Peterson et al., 2004; Stern et al., 2005). Therefore the zebrafish model provides a promising platform which makes drug discovery more feasible and effective.

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