Abstract

PROLACTIN FUNCTION IN ZEBRAFISH DEVELOPMENT

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Extensive studies have implicated a myriad of functional roles for prolactin (PRL) and prolactin receptor (PRLR) across a variety of adult vertebrate species. However, much less is known about the physiological role(s) of PRL during embryonic/fetal development. Previously, we demonstrated that *in vivo* knockdown of PRL produced embryos with multiple morphological abnormalities. In this study, we explored the function of the PRLR family during embryogenesis and established the zebrafish as a useful model organism to examine embryonic functions of PRL. The combined results (1) define a role for PRL during early embryonic development, (2) provide plausible explanation(s) for the observed phenotypes in PRL-knockdown embryos, and (3) provide a foundation for the direction of future research using zebrafish as a model for studying physiological roles and molecular mechanisms of the PRL superfamily in vertebrates.

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AO	acridine orange
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA to RNA
CNS	central nervous system
ddH_2O	double distilled water
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dpf	days post-fertilization
DTT	dithiothreitol
ECD	extracellular domain
ECM	extraceullar matrix
EDTA	ethylene diaminetetraacetic acid
EST	expressed sequence tag
EtOH	ethanol
GFP	green fluorescent protein
GH	growth hormone
GHR	growth hormone receptor
H_2O	water
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
HEPES	hydroxyethlpiperazine ethansulfonate
hpf	hours post-fertilization
ICD	intra-cellular domain
kb	kilo base pair
KCl	potassium chloride
LB	Luria-Bertani medium
MBT	Mid blastula transition
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MO	antisense oligonucleotide morpholino
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
NBT	nitroblue tetrazolium
NCBI	National Centre for Biotechnology Information
nPrlrα	non-functional prolactin receptor alpha
O.C.T	Optimal cutting temperature
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline

PBST	phosphate-buffered saline with 1% Tween-20
PCR	polymerase chain reaction
PFA	paraformaldehyde
PRL	prolactin
PRLRα	prolactin receptor alpha
PRLRβ	prolactin receptor beta
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rpm	revolution per minute
RT	room temperature
RT-PCR	reverse transcriptase mediated polymerase chain reaction
SDS	sodium dodecylsulfate
SL	somatolactin
SLα	somatolactin alpha
SLβ	somatolactin beta
SLR	somatolactin receptor
SSCT	sodium chloride-trisodium citrate solution, Tween-20
TD	transmembrane domain
TEMED	N,N,N',N'-tetramethylethylene –diamine
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end-labeling
WISH	whole-mount *in situ* hybridization
WS	motif containing (Tpr-Ser-X-Trp-Ser)
ZFIN	zebrafish information network

LIST OF PUBLICATIONS

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CHAPTER 1: PROLACTIN-DEPENDENT MODULATION OF ORGANOGENESIS IN THE VERTEBRATE: RECENT DISCOVERIES IN THE ZEBRAFISH

Chapter Summary

The scientific literature is replete with evidence of the multifarious functions of the prolactin (PRL)/growth hormone (GH) superfamily in adult vertebrates. However, little information is available on the roles of PRL and related hormones prior to the adult stage of development. A limited number of studies suggest that GH functions to stimulate glucose transport and protein synthesis in mouse blastocysts and may be involved during mammalian embryogenesis. In contrast, the evidence for a role of PRL during vertebrate embryogenesis is limited and controversial. Genes encoding PRL/GH hormones and their respective receptors are actively transcribed and translated in various animals at different time points, particularly during tissue remodeling. We have addressed the potential function of PRL/GH hormones during embryonic development in zebrafish by the temporary inhibition of *in vivo* PRL translation. This treatment caused multiple morphological defects consistent with a role of PRL in embryonic-stage organogenesis. The affected organs and tissues are known targets of PRL activity in fish and homologous structures in mammalian species. Traditionally, the PRL/GH hormones are viewed as classical endocrine hormones, mediating functions through the circulatory system. More recent evidence points to cytokine-like actions of these hormones through either an autocrine or a paracrine mechanism. In some situations they could mimic the actions of developmentally regulated genes, as suggested by experiments in mammals and fish. In this chapter, we present similarities and disparities between zebrafish and mammalian models in relation to PRL and PRLR activity. We conclude that the zebrafish could serve as a suitable alternative to the rodent model to study PRL functions in development.

Zebrafish as a Model Organism

In recent years, the zebrafish (*Danio rerio*), has become the preferred vertebrate model for the study of early embryonic development. It has become clear that the rapid, optically clear, and external development of zebrafish embryos allows direct *in vivo* observation of many morphogenetic processes associated with early embryogenesis. Daily spawning of sexually mature zebrafish provides an abundant source of materials for experimental manipulations. At the molecular level, resources in genome sequencing, physical mapping of genes, gene expression profiling, and transgenic line development are continuing to advance, thus making genetic analysis easier and faster. The physical characteristics of the zebrafish embryo along with the available genetic resources has fostered the use of zebrafish for large scale mutagenesis, the modeling of human disease processes (Lieschke and Currie, 2007), drug discovery (Zon and Peterson, 2005), and environmental bio-monitoring (Alestrom et al., 2006).

The use of zebrafish has also been extended to the field of endocrinology (McGonnell and Fowkes, 2006). Extensive knowledge has been obtained on the mode of pituitary morphogenesis, patterning and the spatial order of pituitary cell differentiation in zebrafish (Herzog et al., 2003). Moreover, it is easier to isolate and study specific pituitary cell types because, unlike in mammals, pituitary hormones are expressed in defined regions in fish (Herzog et al., 2004). The zebrafish provides an advantage for the current study due to its external development and a reduced number of genes in the PRL/GH family. This eliminates continued maternal contribution through the placenta and the potential effects of large number of placental lactogens, which are unavoidable in mammalian models. Furthermore, cognate receptors for pituitary hormones show greater specificity in fish than mammals; reducing redundancy of function that potentially obscures the true functions of PRL. The advantages afforded by the

zebrafish have led us to use the zebrafish to explore the potential biological function of PRL in relation to developmental endocrinology.

Introduction

The prolactin (PRL) and growth hormone (GH) gene family regulate a variety of physiological processes, including growth and reproduction in juvenile and adult vertebrates (Ormandy et al., 1997; Zhou et al., 1997; Kopchick and Laron, 1999). The role of the PRL/GH gene family in embryogenesis, on the other hand, is controversial due to an absence of developmental defects in either hormone or cognate receptor knockout mice models (Horseman et al., 1997; Ormandy et al., 1997). Nonetheless, the presence of these hormones prior to the onset of pituitary gland ontogenesis and establishment of the circulatory system, in fish and mammals, implies that these hormones may act as local signaling factors, being involved in mechanisms that mediate the actions of developmental regulatory genes (Power and Canario, 1992; Ayson et al., 1994; Yang et al., 1999; Santos et al., 2003). These hormones and their receptors are likely initially maternally-derived and are maintained, fluctuate, or are expressed in specific organs throughout development (de Jesus and Hirano, 1992; Power et al., 2001). In addition, recent knockdown of the PRL/GH family demonstrate their involvement during zebrafish embryogensis (Zhu et al., 2007). In this chapter, we discuss the functions of the PRL/GH superfamily with emphasis on PRL during early embryonic development. For detailed information regarding PRL functions in adults, please refer to recent reviews (Bole-Feysot et al., 1998; Ben-Jonathan et al., 2008). From the perspective of zebrafish as an alternative to mammalian models, we present key similarities between the two models in their PRL's or PRL receptor's (PRLR) structure, function, expression, and signaling to define potential roles of PRL during embryogenesis

Prolactin (PRL)

Prolactin (PRL) is a multifaceted hormone that is involved in the modulation of a wide spectrum of physiological processes (Bole-Feysot et al., 1998; Ben-Jonathan et al., 2008). Based on sequence, structure, binding, and functional conservation, PRL is related to other pituitary-derived hormones, including growth hormone (GH), placental lactogen (PL) of mammalian placenta origin, and two forms of somatolactins (SLs) in teleosts, all of which belong to the PRL/GH superfamily (Niall et al., 1971; Nicoll et al., 1986; Wallis, 2000; Freeman et al., 2000; Zhu et al., 2004). The existence of PRL has been documented in every vertebrate examined, including fish, reptiles, and mammals. The primary structure of PRL is highly conserved within a given class, but sequences from distantly related species show a high degree of divergence (Sinha, 1995; Bole-Feysot et al., 1998). As an example of their conservation, four conserved PRL domains that are believed to be necessary for binding to specific PRLR and play indispensable roles in the expression of PRL-specific activities are present in both fish and mammalian species (Watahiki et al., 1989). Moreover, the secondary structures of the PRLs from fish and mammalian species possess two conserved disulfide bridges in the mid- and C-terminal regions. These conserved disulfide bridges have also been suggested to be critical for specific binding to PRLRs and may be important for PRL-specific activities (Sinha, 1995). Although fish lack the typical N-terminal disulphide bridge found in mammalian PRL, this may simply reflect fundamentally divergent functions between mammals and fish as exemplified by the role of PRL in mammalian lactation or osmoregulation in teleosts. Interestingly, mammalian growth hormone (GH) like the teleost PRL possesses only two disulphide bonds with a four helix bundle motif similar to fish PRL (Nicoll et al., 1986; Goffin et al., 1996). Similarities between the structure and sequence of PRL and GH are hypothesized to confer overlapping functions between these

hormones, which may arise as a consequence of the capacity of GH to bind to PRLR (Somers et al., 1994). By comparison to these generally conserved features, mammalian species possess extensive structural heterogeneity in PRL resulting from alternative splicing of the primary transcript, proteolytic cleavage, and other post-translational modification (Smith and Norman, 1990; Walker, 1994; Sinha, 1995). Structural modifications in the PRL molecule are partly responsible for its functional heterogeneity, providing both unique functions (i.e., 16kDa form of PRL induces anti-angiogenic properties) and regulated PRL activities (i.e., dimerization, polymerization, and glycosylation decreased biological activity of PRL probably resulting from changes in conformation) (Freeman et al., 2000). In fish, on the other hand, few PRL isoforms have been documented. One notable exception is the presence of two distinct PRL isoforms in Nile tilapia and Mozambique tilapia (Specker et al., 1985; Yamaguchi et al., 1988; Rentier-Delrue et al., 1989). One PRL of 188 amino acids long and the other PRL of 177 amino acids long share 70% amino acid similarity in the two tilapia species. Based on a comparison of synonymous/nonsynonymous substitutions between the conserved amino acids in the coding sequences of these two PRL paralogs, it was determined that positive selection followed the gene duplication event that generated these PRL paralogs. This duplication may have been related to the unique maternal care behavior characteristic of these cichlid species (Summers and Zhu, 2008). By comparison, two PRLs found in chum salmon (Yasuda et al., 1986; Kuwana et al., 1988; Song et al., 1988), chinook salmon (Xiong et al., 1992), common carp (Yasuda et al., 1987), and eel (Suzuki et al., 1991) were highly conserved (>95%). These genetic variants in salmon, eel, and carp differ only by a number of substitutions or deletions in amino acids (1-11aa) and their biological significance has not been investigated. In contrast, a novel PRL (named as PRL2 compared to the classical PRL, PRL1) was recently identified in a teleost as

well as non-mammalian vertebrates and possesses low sequence identity with PRL, but is capable of activating prolactin receptor alpha (PRLRα) (Huang et al., 2009). The retention of PRL2 in non-mammalian vertebrates but not in mammals was hypothesized to have occurred as a result of divergent functions that do not overlap with PRL1 (Huang et al., 2009). Further research should aim to provide an understanding of whether teleost PRLs exhibit a degree of genetic heterogeneity similar to their mammalian orthologs and to examine the physiological significance of the newly identified divergent form of PRL2 in relation to PRL1.

Traditionally, production and secretion of PRL in the anterior pituitary was considered the most important and physiologically relevant property of this hormone. However, ample evidence now exists for extra-pituitary production of PRL (Ben-Jonathan et al., 1996 for review), which suggests that PRL may have non-canonical functions. Precursor cells from the zebrafish pituitary gland begin to migrate from the anterior neural ridge at 10-12 hours post-fertilization (hpf) and PRL precursor cells initiate terminal differentiation at 22 hpf (Herzog et al., 2003; Pogoda and Hammerschmidt, 2007), which suggests that PRL may be active during this stage of embryogenesis. The development of the pituitary gland in zebrafish is not complete until 72 hpf when all cells of the anterior pituitary have completed terminal differentiation (Herzog et al., 2003). Using sensitive reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qRT-PCR), we showed the presence of PRL transcripts in zygotic cells at the earliest stages of embryogenesis and much earlier than the appearance of the pituitary precursor cells (Fig. 1-1). Importantly, the circulatory system of the zebrafish does not begin to be established until 24-26 hpf (Isogai et al., 2001), which is indicative that these hormones, while present, cannot function in a canonical endocrine manner; but instead may act as short-range diffusible signals in an autocrine or paracrine fashion at least during this early stage of

embryogenesis (Fig. 1-2). It is worthwhile considering that post-zygotic embryos are physically quite small and that molecules capable of diffusing over distances of a few hundred microns can exert their effects over relatively broad domains. In support of this idea, the recently identified PRL2 in zebrafish was demonstrated to be expressed at 4 hpf and locally within the eye, brain, and kidney (Huang et al., 2009).

Extra-pituitary expression of PRL is not unique to zebrafish nor is the idea of endocrine hormones acting through an autocrine or paracrine mechanism (Ben-Jonathan et al., 1996; Ben-Jonathan et al., 2008). Using *in situ* hybridization, immunohistochemistry, and the sensitive detection technique of RT-PCR, PRL has been detected in a variety of tissues (Bole-Feysot et al., 1998). The distribution of PRL ranges from specific tissues or cells that synthesize PRL to fluid compartments that contain PRL. Some sources of PRL include the mammary gland, myometrium, neurons, myocytes, amniotic fluid, and bone marrow (Bole-Feysot et al., 1998). Furthermore, evidence has shown the presence of transcripts and proteins for the PRL/GH gene family prior to the ontogenesis of a functional pituitary in diverse vertebrates, including: zebrafish (Herzog et al., 2003; Sbrogna et al., 2003; Liu et al., 2006), seabream (Santos et al., 2003), rainbow trout (Yang et al., 1999), chicken (Harvey et al., 2000), mouse (Pantaleon et al., 1997), rat (Garcia-Aragon et al., 1992; Zhang et al., 1997), cow (Joudrey et al., 2003), and human (D'Alfonso et al, 1992; Freemark et al., 1997). The importance of extra-pituitary PRL was first suggested by Nagy and Berczi, who showed that hypophysectomized rats required residual PRL for survival (Nagy and Berczi, 1978, 1991) as demonstrated by the observation that immunoneutralization of PRL decreased lactogenic activity and ultimately resulted in death. Further studies are required to better understand the physiological relevance of extra-pituitary expression of PRL and the non-classical actions of these endocrine hormones. It has been known

for some time that extra-pituitary PRL in the amniotic fluid or transferred from the deciduas (Riddick and Daly, 1982) provides a rich source of PRL important for the developing fetus. More recently, local production of PRL in the mammary gland has been implicated to induce mammary tumor growth through either an autocrine or a paracrine mechanism (Wennbo and Tornell, 2000; Fig. 1-2).

Prolactin Receptor (PRLR)

It is generally accepted that classical PRL action is mediated through a membrane-bound receptor (PRLR), whether the action is endocrine, paracrine, or autocrine. The initial step in PRL signaling requires hormone binding, which induces PRLR homodimerization and produces an active trimeric complex of one ligand and two receptor molecules (Goffin and Kelly, 1997). The same mechanism was demonstrated for trout PRLR (Le Rouzic et al., 2001), indicating that the ligand-receptor mechanism of PRLR activation is conserved in fish. Improper or defective formation of the trimeric complex has been shown to be detrimental to PRLR signaling (Bazan, 1990; Goffin and Kelly, 1997).

The PRLR is divided into an extracellular domain (ECD), transmembrane domain (TD), and intracellular domain (ICD). The PRLR ECD is composed of two pairs of disulfide bonds and a WS motif (Tpr-Ser-X-Trp-Ser), both of which are important for correct folding and cellular trafficking (Rozakis-Adcock and Kelly, 1991; Miyazaki et al., 1991) and ligand-receptor interactions (Rozakis-Adcock and Kelly, 1992; Baumgartner et al., 1994). Unlike the ECD, the ICD is less conserved but contains a region known as Box I (proline-rich motif that is membrane proximal) that is essential for the constitutive association of JAK, an upstream kinase of PRLR (Lebrun et al., 1995). Different forms of the PRLR (long, intermediate, and short) have been suggested to result from differential transcription at alternative initiation sites and from

alternative splicing of non-coding and coding exon transcripts (Hu et al., 1991, 1996). Despite differences in the overall length of each PRLR isoform, each variant is composed of an identical extracellular domain (ECD) (Postel-Vinay et al., 1991; Bole-Feysot et al., 1998). So far only one PRLR, including isoforms with identical ECD but varying ICD, has been reported in vertebrates (Fukada et al., 2005).

Extensive studies have demonstrated the wide distribution of the classical PRLR in both fetal and adult vertebrates. The PRLR is expressed in a wide variety of fetal tissues in mammalian species (Freemark et al., 1993, 1995). PRLR transcripts and proteins were detected in almost all tissues including classic lactogenic tissues (liver, adrenal, pancreas, thymus, lung, intestine, and kidney) and non-lactogenic tissues (ganglia, cochlea, adipose tissue, whisker follicles, facial cartilage, and olfactory epithelium) in mammals (Freemark et al., 1997). In fact, it is nearly impossible to find a tissue that does not express PRLR in mammals (Bole-Feysot et al., 1998). The situation in zebrafish appears to reflect that of the mammalian models. Both PRLRα and PRLRβ transcripts are expressed throughout zebrafish development, from the zygotic up to the adult stage (Fig. 1-1; see Chapter 2.1). Moreover, embryonic expression of PRLRα was detected using whole mount *in situ* hybridization in the primordial adenohypohysis at the anterior neural ridge, pancreas, and pronephric tubules at 24 hpf (Liu et al., 2006), consistent with previous findings indicating roles of PRL in murine pancreas and kidney development (Bole-Feysot et al., 1998; Freemark et al., 2002). It remains to be determined if PRLRs are expressed in other tissues throughout zebrafish embryogenesis in addition to those characterized so far.

Downstream Molecules and Pathways for PRL and PRLR Signaling

The PRLRs are associated characteristically with cytoplasmic kinases, termed Janus kinases (JAKs) (Campbell et al., 1994; Ihle et al., 1994; Lebrun et al., 1995). PRLR dimerization resulting from PRL binding causes the activation of the associated JAK by auto-phosphorylation. The phosphorylation of JAK leads to phosphorylation of PRLR and several other downstream signaling molecules. The major downstream signaling molecules of JAK are a series of signal transducers and activators of transcription (STAT) molecules that serve as transcription factors to promote cell survival, differentiation, and proliferation during zebrafish embryogenesis (Hou et al., 2002). In zebrafish, three JAKs (JAK1, JAK2a, and JAK2b) and four STATs (STAT1, STAT3, STAT5.1, and STAT5.2) have been identified (Conway et al., 1997; Oates et al., 1999a, b; Yamashita et al., 2002; Lewis et al., 2004). Zebrafish JAKs and STATs have a relatively high degree of amino acid sequence similarity to each other and to their mammalian counterparts, with conservation ranging from about 65% to nearly 90% (Conway et al., 1997; Oates et al., 1999a, b; Yamashita et al., 2002; Lewis et al., 2004).

Distinct from its role in the JAK/STAT signal transduction pathway, PRLR signaling can also function through PI3K, the Src family of kinases (SFK; c-Src and Fyn), and other pathways (1. PI3K: Al-Sakkaf et al., 1997; Berlanga et al., 1997; 2. Src: Berlanga et al., 1995; 3. Fyn: Al-Sakkaf et al., 1997; Clevenger and Medaglia, 1994; 4. MAPK: Buckley et al., 1994; Piccoletti et al., 1994; Das and Vonderhaar, 1997; Nohara et al., 1997; 5. IRS: Berlanga et al., 1997, Yamauchi et al., 1998; or novel pathways). However, all of the PRL signaling studies have been performed in mammalian cell lines and the precise nature of the PRLR signaling pathway (JAK/STAT, PI3K/AKT, Src/AKT and/or MEK/ERK) has not been demonstrated in any vertebrate embryo *in vivo*. It is essential to define the PRLR signaling pathways in embryonic

development *in vivo*, especially in major target organs. Most likely, many of the mechanisms mediated in mammals by PRLR are also conserved in zebrafish. The reasons for the likely conservation are: (1) activation of PRLR requires a trimeric complex (Bazan, 1990; Goffin and Kelly, 1997; Le Rouzic et al., 2001), (2) many of the components of the PRLR signaling pathways found in zebrafish have relatively high sequence relatedness with mammalian genes (Conway et al., 1997; Oates et al., 1999a, b; Chan et al., 2002), (3) many of the antibodies specific for mammalian molecules involved in JAK/STAT, Src/AKT, PI3K/AKT, and MAPK/Erk pathways have been successfully used in zebrafish studies (Conway et al., 1997; Wu and Kinsey, 2000; Yamashita et al., 2002; Cha et al., 2006), and (4) inhibitors for these pathways have also been shown to inhibit the same pathways in zebrafish (Montero et al., 2003; Hong et al., 2006).

Embryonic Function of PRL and Growth Hormone (GH)

The functions of PRL can be organized into six categories: 1) water and electrolyte balance, 2) growth and development, 3) endocrine and metabolic regulation, 4) brain function and behavior changes, 5) reproduction, and 6) immunoregulation and protection (Bole-Feysot et al., 1998); many of these functions are shared in all vertebrates. The properties or characteristics that define a function for PRL during embryonic development in any of these categories require the active secretion of PRL and a functional PRLR. It is now generally accepted that PRL is expressed in a wide range of tissues in diverse vertebrate species during early development. In addition, PRLRs are also found during embryogenesis and have been detected in nearly all tissues examined. Intuitively, the presence of hormone and receptor localized in the same tissues/cells of embryos and adults would be expected to function in a similar manner provided that the other components of the pathway required for activity are present. Alternatively, sites of

receptor expression in embryos may have unique functions during development that are independent of their functions in adults. Nonetheless, the presence of both hormone and receptor during embryogenesis strongly suggests functional roles for PRL at a stage prior to the development of a functional pituitary gland. These functions could be mediated either through autocrine, paracrine or even classical endocrine mechanisms provided that the appropriate biological structures or targets are present. While there is evidence that the PRL/GH gene family members and their respective receptors are transcribed and translated in early development, much less is known about the roles of the PRL/GH family in embryonic, larval, or juvenile stages of development. A limited number of studies suggest that GH functions to stimulate glucose transport and protein synthesis in mouse blastocysts (Pantaleon et al., 1997) and that it is involved in various stages in mammalian development (Waters and Kaye, 2002; Markham and Kaye, 2003). Despite these studies, the evidence for a role of PRL during vertebrate development is limited. One explanation for the paucity of information concerning a role of PRL during development involves a dominant paradigm for PRL that has emerged in endocrinology based on the absence of developmental defects in anencephalic fetuses, decapitated mammalian fetuses, hypophysectomized fetuses, fetuses treated with dopamine D2 receptor agonists (repressor of PRL secretion), mutant dwarf mouse strains lacking pituitary lactotrophs, and PRL knockout mice. In all these cases, the absence of gross developmental defects has been interpreted to mean that PRL and the PRL/GH family of hormones do not play a role in embryogenesis (Zhou et al., 1997; Goffin et al., 1999; Kelly et al., 2001).

However, each of these methods has flaws that compromise the interpretation of the results and leave open the possibility that PRL and its related peptides play a role in embryogenesis. The absence of an observed phenotypic effect related to treatments that purport

to inactivate PRL have arisen from various sources, including: 1) the inability to inhibit completely extra-pituitary expression of the pituitary hormones in anencephalic models; 2) the maternal expression of many members of the mammalian PRL/GH gene family at the placental uterine interface (often with overlapping functional roles), such that maternally derived hormone masks the defects induced by physical or pharmacological treatments; 3) cross-talk between multiple ligands and their common receptors; both PL and PRL binds to the PRLR with high affinity (Golos et al., 1993; Freemark et al., 1996; Soares et al., 1998; Herman et al., 2000; Biener et al., 2003; Li et al., 2005); and 4) genetic and biochemical redundancy of PRL and PRL-related hormone effects that have the potential to mask deficiencies in individual genes.

In the following sections, we focus the discussion on potential roles of PRL/GH in early development of representative model organisms.

Mammals

In humans, fetal development consists of early, mid-, and late gestational periods that are characterized by rapid transformations in tissue composition, cellular organization, and biological functions. Maternal serum levels for PRL and PL begin to increase around 10 weeks of gestation and peak near term (Freemark et al., 1999). Similarly, fetal levels for PRL increase at 10 weeks and maintain relatively constant levels until the third trimester where PRL increases dramatically and peaks at term (Freemark et al., 1999; Ben-Jonathan et al., 2008). By comparison, PRL levels in amniotic fluids are 10- to 50-fold higher than those in the maternal or fetal blood and peak at 20-24 weeks of gestation (Ben-Jonathan et al., 1996, 2008). Extremely high levels of PRL in amniotic fluids beginning at early gestation, a period of rapid tissue differentiation and organ development, implicate a role for PRL in organogenesis. Studies on the ontogenesis of the PRLR in human fetuses indicate diverse tissue expression by 7.5 weeks of

gestation (Freemark, 2001). Many of the PRLR immunoreactive sites include derivatives of embryonic mesoderm, the periadrenal and pronephric mesenchyme, the pulmonary and duodenal mesenchyme, the cardiac and skeletal myocytes, and the mesenchymal precartilage and maturing chondrocytes (Freemark et al., 1997). Interestingly, many of these tissues exhibited changes in cellular distribution and the magnitude of PRLR expression throughout development (Freemark et al., 1997). In the fetal bone, adrenal gland, and lung, the receptor is expressed initially in mesenchymal cells and subsequently in maturing chondrocytes, adrenocortical cells, and bronchiolar epithelial cells. In the central nervous system, the PRLR is first detected in the periventricular neuroepithelium and later in mature neurons of the hypothalamus and olfactory bulb. In the pancreas, the PRLR is detected first in the exocrine tissue and ductal epithelium; subsequently, PRLR is predominantly expressed in the pancreatic β-cells in the islet of Langerhans. Changes in PRLR expression in different cell types or regions within the same tissue during development imply developmentally dependent changes in lactogenic functions (PRL or PL actions mediated through the PRLR). Cellular distribution of PRLR expression at mid-gestation is localized in acinar tissue and ductal epithelial cells, suggesting a role for lactogens in the growth and function of the exocrine pancreas (Freemark et al., 1997). However, during late gestation and in postnatal life, PRLR is expressed preferentially in islet cells, consistent with the insulin tropic effects of lactogenic hormones in pancreatic islets of adult humans (Brelje et al., 1993; Sekine et al., 1996; and Weinhaus et al., 1996). Similarly, initial expression of PRLRs in surface mesenchymal cells and neocortical cells suggests a role of lactogens in adrenocortical maturation or growth during fetal development (Freemark et al., 1997); while subsequent expression of PRLRs in differentiated adrenocortical cells suggest a role

in modulating the production of fetal adrenal androgens and glucocorticoids (Ogle et al., 1979; Eldridge et al., 1984; Pepe and Albrecht, 1990; Glasow et al., 1996).

The tissue distribution of PRLR in rodents appears to mirror those observed in human fetuses. PRLR has also been detected in a number of fetal tissues, both in rat and mouse, including derivatives from all three germ layers, although the absolute levels may differ among tissues (Royster et al., 1995; Freemark et al., 1996; Freemark et al., 1997; Tzeng and Linzer, 1997). Rodent PRLR is strictly lactogenic and typically interacts with PRL, PL-I, or PL-II (Ogren and Talamantes, 1988), which is different than human PRLR that binds prolactin as well as growth hormone. The predominant lactogen-mediating PRLR function in rodents during the first half of pregnancy is the daily surges of PRL, while mid- to late gestational stages involve PL as both maternal and fetal PRLs are suppressed (Reusens et al., 1979; Slabaugh et al., 1982; Khorram et al., 1984; Soares et al., 1991; Soares et al., 2004). In fetal rats, the functions of PRLR that mediate tissue differentiation and/or organ development and function correlate closely with its observed effects in humans (Freemark et al., 1997). The fetal mouse shows similar tissue distribution of PRLR and also suggested potential differences in functional roles of PRLR between the fetus and postnatal animals in specific tissues (Tzeng and Linzer, 1997). The results from mouse PRLR knockouts are difficult to reconcile in the context of information about levels of PRL, PRL-related hormones and functional PRLR activities in humans, mice and rats. Results from mouse PRLR knockouts failed to demonstrate fetal lethality or gross morphological defects (Ormandy et al., 1997) but removal of low molecular weight molecules (including PRL and PL) in serum used to incubate rat fetuses significantly reduced embryonic growth and development, which were partially restored by supplementation with either PL or PRL (Karabulut and Pratten, 1998; Karabulut et al., 1999; 2001). While it is difficult to compare the results from knockouts

with small molecule depletion experiments, the restoration of growth and development by PL and PRL supplementation argues for a role of PL and PRL in mouse embryogenesis. The mechanism of PRL and PL-dependent growth promotion has been suggested to involve insulin-like growth factors (IGFs) (Karabulut et al, 1999; 2001), which is essential for fetal survival in rodents (Powell-Braxton et al., 1993). The effects of PL, PRL and PRLR and their interactions with other growth factors will require a detailed analysis of the distribution and activities in each tissue of the PRLR-deficient mouse and would benefit from an examination of their characteristics in an alternative model organism like the zebrafish.

Information concerning the activity of GH in mammalian development is also very limited. GH transcripts or proteins were detected in the rat from ED 12 (Garcia-Aragon et al., 1992; Zhang et al., 1997), in human at 9-16 weeks (D'Alfonso et al., 1992); in mouse at the blastula stage (Pantaleon et al., 1997); and in the cow at the 2-4 cell stage (Joudrey et al., 2003). It is unclear at this time whether the significant discrepancies in stage of embryogenesis at which GH transcripts or products were first identified is the result of improved sensitivity in the detection methods employed or whether they represent authentic species-specific differences in the timing of initial expression. GHR transcripts or proteins were also detected during the early cleavage stage of mouse embryogenesis (Pantaleon et al., 1997; Terada et al., 1996), and in cows from ED 2 onwards (Izadyar et al., 2000; Kolle et al., 2001). These GHRs continued to be expressed in these two species even at later stages of development (Garcia-Aragon et al., 1992; Hill et al., 1992; Scott et al., 1992; Ymer and Herington, 1992; and Werther et al., 1993). The presence of these functional hormones and receptors has been demonstrated to act directly on the early embryo (Waters and Kaye, 2002) by improving the reliability of cleavage and blastocyst formation in the cow (Izadyar et al., 1998) and mouse (Fukaya et al., 1998). One physiological

role of GH at the blastocyst stage may be involved in the stimulation of glucose transport and protein synthesis in the blastocyst (Pantaleon et al., 1997), which suggests that GH activity at this stage of embryonic development, is probably important for successful implantation.

Aves

Historically, the chicken has served as an important model for studies of development because of the size of the embryos and the ease with which they can be manipulated *in ovo* and it is one of a handful of model organisms in which the PRL/GH gene families have been shown to play a critical role during embryogenesis. In chick embryos, GH protein was initially detected at embryonic day (ED) 1-2 (Wang, 1989) while the transcripts were first observed as early as ED 2 (Harvey et al., 2000). Subsequently, GH immunoreactive cells were detected in a host of tissues and in specific cell populations between ED 3-5, particularly within the neural tube, notochord, somites, limb buds, heart, liver, mesonephros, Wolffian duct, and amnion (Harvey et al., 2000). At later stages of embryonic development, between ED 7-8, GH expression was observed to be more restricted and was detected in the chondrocytes of the limb buds, the brain, and the neural retina (Murphy and Harvey, 2001; Harvey et al., 2001, 2004). Interestingly, GH in the embryonic neural retina and the vitreous chamber was shown to be associated primarily with 15-16 kDa proteins although the typically monomeric 22-26 kDa GH was also present in small amounts (Baudet et al., 2003; Sanders et al., 2003). A number of other protein structural variants gave rise to a spectrum of sizes from 15 kDa to >110 kDa during chick development but the functional roles for these various forms, similar to PRL isoforms, are not yet understood (Aramburo et al., 2000). Complementary to the hormones, GHR was also detected beginning at ED 3 and was present in most tissues and cells up to ED 8 in chick embryos (Harvey at al., 2000). Both

hormone and receptor were also present prior to the formation of the pituitary (ED 16) and circulatory system (ED 2-3) during chick development (Kansaku et al., 1994; Porter et al., 1995).

A number of studies have been conducted to understand the physiological roles of GH during development. Deletion of the GHR gene in chickens appeared to be related to dwarfism (Goddard et al., 1996), despite the observation that body weight at hatching was not significantly affected (Decuypere et al., 1991), which was indicative that the effect of GHR gene deletion had a latent effect on growth that may have occurred after hatching. The hypothesis was further supported by lack of hepatic IGF-I in GHR deleted chicken compared to abundant expression of IGF-I in wild-type chicken at 4-weeks after hatching (Tanaka et al., 1996). In addition, growth hormone has been implicated in a variety of embryonic functions, including growth, differentiation, neurogenesis, gliogenesis, adipogenesis, chondrogenesis, angiogenesis, cell proliferation, cell survival, and eye development (Sanders and Harvey, 2004). While results from GH and GHR suggest that these genes have diverse functions during embryogenesis, the absence of information on PRL activity in chickens precludes comparisons to other vertebrates and represents an important area for future comparative molecular physiological research.

Amphibians

In amphibians, PRL is widely known for its anti-metamorphic effects. Numerous *in vivo* (Clemons and Nicoll, 1977; Kikuyama et al., 1980; Eddy and Lipner, 1975) and tissue culture (Derby and Etkin, 1968; Tata et al., 1991) studies have demonstrated the ability of PRL to inhibit endogenous or exogenous thyroid hormone that stimulates tail re-absorption resulting from extensive cell apoptosis and the dismantling of largely collagen-based extracellular matrix. In a seemingly paradoxical series of results it has been demonstrated that PRL is able to stimulate collagen synthesis in tadpole tail fins (Yoshizato and Yasumasu, 1970) and to stimulate the

activity of two *Xenopus* collagenases, XCL3 and XCL4 (Jung et al., 2004). Further, the over-expression of PRL or a combination of PRL and PRLR in transgenic *Xenopus laevis* prevented tail re-absorption (Huang and Brown, 2000a). These seemingly opposing roles of PRL during amphibian development suggest a complex function of PRL involving multiple factors regulating metamorphosis and tissue remodeling. The preservation of the tail in tadpoles of PRL transgenic frogs is consistent with its ability to stimulate fibroblast growth factor to counter the activation of proteolytic enzymes by thyroid hormone during metamorphosis (Yoshizato and Yasumasu, 1970; Berry et al., 1998; Huang and Brown, 2000a). Thyroid hormone has been demonstrated to induce XCL3, XCL4, or other collagenases in the metamorphic tadpole tail when massive collagen degradation is occurring (Patterton et al., 1995; Stolow et al., 1996; Berry et al., 1998; Damjanovski et al., 2000; Jung et al., 2002). In addition to the expression of XCL3 and XCL4 during thyroid hormone-dependent metamorphosis, these collagenases have also been detected at early developmental stages during *Xenopus* embryogenesis (Stolow et al., 1996; Damjanovski et al., 2000). Both expression of PRL (Buckbinder and Brown, 1993) and PRLR (Yamamoto et al., 2000) coincide with collagenase expression *in vivo*, and have been suggested to regulate the thyroid hormone-independent phase of *Xenopus* organogenesis and metamorphosis (Jung et al., 2004). Studies in various mammalian cell lines have recently reported that PRL also stimulates collagenase activity and is likely to be mediated by PKC and PKA, which suggests the possibility that PRL is involved in tissue remodeling in mammals as well as in amphibians (Jung et al., 2004).

Unlike PRL, the primary activity of GH during amphibian development is to stimulate tadpole and frog growth rather than metamorphosis (Bern et al., 1967; Clemons and Nicoll, 1977). Although the expression pattern of GH (Buckbinder and Brown, 1993) during *Xenopus*

development would be consistent with a juvenilizing hormone that inhibits metamorphosis, recent establishment of transgenic frogs over-expressing *Xenopus* GH (xGH) clearly demonstrated no alteration in the developmental programs involved in metamorphosis (Huang and Brown, 2000b). These transgenic frogs grew at an accelerated rate with typical skeletal abnormalities reminiscent of acromegaly in mammalian models. Furthermore, over-expression of Xenopus PRL (xPRL) failed to affect their normal weight (Huang and Brown, 2000a). Transgenic frogs over-expressing ovine PRL (oPRL) did, however, increase the tadpole weight by 30-50% but it was due to the ability of oPRL, but not xPRL, to cross-react with and activate the xGHR (Huang and Brown, 2000b).

Teleosts

Fish provide particularly useful model organisms for investigating the functional roles of the PRL/GH superfamily during embryonic development. We have established the zebrafish as a model to study PRL functions during embryogenesis because of the various advantages over other fish species and mammalian models mentioned previously. In addition to these advantages, PRL and PRLR have been detected throughout embryogenesis in many species of teleosts, which suggests that the expression of these hormones and their receptors during early embryogenesis has a deep evolutionary history and that they likely play a functional role prior to their synthesis by the pituitary gland.

PRL has been detected in larval stages after hatching, particularly in the pituitary, in a number of teleost species, including sea bass (*Dicentrarchus labrax*), coho salmon (*Onchorynchus kisutch*), chum salmon, sea bream, ayu (*Plecoglossus altivelis*) and Japanese eels (*Anguilla japonica*) (Arakawa et al., 1992; Cambre et al., 1990; Leatherland and Lin, 1975; Naito et al., 1993; Power and Canario, 1992; Saga et al., 1999). PRL transcripts have also been

detected in rainbow trout (Yang et al., 1999), Mozambique tilapia (Ayson et al, 1994), seabream (Santos et al., 2003; Power, 2005), and zebrafish (Herzog et al., 2003) during early teleost development and as early as 22 hpf in zebrafish. Studies showed PRL proteins are expressed by 30 hpf using immunohistochemical methods (Sbrogna et al., 2003) and 18 hpf using a PRL promoter-driven green fluorescent protein in zebrafish (Liu et al., 2006). Interestingly, PRL in both seabream (Santos et al., 2003) and zebrafish are expressed during somitogenesis and levels gradually increase at gastrulation (Song et al., unpublished data). Seabream and Mozambique tilapia PRLR transcripts were present after fertilization and mRNA levels also increased at gastrulation, suggesting roles for PRL in subsequent organogenesis (Santos et al., 2003; Shiraishi et al., 1999). Although levels of PRLR during zebrafish embryogenesis do not appear to increase dramatically, likely due to analysis of whole embryos rather than specific tissues, both the classical PRLR and the recently identified PRLRβ transcripts are detected soon after fertilization and throughout embryogenesis (Fig. 1-1). Furthermore, immunocytochemistry of 1 dpf seabream embryos indicate PRLR immunoreactive cells are present in the developing brain, eye primordium, and olfactory lobe (Santos et al., 2003). The combined results from various teleosts that possess both PRL and PRLR during early development, show changes in expression patterns at certain developmental stages, and the expression of functional PRLR in multiple organ placodes strongly suggest functional roles for PRL in regulating various aspects of development in teleosts.

Using zebrafish and antisense morpholino (MO) gene knockdown, we have circumvented many of the obstacles present in mammalian models and provide data to suggest a functional role for PRL during embryogenesis. Morpholino knockdown of PRL (PRL-MO) yielded several significant phenotypes in larvae including the lack of a gas bladder, short body length, fewer

proliferating cells, and reduced head, brain ventricle, and eye sizes (Zhu et al., 2007; unpublished data). These morphological changes were dependent on the dosage of PRL-MO injected, with increasing concentrations of PRL-MO resulting in greater effects (Zhu et al., 2007). The morphological defects observed in multiple tissues/cells in PRL-knockdown (PRL-KD) embryos are not surprising as the wide distribution of PRLR contributes to the functional diversity of PRL. The specific effects of PRL-KD on the development of zebrafish embryos have been verified by rescue using *in vitro* transcribed *prl* mRNA (Zhu et al., 2007) and with two additional morpholinos targeted to different regions of the *prl* gene that resulted in similar phenotypic abnormalities (Zhu et al., 2007; unpublished observation).

These are only some of the phenotypes examined thus far. With the generation of PRLRα antibody, new sites of receptor expression have been identified (see Chapter 2.1). The abundant expression of PRLRα in the olfactory system, as recently determined, suggests either structural or functional defects in the olfactory system in these PRL-KD embryos. Detection of PRLR in osmoregulatory organs such as the kidneys, gills, and intestine in zebrafish is consistent with other fish species (Shiraishi et al., 1999; Santos et al., 2003; Liu et al., 2006); and PRL probably regulates osmoregulation as described in these fish (Power, 2005). Similarly, PRL effects on calcium balance, metamorphosis, and possibly immunoregulation suggested in other fish during early development is also expected in zebrafish (Power, 2005). Further studies on the morphological defects of other tissues/organs and the physiological consequences of PRL depletion in PRL-KD embryos require more detailed analysis of each phenotype. In addition, knockdown of PRLRα and PRLRβ should be complemented with other approaches such as dominant negative receptors and mutants to further verify the specificity of PRL functions during zebrafish embryogenesis.

Rationale and Objectives of the Study

Despite a wide spectrum of physiological functions of prolactin in adults and postnatal growth effects of growth hormone, the effects of the PRL/GH family in embryonic development and organogenesis remain controversial (Bole-Feysot et al., 1998). The results from PRL or GH knockout mouse models suggested non-essential functions of members of PRL/GH during early development. However, the redundancy of PRL/GH/PL genes, ligand binding capacities, and biochemical pathways complicated the investigation of the true functions of these hormones in embryonic development. In our previous studies, we used antisense oligonucleotides morpholino to disrupt targeted *in vivo* mRNA translation to investigate the involvement of PRL, GH, SLα, and SLβ in zebrafish development. We found that the members of the PRL/GH family, especially PRL, affect the normal development and growth of the gas bladder, head, body, and eyes (Zhu et al, 2007). Our research provided the first evidence that a PRL family member plays a role in vertebrate embryogenesis, and suggests that redundant effects of the PRL/GH family observed in mammalian models poses fewer complications to identify functions of these hormones in zebrafish development than comparable research in mammals. Nevertheless, the mechanisms of PRL leading to these morphological defects are unknown. Characterization of the entire cognate receptor set for the PRL/GH family that mediate the biological effects of these hormones and target tissues of PRLRs throughout zebrafish embryogenesis is lacking. Although it was demonstrated that PRLRα was expressed in the pancreas and kidney at 24 hpf (Liu et al., 2006), there is no illustration of their target sites prior to and after 24 hpf, the cell-specific expression of PRLRα within the pancreas, or the expression profile of PRLRβ. Analysis of tissue-specific expression of PRLRs would allow the differentiation between direct and indirect actions of PRL and possibly assign different functions of PRL that are likely dependent on the

receptor type-mediated signaling. Furthermore, the biological or developmental events leading to the morphological defects associated with PRL-KD have not been investigated. Identifying a biological process disrupted in PRL-KD embryos with a characterized function of PRL would strengthen support for the PRL morphant phenotypes. Lastly, using an alternative method to complement the morpholino technology would help distinguish potential non-specific or off-target effects often seen with MOs and would greatly help define PRL function(s) in zebrafish embryogenesis.

In the subsequent chapters, we establish zebrafish as a useful model to explore the role of PRL by 1.) identifying the members of the PRL/GH receptor family, 2.) demonstrating that PRL acts as an anti-apoptotic factor during embryogenesis, and 3.) establishing the first stable transgenic line expressing a non-functional prolactin receptor α (*nPrlrα*) within the pancreas to examine its role during pancreatic development.

Fig. 1-1. Reverse-transcriptase PCR analysis for expression of hormone and receptors of the PRL/GH superfamily throughout early zebrafish development. Primers were directed towards two different exons to eliminate amplification of genomic DNA. β-actin was used as a loading control. Abbreviations at the end of each row are as follows: cell (c), hour (h), day (d), ovary (ov), prolactin receptors (PRLRα, PRLRβ), growth hormone receptors (GHR-I, GHR-II), prolactin (PRL), growth hormone (GH), somatolactin alpha (SLα), and somatolactin beta (SLβ).

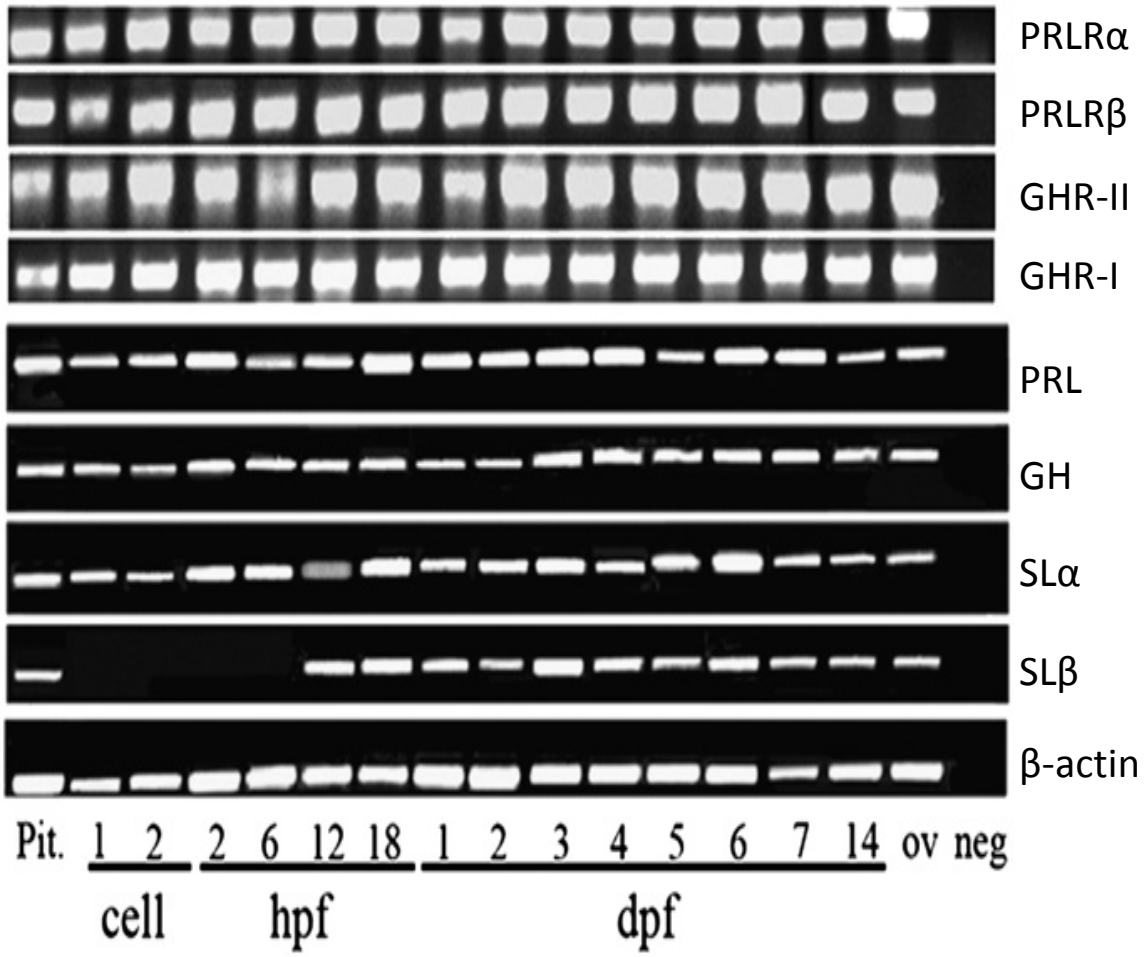
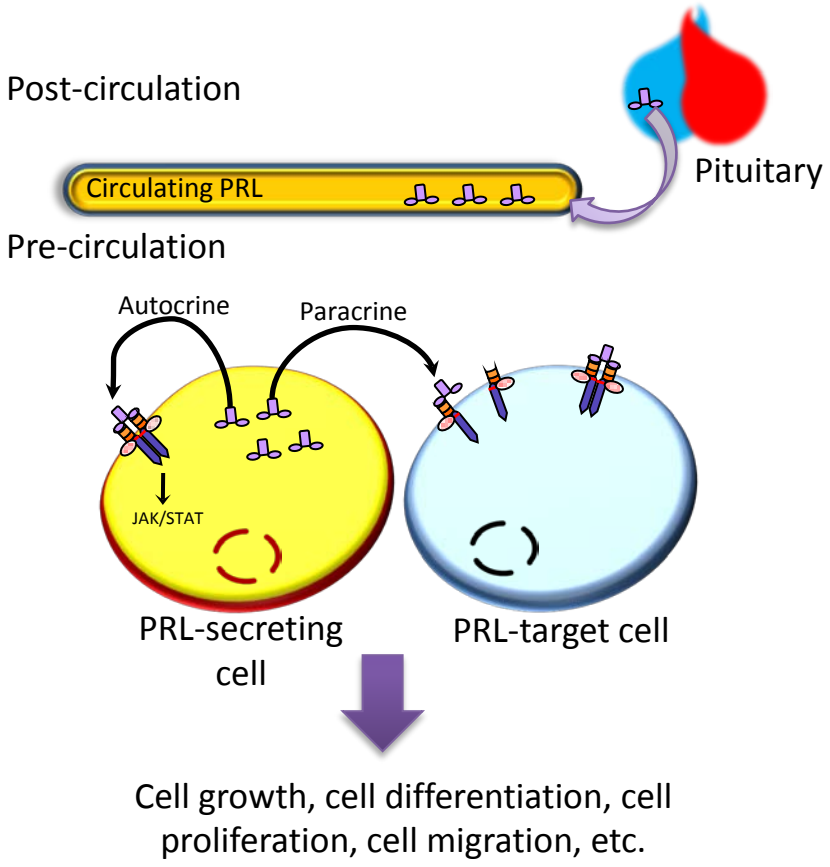


Fig. 1-2. Proposed mechanism of PRL actions during development. Two different populations of cells are illustrated by two different colors, representing cells capable of producing local PRL and those that are responsive to PRL (presence of PRLRs on the cell membrane). Prior to the development of the circulatory system, PRL functions through either an autocrine or paracrine mode of action. Establishment of the circulatory system by 24-26 hours post-fertilization (hpf) enables PRL to function through an endocrine mechanism by releasing PRL into the blood stream. Local production and central secretion of PRL by the pituitary gland may exhibit functions including but not limited to cell migration, survival, proliferation, and growth.



CHAPTER 2.1: CLONING, STRUCTURAL CHARACTERIZATION AND EXPRESSION OF THE PRL/GH RECEPTOR FAMILY

Chapter Summary

Functional roles for the pituitary-derived hormones prolactin (PRL), growth hormone (GH), and somatolactins (SLs) during early embryogenesis are limited and controversial. Pituitary hormones mediate specific functions through membrane bound receptors on target tissues, indicating the need to identify cognate receptors for these hormones in order to gain insight into the potential roles for the PRL/GH superfamily in development. In zebrafish, we identified two genes that closely resemble growth hormone receptors (GHR), namely GHR-I and GHR-II, and two distinct subtypes of prolactin receptors (PRLR; PRLRα and PRLRβ). It appears that GHR-I is more structurally similar to the characterized fish somatolactin receptors (SLRs) and GHR-II is likely the ortholog of human GHRs, which are more divergent in teleost species. The duplication of zebrafish GHRs and PRLRs seems to have arisen through the fish-specific whole genome duplication event. Quantitative real-time PCR demonstrated that all four receptors (GHR-I, GHR-II, PRLRα, and PRLRβ) were expressed throughout early zebrafish embryogenesis. *In situ* hybridization analyses of PRLRs illustrated both overlapping and unique expression patterns for the two different forms. PRLRα appears to have acquired a novel function in pancreas development while PRLRβ maintains the ancestral role in osmoregulation. Expression of PRLRs within the kidney and pancreas suggest that these two tissues are prime targets for PRL action during embryogenesis. Taken together, the existence of both hormones and receptors for the PRL/GH family suggests functional roles for pituitary hormones during zebrafish embryogenesis.

Introduction

The prolactin (PRL) and growth hormone (GH) superfamily, including the teleost-specific somatolactin alpha (SLα) and somatolactin beta (SLβ), regulate a diverse range of biological activities. GH regulates growth (Wood et al., 2005), PRL controls osmotic equilibrium (Sakamoto and McCormick, 2006), SLα regulates fat metabolism (Fukamachi et al., 2005), and SLβ has been proposed to control melanophore aggregation (Nguyen et al., 2006). The functions of these pituitary-derived hormones are mediated by initially binding to their cognate membrane bound receptors (growth hormone receptor, GHR; prolactin receptor, PRLR; and somatolactin receptor, SLR, respectively) that subsequently triggers a phosphorylation cascade leading to a multitude of signaling events.

The identities and structures of these hormone-specific receptors have been extensively studied. Since the initial discovery of GHR in humans (Leung et al., 1987), cDNA sequences for GHRs have been described for a variety of vertebrate species (Edens and Talamantes, 1998; Huang and Brown, 2000b; Calduch-Giner et al., 2001; Lee et al., 2001; Tse et al., 2003; Kajimura et al., 2004; Fukada et al., 2004; Very et al., 2005; Benedet et al. 2005). Orthologs of the human PRLR has also been identified in numerous species (Bole-Feysot et al., 1998). However, SLRs have only been cloned recently in a limited subset of teleost species: masu salmon (Fukada et al., 2005), Atlantic salmon (Benedet et al., 2008), Japanese medaka and Takifugu rubripes (Fukamachi et al., 2005). Due to a lack of studies comparing the binding affinities of a complete homologous set of hormone-receptor members and limited SLR sequences, classification of GHRs and SLRs remains controversial. Nonetheless, current information on the functional and structural similarities between these receptors places the PRLRs, GHRs, and SLRs among the class I cytokine receptor family (Huising et al., 2006).

We hypothesized that in parallel with the existence of PRL/GH family of hormones, their cognate receptors were also expressed during zebrafish embryogenesis. We further hypothesized that, the actions of these pituitary hormones would be mediated by their specific cognate receptors and would be expressed in specific target tissues important for hormone function. The objective of this study was to identify all the members of the PRL/GH receptor family, and examine the target tissues of each receptor during embryogenesis. In our experiments, we developed molecular tools for the quantitation of receptor levels (transcripts and protein). Investigating the differential distribution of these hormone receptors will provide a better understanding of target tissues and define potential roles of the PRL/GH family during early development and in adult zebrafish.

Methods and Materials

Fish Maintenance and Staging

Zebrafish, *Danio rerio*, were maintained according to standard protocols (Westerfield et al., 1993). Zebrafish were purchased from a local pet store and maintained at 28.5°C on a 14-hour light and 10-hour dark cycle. Embryos were staged in hours post-fertilization (hpf) and days post-fertilization (dpf) with reference to morphological features as previously described (Kimmel et al., 1995).

Cloning of the Zebrafish PRLRβ Gene

Zebrafish embryos at 24 hpf were collected and immediately placed in 1 ml TRIzol reagent (Gibco). Following homogenization by sonication, 200 µl of chloroform was added and the solution was vortexed vigorously. The mixture was centrifuged, and the aqueous layer was transferred to a clean RNase-free microcentrifuge tube. Total RNA was precipitated and redissolved in 100 µl of water. First-strand cDNA was synthesized from 1 µg total RNA using

the GeneRacer kit (Invitrogen), in accordance with the manufacturer's instructions. Nested PCR was performed using zfPRLR2nestedF2, 5'-CCGTTCCCTTTGCTGCTTTCTG-3', and zfPRLR2nested R2, 5'-ACCTGTGATTCTCCCATAAACCGC-3', designed from sequences similar to known zebrafish PRLRα identified from the zebrafish genome (Ensemble Zv 7). The first PCR was performed in 50 µl aliquots using a gradient Eppendorf Mastercycler with a 2 min denaturation at 94°C for 30 sec, annealing at 40-55°C for 30 sec and elongation at 72°C for 1 min. The product of the first PCR reaction was diluted 1:10 with sterilized deionized water and 1 µl was used as the template for the second round of PCR. Using the nested primers zfPRLR2gspF2, 5'-TCTTTGGTTCTGGAACTGGTGGCA-3', and zfPRLR2gspR2, 5'-TCTCTCATTGTGTCCTGGATCC-3', a 20 µl reaction was prepared. The reaction conditions for the second PCR were a 2 min denaturation at 94°C, the PCR cycle was repeated 25 times with the following conditions: 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min.

Gene-specific primers were designed from the partial PRLRβ sequences. 5' and 3' RACE were performed using the GeneRacer kit following the manufacturer's instructions, for both PRLRα and PRLRβ. The products were separated on a 2% agarose gel by electrophoresis at 150V for 15 min, ligated into the TOPO TA vector (Invitrogen) and sequenced with vector-specific primers using the Big Dye Terminator kit and ABI Prism DNA sequencer 377 (Perkin-Elmer, Willesleg, MA, USA). Sequence data were compiled using Sequence Navigator (ABI, Foster City, CA, USA). Gene-specific primers were designed from the compiled sequences and the purified full-length sequence of PRLRβ and PRLRα PCR products were subcloned into pGEM-T Easy vector (Promega, Madison, WI) using T4 DNA ligase (Promega, Madison, WI) with the reaction incubated overnight at 4°C.

Whole Mount *In Situ* Hybridization (WISH) and Sectioning

Whole mount *in situ* hybridization (WISH) using digoxigenin (DIG)-labeled riboprobes were carried out as previously described (Korzh et al., 1998). PRLRα and PRLRβ in the pGEM-T Easy vector were linearized with Not I and Sac II respectively, followed by *in vitro* transcription reaction with T7 or SP6 RNA polymerase (Ambion, TX, USA) for synthesis of the DIG-labeled anti-sense RNA probes. The embryos were fixed with 4% paraformaldehyde (PFA) for 24 hpf at room temperature (RT), hybridized with the DIG-labeled probe in hybridization buffer [50% formamide, 5X standard saline citrate (0.75 M NaCl, 0.075 M sodium citrate), 50 μg/ml heparin, 500 μg/ml yeast tRNA, and 0.1% Tween-20] at 68°C, followed by incubation with 1:2000 anti-DIG antibody conjugated with alkaline phosphatase at 4°C overnight. Hybridization of the probe was detected by incubating with NBT (nitroblue tetrazolium; 0.03%) and BCIP (5-bromo, 4-chloro, 3-indolyl phosphate; 0.02%) in 0.1 M TBS at pH 9.5 until desired color development occurred (30 min to 1 hr) at RT. For sectioning, the stained embryos were embedded in 1.5% bacto-agar and incubated in 30% sucrose at 4°C overnight. The embedded embryos were sectioned with a cryostat microtome (Microm HM 505E, Zeiss) in cross section orientation at 12 μm thickness and collected on polysine microscope slides (Thermo Scientific). Sections were fixed with 4% PFA in phosphate buffered saline (PBS) for 10 min, washed with PBS, and mounted in 1:1 PBS:glycerol under a glass cover slip and sealed with nail polish to prevent drying. Photographs were taken using a camera mounted to an Olympus AX-70 microscope (Olympus, Japan) using bright field illumination.

Reverse Transcriptase PCR and Quantitative Real-Time PCR (qRT-PCR)

Total RNA isolation was performed as described above for all tissues and larvae. First-strand cDNA synthesis was conducted as described previously, but gene-specific primers were

used to synthesize the templates for PCR (Table 2.1-1). PCR amplification of PRLRα cDNA from these samples were achieved with a 2 min denaturation at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 58°C and 1 min at 72°C; PRLRβ conditions were the same as PRLRα except with an annealing temperature of 54°C; for GHR-I, a 2 min denaturation at 94°C, then 35 cycles of 30 sec at 94°C, 30 sec at 63°C and 1 min at 72°C. GHR-II cDNA was amplified with a 2 min denaturation at 94°C, then 35 cycles of 30 sec at 94°C, 30 sec at 63°C and 1 min at 72°C. All conditions ended with a 10 min extension at 72°C and primers (Table 2.1-1) for each gene were produced on two different exons to prevent amplification of genomic DNA. Water was used in place of cDNA to serve as a negative control and β-actin was used as a positive and loading control.

For qRT-PCR, the same primers (Table 2.1-1) were used with shorter program times and slight changes to annealing temperature. Briefly, plasmid DNAs containing the full-length gene of interest were quantified by using a Pharmacia DNA/RNA calculator to set up the standard curve. Each plasmid was serially-diluted (10^3 to 10^9). SmartCycler software created a growth curve based on the amount of fluorescence detected at each cycle number. The critical threshold (Ct) was empirically determined and calculated from this growth curve. The Ct value and the Log concentrations of a serially-diluted plasmid standard were used to create a standard curve. The cDNA standards were measured by quantitative real-time polymerase chain reaction (qRT-PCR) with three independent experiments to check reproducibility, and to determine the linear range of the standard curve.

Candidate cDNAs from the different developmental stages and ovarian tissues were measured using qRT-PCR with SYBR green dye (Stragtagene, La Jolla, CA) in a Cepheid Smart Cycler MX4000 (Cepheid, Sunnyvale, CA). The PCR mixture (25 μl) consisted of a 1X Cepheid

enhancer additive (1 mM Tris, pH 8.0; 0.1 mg/ml bovine serum albumin, non-acetylated; 0.75 M trehalose; 1% tween-20), 10 μl Master Mix (2.5X) (Eppendorf), 500 nM forward and reverse primers and 0.25X SYBR green dye. Primers for PCR were produced to yield between 200-400 base pair amplification products.

Sample Ct values were determined by the Smart-Cycler program. The initial concentrations of candidate cDNAs were interpolated from the standard curve. Then these concentrations of the sample cDNAs, which should be equal to the concentrations of the sample candidate mRNAs, were converted to fmol/μg total RNA.

Construction of Recombinant Expression Vector for the Extracellular Domain of Zebrafish GHR-I, GHR-II, and PRLRα

The extracellular domain (ECD) including the transmembrane domain of PRLRα, PRLRβ, GHR, and SLR were amplified by polymerase chain reaction (PCR) from the full-length cDNA sequence for each receptor in the pGEM-T Easy vector. The PCR reaction was carried out in a 50 μl volume, which included 5 μl of 10X PCR buffer, 1.5 μl of 10 mM dNTP, 1 μl (10 μg) of cDNA template, 2.5 units of *Pfu* DNA polymerase, and 0.3 μM of forward and reverse primers (Table 2.1-2). The PCR conditions were as follows: a 2 min denaturation at 94°C, 25 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 68°C followed by a 15 min extension at 68°C. The PCR products were subcloned into pET-100 expression vector (Invitrogen, Carlsbad, California) and transformed into TOP 10 competent *E. coli* cells by heat shock at 42°C. Clones were verified for correct ligation by sequencing with forward and reverse universal primers using the Big-Dye Terminator kit and an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Willesleg, MA, USA). The plasmid constructs were amplified and purified using Qiagen Plasmid

Purification kit (Qiagen, USA), then transformed into BL21 Star (DE3) competent cells for the production of the recombinant proteins.

Expression and Solubilization of Recombinant Zebrafish GHR-I, GHR-II, and PRLRα Proteins

Production of recombinant protein for GHR-I-ECD, GHR-II-ECD, and PRLRα-ECD were initiated by inoculating BL21 Star (DE3) cells containing the appropriate expressing vectors into 10 ml Luria Broth (LB). The cells were grown overnight at 37°C in a C24 incubator shaker (New Brunswick Scientific, Edison, NJ). Then, the entire 10 ml of the bacteria cells were inoculated into 500ml LB and incubated at 37°C shaker for 2 to 4 h. A 500 µl 1M IPTG was added when the $O.D_{600}$ of the bacterial suspension reached between 0.5 and 0.8 to induce the production of the recombinant protein. The culture was incubated further for an additional 24-28 h to accumulate recombinant protein. The recombinant proteins were isolated and purified from inclusion bodies using a procedure modified from the protocol described previously (Nguyen et al., 2006). Briefly, bacterial cells were collected from the culture suspension by centrifugation at 4°C for 10 min at 7974 x g using a Sorvall RC-5B Refrigerated Superspeed centrifuge (DuPont Instruments, USA). The supernatant was discarded and cell pellets were collected and washed in a suspension buffer (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Then, the bacterial cells were disrupted by sonication in a homogenizing buffer (20 mM Tris-HCl, 5 mM EDTA, 1 mM PMSF, 1% Triton X-100, pH 8.0). Inclusion bodies with cell membranes were collected in pellets by centrifuging the mixture for 15 min at 3645 x g. Partial removal of cell membranes and bacterial proteins was carried out by stirring the suspension overnight at 4°C in 20 ml stirring solution (20 mM Tris-HCl, 5 mM EDTA, 4% Triton X-100, pH 8.0). Then, the suspension was centrifuged as described previously and the pellets were

collected and sonicated (at power 3) for 3 secs on ice in the stirring buffer. The bacterial pellets were collected again by centrifugation and washed with three 20 ml washes using the suspension buffer. Finally, the inclusion bodies were solubilized by stirring the pellets for 2 days in 10 ml solubilization buffer (8 M urea, 20 mM $NaPO_4$, 500 mM NaCl, pH 7.8).

Metal Affinity Column Purification of Recombinant Zebrafish GHR-I, GHR-II, and PRLRα Proteins

The recombinant proteins were purified using ProBond purification system (Invitrogen) at RT. Briefly, 10 ml of solubilized proteins were added to the purification column containing 5 ml ProBond^{TM} resin. The recombinant proteins containing a His-tag were allowed to bind to the resin by gently stirring the suspension for 30 min on a shaker. The resin was pelleted by centrifuging for 1 min at 180 x g or by gravity. The supernatant was discarded. The resin was washed twice with 5 ml of a denaturing binding buffer (8 M urea, 20 mM $NaPO_4$, 500 mM NaCl, pH 7.8) for 5 min. Subsequently, the resin was washed five times with 5 ml of a denaturing wash buffer with the same composition as the binding buffer but with pH 5.0, for 5 min each time by gently inverting the column. Finally, the protein was recovered with two washes using 4 ml elution buffer (8 M urea, 20 mM $NaPO_4$, 500 mM NaCl, pH 4.0). The recovered recombinant protein elution was transferred to a dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA), suspended in 1 L of ammonium bicarbonate buffer (0.05 M), and stirred overnight at 4°C. The next morning, the protein solution was transferred to a new 1 L ammonium bicarbonate buffer and stirred for an additional 4-6 h at 4°C. The protein solution was subsequently added to a clean 15 ml centrifuge tube, solidified by storage at -20°C and lyophilized using Freezone6 (Labconco) at -20°C.

Production of Antibodies for the Extracellular Domain of Zebrafish GHR-I, GHR-II and PRLRα

Antisera to recombinant zebrafish GHR-I, GHR-II, and PRLRα were produced in three female rabbits. Each rabbit received injections subcutaneously at multiple locations on the back. For the initial immunization, each rabbit received 0.5 ml of emulsion containing 50-100 μg recombinant extracellular domains of GHR-I, GHR-II, or PRLRα proteins and complete Freund's adjuvant to elicit a rapid immuno-response to the antigens. At 38 days, each rabbit was boosted with 0.5 ml of emulsion containing 50 μg of the respective receptor recombinant proteins and incomplete Freund's adjuvant. Booster injections were repeated at a 2-week interval and test bleeds were conducted prior to the final bleeding. After the final bleed, the serum was allowed to coagulate overnight at 4°C, followed by centrifugation at 10,000 x g for 10 min. The coagulated particles were removed with a glass Pasteur pipette and the anti-sera was stored at -20°C. Specificity and affinity of antibodies were determined by Western blotting and immunohistochemistry.

Western Blot Analysis using Zebrafish Specific GHR-I, GHR-II and PRLRα Antibodies

Western blot analysis was performed as previously described (Nguyen et al., 2006) with a few modifications. Tissue samples were collected from anesthetized adults (MS-222; 200 mg/L in buffered solution) and immediately transferred to ice cold PBS. Samples were sonicated with 10 short bursts (2 sec each) with a sonicator (Sonic Dismembrator, Fisher Scientific) at power 3, followed by centrifugation for 10 min at 20,000 x g. The resulting pellet was resuspended in 1X SDS buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol), boiled for 10 min and then cooled on ice. Each sample was loaded with 40 μg of total protein, estimated by the Bradford assay, onto a 12% SDS/PAGE gel in a Bio-Rad apparatus (Bio-Rad

Laboratories, CA) and electrophoresed at 200V for 1 h on ice. The protein was transferred to a nitrocellulose membrane (Whatman) in transfer buffer (25 mM Tris-base, 192 mM Glycine, 20% v/v Methanol, pH 8.3) at 100V for 1 h. The membrane was blocked with 5% nonfat milk in TBST (50 mM Tris, 100 mM NaCl, 0.1% Tween 20, pH 7.4) for 30 min at RT and incubated with primary antibody against the respective receptors (1:2000) in 10 ml of 5% nonfat milk in TBST overnight at 4°C. Subsequently, the membrane was washed with 15 ml of TBST five times for 5 min at RT, incubated for 1 h with horseradish peroxidase conjugated to goat anti-rabbit antibody (1:5000; Cell Signaling, Beverly, MA) at RT, and finally washed five times for 5 min each with 15 ml of TBST at RT. The Western blots were then treated with a chemiluminescent substrate (Super Signal West Extended Dura Substrate, Pierce, Rockford, IL, USA) at RT for 5 mins. The signals were digitally recorded using a chemiluminescence image system (FluorChem^{TM} 8800, Alpha Innotech, San Leandro, CA). Protein size was determined by comparing blotted protein size to a biotinylated protein ladder (Cell Signaling, Beverly, MA) following the manufacturer's instructions. The specificity of each antibody was assessed using the same recombinant extracellular domain for the respective receptors used to inject the rabbits for antibody production to serve as a positive control.

Immunohistochemistry (IHC) using Zebrafish Specific PRLRα Antibodies

Twenty zebrafish larvae and twenty 1-month old juveniles were fixed overnight or for one week, respectively, in 20 ml of 10% Bouin's fixative (Fisher Scientific). Three 1-month old juveniles were fixed per 20 ml of 10% Bouin's and twenty larvae were fixed in 20 ml. Samples were then dehydrated using 10 ml of the following solutions for 30 mins unless indicated otherwise: washing 2X with 70% ethanol (EtOH), 1X with 95% EtOH, 1X with 100% EtOH, 1X with Xylene, 1X with Xylene:Methyl Salicylate (1:1), and finally 1X with Methyl Salicylate for

1 h. Then samples were mounted in paraffin using cassettes, sectioned at 8 μm thickness, and collected onto a pre-frosted glass microscope slide (Fisher Scientific, Pittsburgh, PA). The sections were then deparaffinized by washing 2X for 5 min with Xylene and rehydrated by washing the sections 1X with 100% ethanol (EtOH), 1X with 95% EtOH, and 1X with 70% EtOH for 5 min each and finally washed 3X with PBS for 5 min. Then, the sections were incubated in blocking solution (PBS with 3% BSA, and 1% Normal goat serum) for 1 h at room temperature. Finally the sections were incubated with anti-zebrafish PRLRα antibody (1:1000 diluted in blocking solution) overnight at 4°C. Next, the primary antibody was washed away with PBS for 4X for 5 min and incubated with horseradish peroxidase conjugated to goat anti-rabbit (1:2000; Cell Signaling, Beverly, MA). The secondary antibody was detected using Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol.

Statistical analysis

The significance of the mean differences between various experimental groups was determined by one-way ANOVA followed by Tukey test analysis. A *P* value <0.05 was considered statistically significant.

Results

Identification of Zebrafish GHR-I and GHR-II Genes

In an attempt to identify the cognate receptors for the PRL/GH family of hormones, known PRLR, GHR, and SLR cDNAs from selected fish species were used to conduct a BLAST search (Basic Local Alignment Search Tool) against the zebrafish genome (Ensembl genome browser: http://www.ensembl.org/index.html and NCBI database: http://www.ncbi.nlm.nih.gov). Two expressed sequence tag (EST) clones were identified to be similar to GHRs, one located on

chromosome 8 (zfGHR8; Image:6896869) and the second on chromosome 21 (zfGHR21; Image:7428125). Complete sequencing and assembly of the full-length EST clones, indicated that zfGHR8 was more similar to the well established goldfish GHR, and was re-named zfGHR-I; while we designated zfGHR21 as zfGHR-II. Both zfGHR-I and zfGHR-II possess conserved functional domains with the class 1 cytokine receptor family and other vertebrate GHRs: a signal peptide (predicted by SignalP 3.0, http://www.cbs.dtu.dk/services/SignalP), an FGEFS motif in the extracellular domain, a single transmembrane domain (predicted by using TMHMM Server v2.0, http://www.cbs.dtu.dk/services/TMHMM), Box 1 and Box 2 in the intracellular domain (Fig. 2.1-1; Fig. 2.1-2). zfGHR-I is composed of 571 aa consisting of 9 exons, 7 cysteine residues in the ECD, and 9 tyrosine residues. zfGHR-II is shorter in overall length, with 8 exons spanning 555 aa. zfGHR-II only possesses 5 cysteine residues for two potential disulfide bridges compared to zfGHR-I and retains 5 tyrosine residues in the intracellular domain. Another key difference between the two receptors is the B site; zfGHR-I maintains the typical TVEN sequence observed in classical GHRs across vertebrate species, while there is a modification of the first two amino acids in zfGHR-II, NIEN.

Comparison of amino acid similarities of zfGHR-I with other GHR cDNAs reveals a high level of conservation within the cyprinids (Table 2.1-3). zfGHR-I shares an approximate 88.2% amino acid similarity with flathead minnow GHR and between 81.1-83.3% similarity with other members of the carp family. In contrast, sequence comparison between zfGHR-I with more distantly-related fish species or higher order vertebrates indicated fewer similarities, 53.5-61.1% and 39-42% respectively. Despite low sequence similarities with non-teleost GHRs, all characteristic landmarks of GHRs were present. Phylogenetic analysis groups zfGHR-I with SLRs and zfGHR-II in the GHR clade with other known teleost GHRs and SLRs (Fig. 2.1-3).

Zebrafish Prolactin Receptor (zfPRLR) Genes

The zebrafish PRLRα (zfPRLRα) sequence was previously identified (NCBI accession no. NM_001128677.1) but only the coding region was presented. RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was employed to identify the complete sequence for zfPRLRα. The results indicated that zfPRLRα possesses two distinct 5' UTR. The different 5' UTR consisted of 186 and 199 bases, representing type-I and type-II, respectively (Fig. 2.1-4). The two types of 5' UTR were distinct but 31 bases immediately upstream of the start codon were identical. In contrast, only one 3' UTR was present, consisting of a short sequence of 51 nt that included the poly-A tail sequence. The zfPRLRα cDNA demonstrates the presence of 8 exons, 5 cysteine residues for two potential disulfide linkages, and 14 tyrosine residues in the intracellular domain with two located within Box 2.

The complete zfPRLRα cDNA encodes a receptor protein of 605 amino acids. Similar to all class I cytokine receptor superfamily members, zfPRLRα is divided into three domains, an extracellular, transmembrane, and intracellular domain. The extracellular domain consists of 230 amino acids with a putative signal peptide represented by the first 22 aa of the coding sequence and a WSEWT motif in the membrane proximal region. The transmembrane domain spans amino acid 230-253 with a sequence, RSLWIMITIFSVFIVFILTWMLK. The intracellular domain contains 352 aa and possesses the conserved functional domains common to GHR-I and GHR-II: a proline rich region Box 1 (PPVPGPKI) and Box 2 (DLLVEYLEVY). All conserved functional domains for zfPRLRα are shown in Fig. 2.1-4.

Interestingly, while data mining for all the potential hormone receptors of the PRL/GH superfamily, one genomic contig had low homologies with all other receptors, but possessed features of zfPRLRα, and was named zfPRLRβ. Further data mining using the BLAST search

algorithm to examine the zebrafish genome at the Ensemble genome browser with PRLR sequences from similar fish species provided evidence for a full-length gene. Subsequent PCR-based cloning of cDNAs obtained from 24 hours post-fertilization (hpf) zebrafish embryos generated full-length clones (Fig. 2.1-5A-B), indicating that the zfPRLRβ sequence was actively transcribed and not a pseudogene. The full-length sequence of zfPRLRβ was later obtained from RLM-RACE and identified to be a single distinct gene located on chromosome 5.

Analysis of the full-length cDNA for zfPRLRβ shows a sequence of 1544 bp encompassing a 5' UTR of 190 nt, a coding region of 1341 bp, and a short sequence of 13 adenines following the stop codon (Fig. 2.1-5A). The coding region is composed of 447 aa with a putative signal peptide of 22 aa and a single transmembrane domain, QNTVVICAVTLTVVIFMLTAGVMT. The extracellular domain contains five cysteine residues and the conserved WSDWS motif. Within the intracellular domain, Box 1 and five potential sites for tyrosine phosphorylation were identified, but Box 2 appeared not to be conserved. All conserved functional domains for zfPRLRβ are represented in Fig. 2.1-5A.

Both PRLRα and PRLRβ share conserved functional domains with human PRLR (hPRLR) including the two disulfide bonds within the extracellular domain, the WS motif, and Box 1 (Fig. 2.1-6). PRLRβ did not maintain a conserved Box 2 with hPRLR.

Distribution of GHR-I and GHR-II Proteins in Zebrafish

The temporal distribution of zfGHR-I and zfGHR-II during development was analyzed to gain a better understanding of hormone functions in zebrafish. Both zfGHR-I and -II were expressed throughout early zebrafish embryogenesis (Fig. 2.1-7A and Fig. 2.1-7C). Levels of either of the receptor types were not significantly different among any developmental stages or times from one-cell to 14 days post-fertilization (dpf). In contrast, transcripts for both receptors

were abundant in the ovaries.

In adult zebrafish, both zfGHR-I and zfGHR-II were co-expressed in most tissues, albeit at different levels (Fig. 2.1-8A). zfGHR-II expression was highest in the eyes, brain, liver, spleen, and gills. On the other hand, zfGHR-I was expressed most abundantly in the ovary, eye, muscle, heart, liver, and spleen. Although these receptors share common target tissues, zfGHR-I was more abundant in the ovaries, muscle, and intestine, while zfGHR-II levels were higher in the brain, kidney, and gills. At the protein level, both zfGHR-I and zfGHR-II were confirmed in all tissues examined, with the addition of the olfactory tissue (Fig. 2.1-8B). The specificity of the antibodies was verified using the recombinant extracellular domain of either zfGHR-I or zfGHR-II as a positive control, demonstrating the detection of the respective receptors in native zebrafish tissues.

Developmental Expression of PRLRα and PRLRβ Transcripts and Proteins

Similar to the zfGHRs, both zfPRLRα and zfPRLRβ transcripts were expressed throughout zebrafish development, from the zygote to juvenile stages (Fig. 2.1-7B, Fig. 2.1-7D). Embryonic expression of PRLRα was detected using whole mount *in situ* hybridization (WISH) as early as 15 hpf in the pronephric ducts and tissues undergoing morphogenetic events (Fig 2.1-9A-C). By 17 hpf, PRLRα was detected in multiple clusters of cells located between the bilateral rows of pronephric ducts (Fig. 2.1-9E). The location of these cell clusters suggest that some are insulin-positive while the other cells may be β-cell progenitors or other endocrine precursor cells that have not completed differentiation. At 20 hpf, PRLRα expressing cells migrate to the midline and form a single layer of clustered cells (Fig. 2.1-9I). By 24 hpf, the cluster of PRLRα positive cells expands and is expressed in β-cells (Fig. 2.1-9M; Fig. 4-2D-F). The expression of PRLRα is maintained in pancreatic β-cells up to 5 dpf and follows the same migratory and

expansion pattern of insulin-producing cells (Argenton et al., 1999; Biemar et al., 2001). Unlike the pancreas, the expression of PRLRα in the kidney is restricted to particular regions of the kidney as development proceeds. PRLRα is initially expressed during early somitogenesis, throughout the entire pronephric tubule (Fig. 2.1-9C, F-G, J) but never fused to the cloaca (Fig. 2.1-9F). By 24 hpf, PRLRα is expressed strongly in all segments of the primitive kidney: proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early (DE), distal late (DL), and pronephric duct (PD) (Fig. 2.1-9K). At 48 hpf, PRLRα expression is restricted to PCT and the anterior region of the PCT (Fig 2.1-10A-B). Expression of PRLRα is further restricted to the region that defines the onset of PCT coiling at 72 hpf, and by 5 dpf PRLRα is only detected in the PCT coils of the zebrafish pronephric tubule (Fig. 2.1-10E, I). PRLRα was also expressed in various regions of the eye (Fig. 2.1-10K), ionocytes, optic vesicle (Fig. 2.1-10F), and intestine (Fig 2.1-10D, H).

In contrast, PRLRβ was expressed most strongly in the kidney and the expression was maintained throughout embryogenesis (Fig. 2.1-11B, D, F-J). PRLRβ expression was initially observed at 17 hpf in both tissues undergoing morphogenesis around the eyes and within the pronephric ducts (Fig 2.1-11A-C). At 19 hpf, PRLRβ was expressed throughout the entire kidney, from the PCT to the distal PD (Fig. 2.1-11D). The expression pattern persisted even at 3 dpf in all regions (Fig. 2.1-11I-J). Additionally, PRLRβ was observed in the optic vesicle, heart, and within different parts of the brain, though staining was weak (Fig. 2.1-11E, G; data not shown).

Immunohistochemistry using a zebrafish-specific PRLRα antibody demonstrated abundant expression of PRLRα in olfactory epithelium and bulbs by 48 hpf and in 1 month post-fertilization zebrafish (Fig. 2.1-12A-B); as well as in the levator arcus palatine (Fig. 2.1-12C),

intermandibularis anterior and posterior, and the kidneys of one month old zebrafish (Pereira et al., unpublished data). In addition, Western blotting showed PRLRα expression in the olfactory organs, brain, eye, intestine, ovaries, and spleen of adult tissues (Fig. 2.1-8B). The recently identified PRLRβ transcript was also demonstrated to be expressed in a number of adult tissues, including the gills, kidneys, testes, ovaries, intestines, liver, scales, heart, lipids, brain, muscles, eyes, and spleen (Fig. 2.1-8A).

Discussion

In this study, we identified four cognate receptors for the PRL/GH family in zebrafish and examined the potential targets of hormone receptor action during zebrafish embryogenesis. Analysis of the zebrafish genome revealed the existence of two growth hormone receptors (GHRs; GHR-I and GHR-II) and two distinct prolactin receptors (PRLRs; PRLRα and PRLRβ) that share multiple conserved functional domains with known vertebrate GHRs and PRLRs. The GHRs were expressed most abundantly in the brain, liver and muscles as expected for their well characterized function in postnatal growth and metabolism (Rousseau and Dufour, 2007). Transcripts for the PRLRs were highest in the gills, kidney, brain and eyes, consistent with their reported roles in regulating ion balance and development (Manzon, 2002; Nguyen et al., 2008). Furthermore, all four receptors were expressed within the ovaries and the overall receptor levels were maintained from the one-cell stage throughout the first 14 days post-fertilization (dpf). At the cellular level, PRLRs were expressed predominantly in the pancreas and pronephric tubule during early zebrafish embryogenesis.

In previous studies, it was initially observed that fish exhibited two divergent GHRs that represented two distinct lineages of GHRs in fish evolution (Tse et al., 2003). The two groups of GHRs were divided into the salmonid GHRs (GHR type I) and the non-salmonid GHRs (GHR

type II) based primarily on the number of conserved extracellular cysteine residues. However, this view was subsequently challenged by the identification of both GHR types within a single species in several teleosts: gilthead seabream, black seabream, southern catfish, Nile tilapia, and eel (Saera-Vila et al., 2005; Jiao et al., 2006; Ozaki et al., 2006). Furthermore, the two GHR-like genes in masu salmon were characterized as a GHR and a SLR based on their preference for binding to GH and SL, respectively (Fukada et al., 2004, 2005). Phylogenetic analysis revealed that masu salmon SLR is orthologous to GHR-I of non-salmonids along with medaka and fugu SLRs, suggesting that non-salmonid GHR-I are potentially SLRs rather than GHRs (Fukamachi et al., 2005).

Two genomic contigs representing both putative GHR subtypes were recently identified in the zebrafish genome database. Our physical sequencing of EST clones and real-time PCR analysis demonstrated that both genes are functionally expressed in zebrafish. Consistent with other fish possessing two distinct genes similar to tetrapod GHRs, the zebrafish GHR-like genes can also be classified based on amino acid (aa) sequence comparison into zfGHR-I and zfGHR-II. Examination of the zfGHR-I and zfGHR-II demonstrated distinct amino acid sequence and structural differences of the two GHR-like genes similar to other teleost (Saera-Vila et al., 2005; Jiao et al., 2006). Zebrafish GHR-I, like all vertebrate GHRs except for the salmonids (Very et al., 2005), possesses 7 conserved cysteine residues. Biochemical analysis of the human GHR demonstrates that disulfides are paired sequentially to produce short loops, 10-15 residues long, with one cysteine residue un-paired (Fuh et al., 1990). Applying the same conceptual framework suggests that in zfGHR-I, Cys at position 44 (Cys_{44}) likely forms a disulfide bond with Cys_{54}, Cys_{86} links with Cys_{97}, and a third disulfide linkage between Cys_{111} and Cys_{127}. In contrast, zfGHR-II only possesses two of the conserved N-terminal disulfide bonds (Cys_{37} with Cys_{47} and

Cys_{83} with Cys_{93}) and lacks a third intramolecular disulfide bond. Although zfGHR-II lacks a third disulfide cross bridge, both zfGHRs retain the conserved second disulfide bond that has been demonstrated to be important for GH ligand binding (Van den Eijnden, 2006). Another key difference is the position of the unpaired cysteine residues between the two zfGHRs. In zfGHR-I, Cys_{219} is positioned 8 aa upstream of the conserved FGEFS motif compared to the free cysteine residue located 73 aa upstream in zfGHR-II. The unpaired cysteine residue has been suggested to be involved in intermolecular disulfide bonds with a dimerizing GHR (Zhang et al., 1999), and the membrane proximal cysteine in zfGHR-I may provide more structural flexibility of the receptor for ligand binding compared to zfGHR-II which may possess strict affinity for GH. Together, these characteristic may provide important clues into the potential ligands that may bind to the zfGHRs.

The masu salmon GHR (msGHR), which is structurally similar to zfGHR-II, has strict binding capacity for GH and not SL or PRL (Fukada et al., 2004). On the other hand, masu salmon SLR (msSLR), which is structurally similar to zfGHR-I, has a preference for SL but GH also has the capacity to bind to msSLR (Fukada et al., 2005). Based on the residual and structural similarities and phylogenetic position of zfGHR-I with other known teleost SLRs while zfGHR-II belongs to the teleost GHR clade (Fig. 2.1-3), it is speculated that zfGHR-I is the ortholog of teleost SLRs; and zfGHR-II represents the teleost-specific GHR. In this scenario, zfGHR-I and zfGHR-II are likely to show a similar binding capacity to zebrafish GH and SL as observed in the masu salmon. Although our binding assays failed, it was recently demonstrated that zebrafish PRLs did not bind to either zfGHR-I or zfGHR-II (Huang et al., 2009) consistent with the inability of sPRL to bind to masu salmon GHRs, leaving GH and SLs as the remaining ligands for these receptors. The identity of the zebrafish SLR awaits future binding studies for the two

GHR-like genes using a homologous system encompassing the entire hormone-receptor set.

Recently, zfGHR-I and zfGHR-II were demonstrated to possess conserved synteny with the human GHR loci, indicating that zfGHR-I and zfGHR-II are true orthologs to human GHR (Fukamachi and Meyer, 2007). Thus, the emergence of zfGHR-I in the zebrafish lineage is likely due to duplication and divergence from the ancestral GHR gene during the fish-specific genome duplication (FSGD) event in the stem lineage of actinopterygians (ray-finned) leading to the modern day teleosts. The lack of a second GHR-like gene in lungfish and sturgeon, lineages that did not experience FSGD, further supports the duplication of fish GHR through the FSGD event (Fukamachi and Meyer, 2007), possibly giving rise to the teleost SLR. However, lungfish and sturgeon GHRs show higher amino acid similarity with teleost SLRs then GHRs of mammals, birds, and teleosts. It is possible that the ancestral GHR gene duplicated and underwent functional switching in fish to account for the emergence of a second SL, SLβ, present in some teleosts (Zhu et al., 2004). Nonetheless, further studies to identify additional SLRs across teleost species complemented with competitive binding studies would greatly facilitate the proper nomenclature of the GHR isoforms as either a SLR or GHR in teleosts. The identities of the GH family of peptide hormones and receptors in the lamprey (*Petromyzon marinus*) would be particularly useful in determining the timing of events that led to the expansion of this hormone-receptor family because it did not experience the FSGD (3R) or 2R (Daza et al., 2009; Panopoulou and Poustka, 2005). Currently, only GH has been found to exist in the sea lamprey and possibly represents the most ancient gene of this hormone family, but no evidence for the receptors are available (Kawauchi et al., 2002; Moriyama et al., 2006).

Similarly, the prolactin receptor (PRLR) also appears to have undergone gene duplication during the FSGD event. Although less studied, two distinct genes for PRLR have been isolated

or described by *in silico* data mining in several teleost species, but only a single PRLR gene have been found in non-teleost species, suggesting a unique phenomenon found only in fish (Huang et al., 2007; Fiol et al., 2009). Unlike mammalian species where extensive heterogeneity of the PRLR is observed (Freeman et al., 2000; Nguyen et al., 2008), the zebrafish complement consists of two PRLR genes that have been designated as PRLRα and PRLRβ or the equivalent PRLR1 and PRLR2 in other teleosts. Compared to PRLRα, PRLRβ shares relatively low overall sequence homologies with non-teleost PRLRs. Furthermore, PRLRβ differs with respect to the length and composition of their intracellular domain (ICD). PRLRβ lacks several tyrosine residues conserved in the ICD of the classical PRLRα and the conserved Box 2 domain found in most class 1 cytokine receptor families, suggesting differences in the activation of post-receptor signaling pathways.

It appears that the retention of the duplicated PRLR genes in zebrafish was a consequence of both sub- and neo-functionalization. Developmental expression profiles indicate that both PRLRα and PRLRβ are expressed during early somitogenesis throughout the primitive pronephric tubule. However, as the kidney develops, PRLRα expression becomes restricted to the proximal convoluted tubules (PCT), while PRLRβ maintains expression in all regions of the pronephric tubule. This suggests that initially, the overlapping of both PRLRα and PRLRβ signaling are important for the regulation of osmotic equilibrium, as is expected for the well defined role of PRL as a freshwater adapting hormone (Sakamoto and McCormick, 2006). The subsequent differential expression of zebrafish PRLRs within the pronephric tubules is possibly an example of sub-functionalization of the ancestral function as an osmoregulating hormone (Manzon, 2002). While PRLRβ continues to maintain general hydromineral balance throughout zebrafish embryogenesis, PRLRα acquired more specific functions related to the anterior

structure of the kidneys, such as blood filtration or tubular resorption. The expression pattern of PRLRα and PRLRβ also appears to be different in the anterior region of kidney (Fig. 2.1-10E and 2.1-11J), possibly indicating the regulation of different cell types. In contrast, PRLRα may have acquired novel functions, possibly involved in developmental aspects related to the onset of coiling within the PCT region as suggested by its restricted expression. In addition, only PRLRα was transcribed in the endocrine pancreas during embryogenesis, suggesting a unique role of PRLRα in regulating pancreas development and indicating a new function acquired by PRLRα to secure its existence in the zebrafish genome.

Intriguingly, PRLR was expressed abundantly in the olfactory system in the rat fetus (possibly for regulation of food intake), despite its low expression in adult rat olfactory bulbs (Freemark et al., 1996). Although PRLR transcripts and proteins were localized in olfactory sensory neurons (OSN), the specific cell types have not been identified (Freemark et al., 1996). We found PRLRα expressed abundantly in the olfactory system of zebrafish (Fig. 2.1-12A-B). Using immunohistochemical methods, PRLRα was detected as early as 36 hpf and sustained up to the juvenile stage in both the olfactory epithelium and olfactory bulb in zebrafish (Fig. 2.1-12A-B). Similar results reporting persistent high level expression of PRLR throughout larval development in the olfactory nerve and neurons in the sea bream have been reported (Santos et al., 2003). To date, not a single study has demonstrated a role for PRLR during embryogenesis, even within the olfactory system in vertebrates, despite the extremely important role of olfaction for social and environmental interaction, as well as for survival. Future studies should be conducted to analyze the consequences of PRLR expression on development of the olfactory system and its physiological relevance during development.

Here, it is important to note the discrepancies between PRLRα expression between

immunohistochemistry (ISH) and whole mount *in situ* hybridization (WISH). At first glance, the differences in PRLRα expression may reflect the detection of two fundamentally different aspects of cellular biology, protein and transcripts respectively. Due to the fact that our WISH data is consistent with an independent study (Liu et al., 2006), it is likely that our IHC results may require additional validation. Our lab produced polyclonal anti-sera against the PRLRα using recombinant protein of the zebrafish extracellular domain of PRLRα. As with all novel antibodies, we tried several types of fixatives, time of fixations, antibody dilutions, among other factors to optimize the staining conditions for the PRLRα antibody. However, only 10% bouins fixative produced staining at a dilution factor of 1:1000 without producing severe background. The anti-PRLRα antibody was expected to detect the ECD of PRLRα without complication due to the fact that the ECD is exposed on the cellular membrane. However, the requirement of a relatively low antibody dilution to detect the antigen when the anti-sera was produced against zebrafish PRLRα suggests that our antibody titer was not optimal, and may have reduced antibody stability and affinity for the antigen. The PRLRα antibody detected PRLRα in various adult tissues under a denatured state by Western blot analysis. IHC may have failed to detect PRLRα in the pancreas and kidney due to the native conformation of the PRLRα extracellular domain which masks PRLRα antibody-specific epitopes. Alternatively, our PRLRα antibody may have cross-reacted with other structurally similar receptors. This is suggested by the fact that strong expression of GHR-I was also detected by IHC in the olfactory epithelium and bulb (Pereira et al., unpublished data) which would be consistent with an absence in olfactory epithelium staining in our WISH data. Consequently, additional work will be required to optimize the efficiency and specificity of the PRLRα antibody.

In summary, we demonstrated that the zebrafish PRL/GH receptor family is composed of

zfGHR-I, zfGHR-II, zfPRLRα, and zfPRLRβ. All four receptors are expressed throughout zebrafish embryogenesis, providing targets for hormone action and supportive role for these proteins in early development as we previously suggested (Zhu et al., 2007). Expression of PRLRs within the endocrine pancreas and embryonic kidney indicates that PRL has direct physiological or developmental functions on these organs during development. Future studies will aim to identify the role of PRLRs in these tissues and explore the molecular mechanisms leading to embryonic functions of PRL and PRLRs.

Table 2.1-1. Primers used for quantitative real-time PCR (qRT-PCR) of *ghr-I*, *ghr-II*, *prlrα*, *prlrβ*, *ins*, and *ef1α*.

Gene Name	**Primer Name**	**Direction**	**Target Sequence**
ghr-I	zfGHR cDNA Synthesis	**Reverse**	5' AGGAAGGAGGATTTGGAG 3'
	zfGHRRealTF1	**Forward**	5' GGTGACTTTTCGTTGCTG 3'
	zfGHRRealTR1	**Reverse**	5' TGTAAAGCAGGCCTCATC 3'
ghr-II	21Ests cDNA Synthesis	**Reverse**	5' GGCTGTTGGTGTATTAGG 3'
	21EstsRealTF1	**Forward**	5' TTCAACACGGCCTCATCT 3'
	21EstsRealTR1	**Reverse**	5' GCAGCTGGATCACATAAG 3'
prlrα	zfPRLR cDNA Synthesis	**Reverse**	5' GGCATTTGGACTGTTGTG 3'
	zfPRLRRealTF1	**Forward**	5' TCTGCCCACTACATATGC 3'
	zfPRLRRealTR1	**Reverse**	5' ACCGCTTTGACGTTTTCC 3'
prlrβ	zfPRLR5 Coding R1	**Reverse**	5' GACCTCTTTGTGTTCCTGTA3'
	PRLR5 RT R1	**Forward**	5' GTGCTCTGGGATATTTGC 3'
	PRLR5 RT F1	**Reverse**	5' GCCTGTGGAAGTTGATGT 3'
ins	Insa RT F1	**Forward**	5' TAAGCACTAACCCAGGCACA 3'
	Insa RT R1	**Reverse**	5' GATTTAGGAGGAAGGAAACC 3'
elf1α	Ef1a RT F1	**Forward**	5' AGACTGGTGTCCTCAAGCCT 3'
	Ef1a RT R1	**Reverse**	5' TGAAGTTGGCAGCCTCCATG 3'

Table 2.1-2. Primers used for construction of recombinant expression vectors of *ghr-I*, *ghr-II*, *prlrα*, and *prlrβ*.

Gene Name	**Primer Name**	**Direction**	**Target Sequence**
ghr-I	zfGHRpET100R1	Reverse	5’ TCACGCCATCGGAGACTG 3’
	zfGHRpET100F1	Forward	5’ CACCCAAGGATCTGAGCTGTTT 3’
ghr-II	21EstspET100R1	Reverse	5’ TCATTTGTTAGGTATAAGTATAAA 3’
	21EstspET100F1	Forward	5’ CACCACACAAAATGTGCTT 3’
prlrα	zfPRLRpET100R1	Reverse	5’ TCATCTGGGAATATAGTTGGG 3’
	zfPRLRpET100F1	Forward	5’ CACCGTCAGTCCTCCA 3’
prlrβ	PRLR5 pET100 R1	Reverse	5’ TCAATTCTGCATGACAGTCATATTG 3’
	PRLR5 pET100F1	Forward	5’ CACCGAGGAGTGTGATCCCCCAATA 3’
prlrα	dnPRLRaF2	Forward	5’ AGAGACCGCGGGAACAACAGATC TGAGGAGTTTGG 3’
	dnPRLRaR2	Reverse	5’ CTCTCCGCGGCAGCAAACAAAGC TTCACACTGTTGTG 3’

Fig. 2.1-1. Organization of zebrafish growth hormone receptor type I (zfGHR-I) with respect to functional domains. The full-length cDNA was determined by sequencing EST clone Image:6896869. The conserved cysteine residues in the extracellular domain are represented by green bars. The FGEFS motif is represented by a red bar, transmembrane domain by a black box, Box 1 by a purple bar, and Box 2 by a blue bar. Specific to GHR is the B site highlighted with a grey bar. Potential tyrosine phosphorylation sites are indicated by a bolded letter Y highlighted in red. Sequences that are not highlighted represent the 5' and 3' untranslated regions; while those that are either highlighted by yellow and sky blue represent exons.

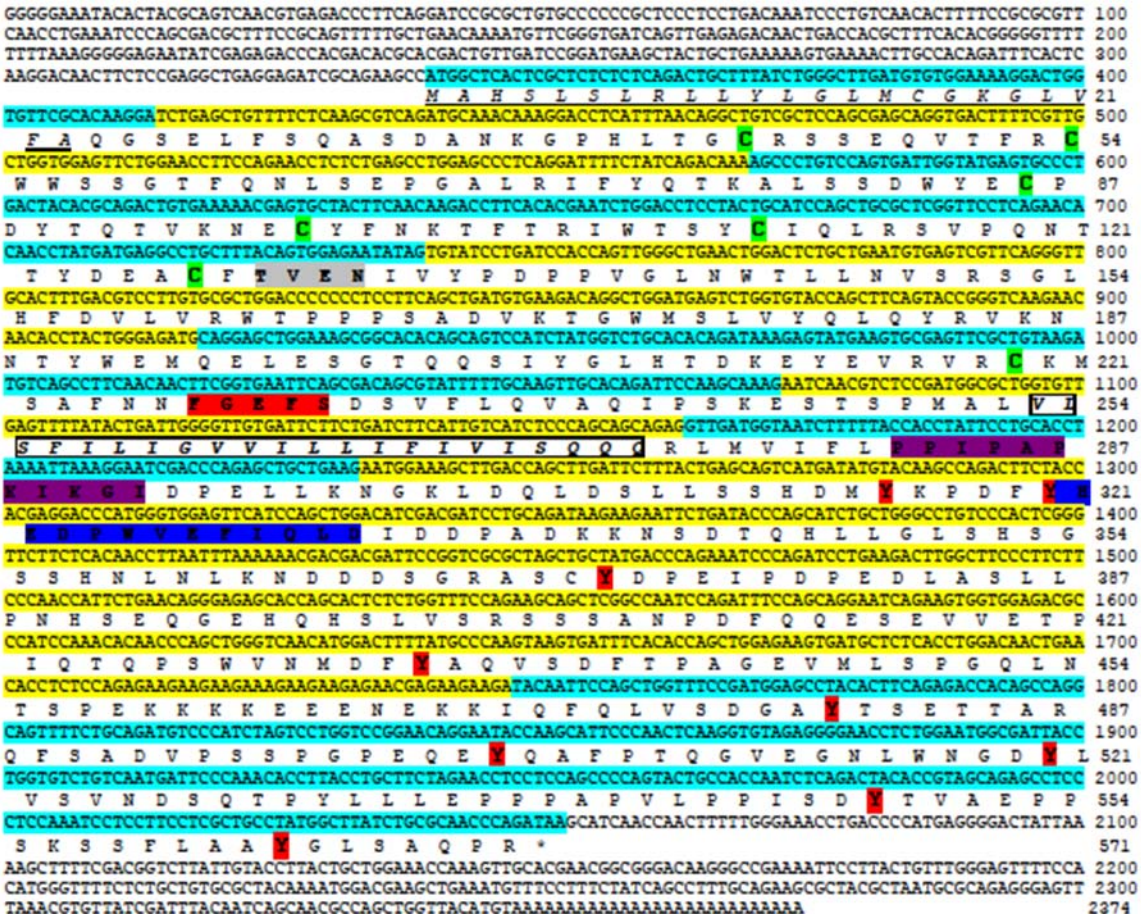


Fig. 2.1-2. Organization of zebrafish growth hormone receptor type II (zfGHR-II) with respect to functional domains. The full-length cDNA was determined by sequencing of an EST clone Image:7428125. The conserved cysteine residues in the extracellular domain are represented by green bars. The FGEFS motif is represented by a red bar, transmembrane domain by a black box, Box 1 by a purple bar, and Box 2 by a blue bar. Potential tyrosine phosphorylation sites are indicated by a bolded letter Y highlighted in red. Sequences that are not highlighted represent the 5' and 3' untranslated regions; while those that are either highlighted by yellow and sky blue represent exons.

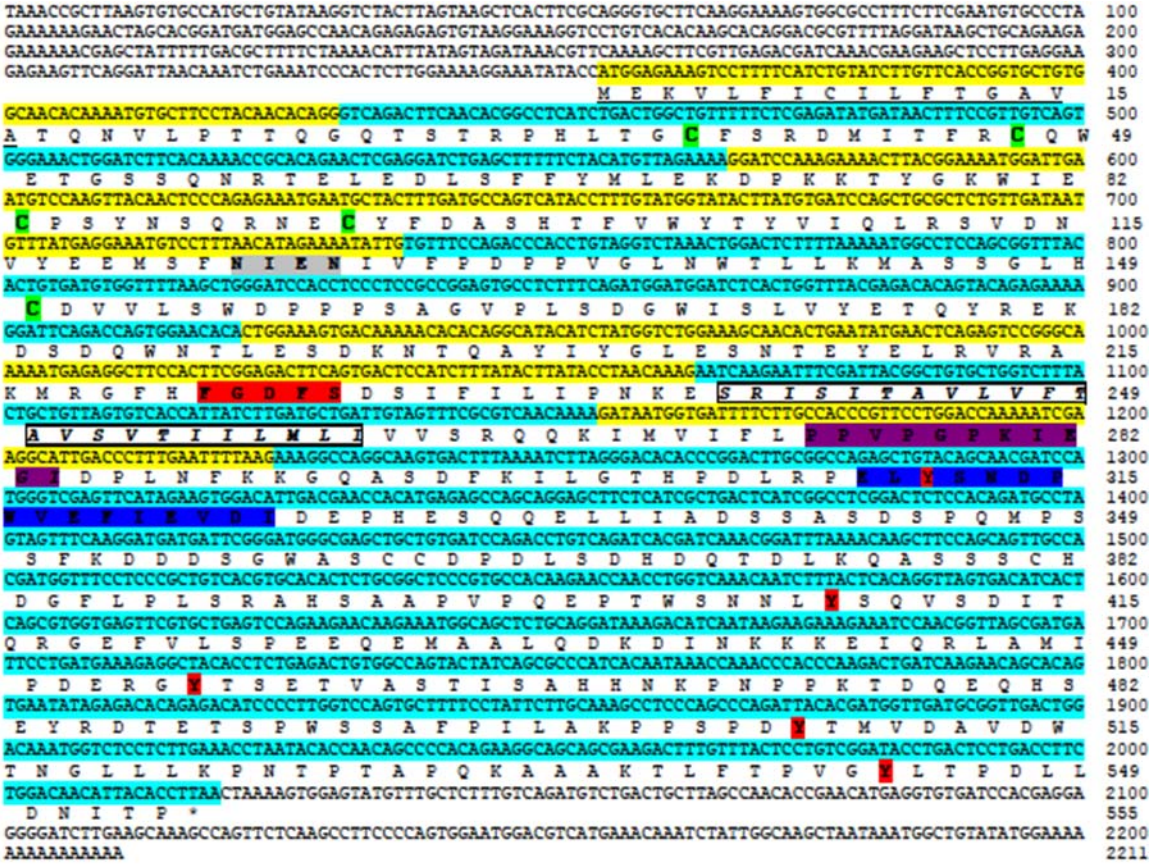


Table 2.1-3. Amino acid similarity of zfGHR-I and zfGHR-II to other vertebrate GHRs.

Species	**zfGHR-I**	**zfGHR-II**
Zebrafish GHR-I	-	52.9
Zebrafish GHR-II	52.9	-
Fathead minnow GHR (AAY63802)	88.2	65.9
Catla GHR (AAU93896)	83.1	52.6
Common carp GHR (AAU95675)	83.3	53.6
Grass carp GHR (AAP37033)	82.7	53.5
Goldfish GHR (AAK60495)	81.5	52.9
Turbot GHR (AAK72952)	61.1	49.5
Rainbow trout GHR1 (AAW56611)	56.7	55.1
Rainbow trout GHR2 (AAT76435)	56.0	55.3
Black seabream GHR (AAN77286)	60.5	50.1
Black seabream GHR2 (AAV83932)	53.5	53.5
Human GHR (NP000154)	39.0	29.0
Sheep GHR (AAP49814)	41.0	29.0
Frog GHR (AF193799)	40.0	29.0
Turtle GHR (AAF05775)	42.0	30.0
Chicken GHR (AAA48781)	41.0	30.0
Rabbit GHR (AAB67613)	40.0	30.0
Mouse GHR (NP034414)	39.0	29.0
Rat GHR (NP058790)	42.0	30.0

Fig. 2.1-3. Phylogenetic analysis of zfGHR-I and zfGHR-II sequences. Multiple sequence alignment and construction of the phylogenetic tree was performed with ClustalW using the mature proteins of four teleost species possessing a single GHR and SLR; frog PRLR was used as an out group. All nodes are supported by bootstrap values of 1000. Branch lengths indicate proportionality to amino acid changes on the branch. GenBank accession numbers: masu SLR (BAD51998); medaka SLR (DQ002886); zebrafish GHR-I (zfGHR-I, BC134903); masu GHR (BAB64911); medaka GHR (DQ010539); zebrafish GHR-II (zfGHR-II, EU649775); and frog PRLR (AF193801). Fugu SLR and GHR sequences were obtained as described by Fukamachi and Meyer (2007).

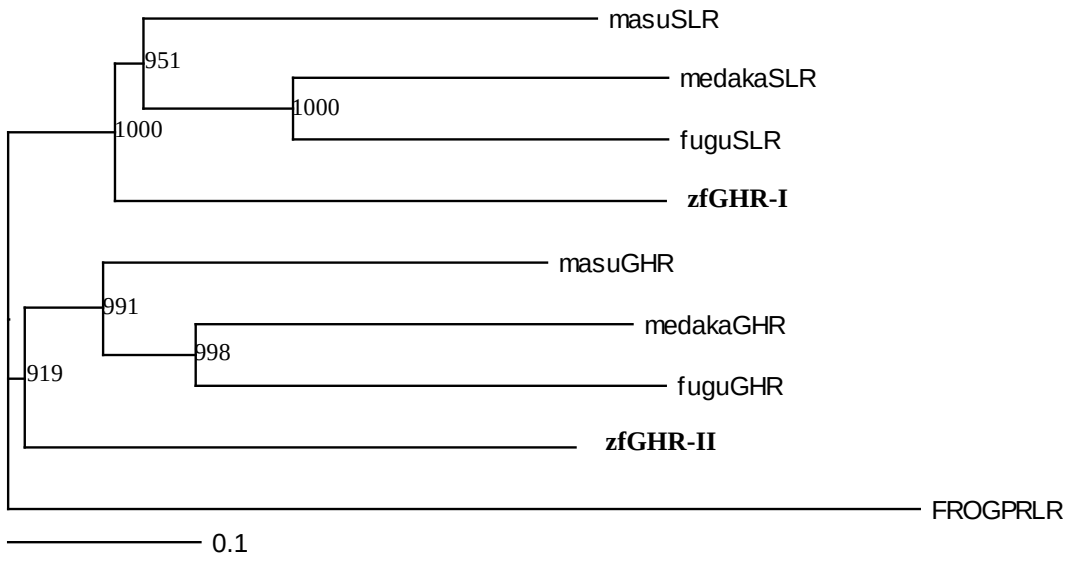
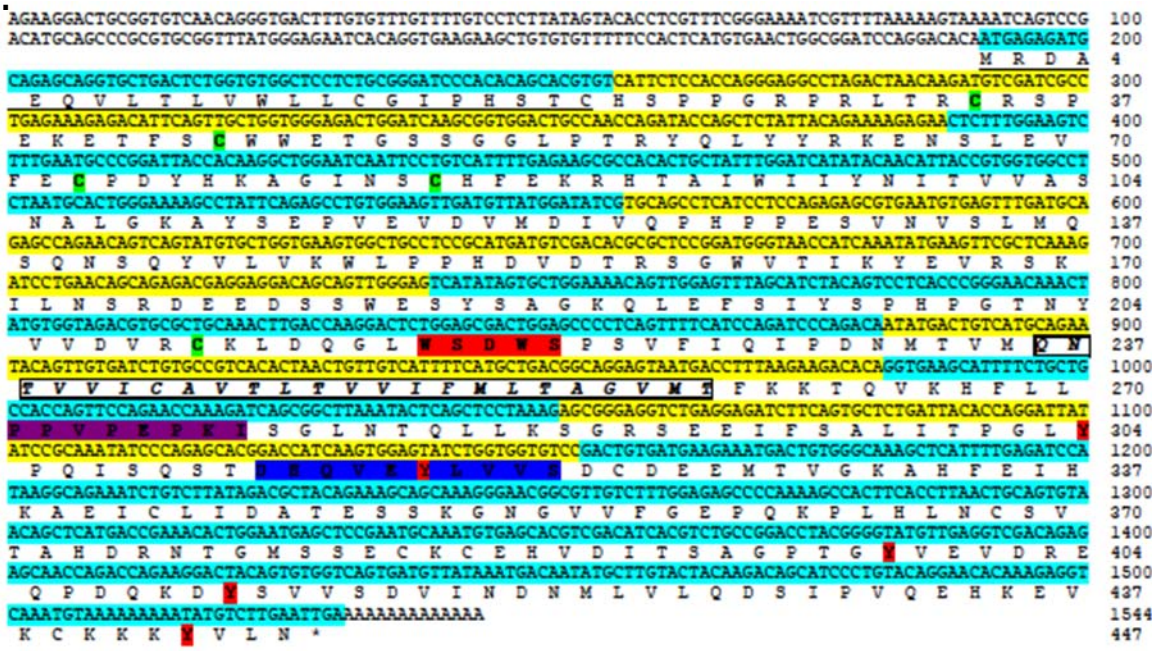


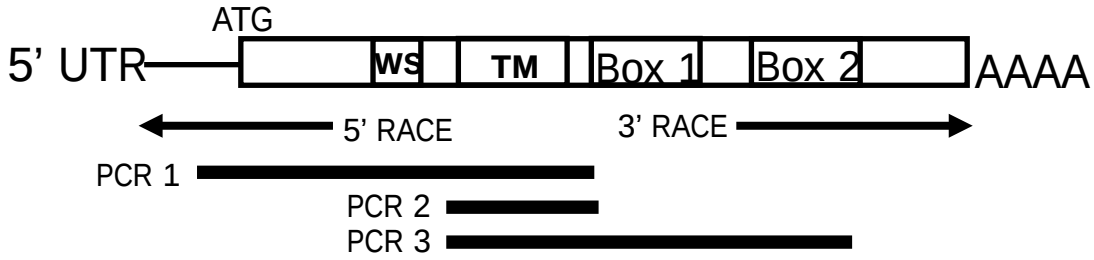
Fig. 2.1-4. Organization of zebrafish prolactin receptor α (PRLRα) with respect to functional domains. The full-length cDNA was determined by rapid amplification of cDNA ends (RACE). The five conserved cysteine residues are represented by bolded C's highlighted in green. The WS motif is represented by a red bar, the transmembrane domain by a black box, the Box 1 sequence by a purple bar, and the Box 2 sequence by a blue bar. Potential tyrosine phosphorylation sites are indicated by a bolded letter Y highlighted in red. Sequences that are not highlighted represent the 5' and 3' untranslated regions (UTR). The two different 5' UTR are labeled. Sequences highlighted in yellow and sky blue represent exons.

Fig. 2.1-5. Organization of zebrafish prolactin receptor β (PRLRβ) with respect to functional domains. (A) The full-length cDNA was determined by RACE. The five conserved cysteine residues are represented by bolded C's highlighted in green. The WS motif is represented by a red bar, transmembrane domain by a black box, the box 1 sequence by a purple bar, and the box 2 sequence by a blue bar. Potential tyrosine phosphorylation sites are indicated by a bolded letter Y highlighted in red. Sequences that are not highlighted represent the 5' and 3' untranslated regions; while those that are either highlighted in yellow and sky blue represent exons. (B) The diagram shows corresponding PCR products from different sets of primers produced to target different regions of the PRLRβ gene, (C) amplified by reverse-transcriptase PCR using cDNAs obtained from embryos at 24 hours post-fertilization.

A.



B.


ATG
5' UTR
WS
TM
Box 1
Box 2
AAAA
5' RACE
3' RACE
PCR 1
PCR 2
PCR 3

C.

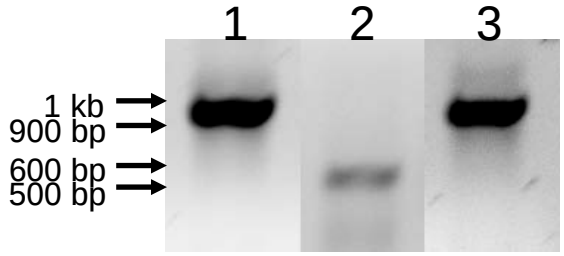

1
2
3
1 kb
900 bp
600 bp
500 bp

Fig. 2.1-6. Comparison of human prolactin receptor (hPRLR), and the two zebrafish prolactin receptor subtypes (zfPRLRα and zfPRLRβ). The full-length sequence of PRLRα and PRLRβ were obtained by rapid amplification of cDNA ends (RACE) from 24 hpf zebrafish embryos, and the hPRLR sequence was obtained from NCBI (NP_000940). The two conserved disulfide bridges in the extracellular domain are represented by red bars. The WS motif is represented by a purple bar, transmembrane domain by a black bar, the Box 1 sequence by a yellow bar, and the Box 2 sequence by a green bar. Sequence alignments of each conserved domain are shown. N-terminal (NH2), C-terminal (COOH), and the total number of amino acids in the mature hormone are indicated.

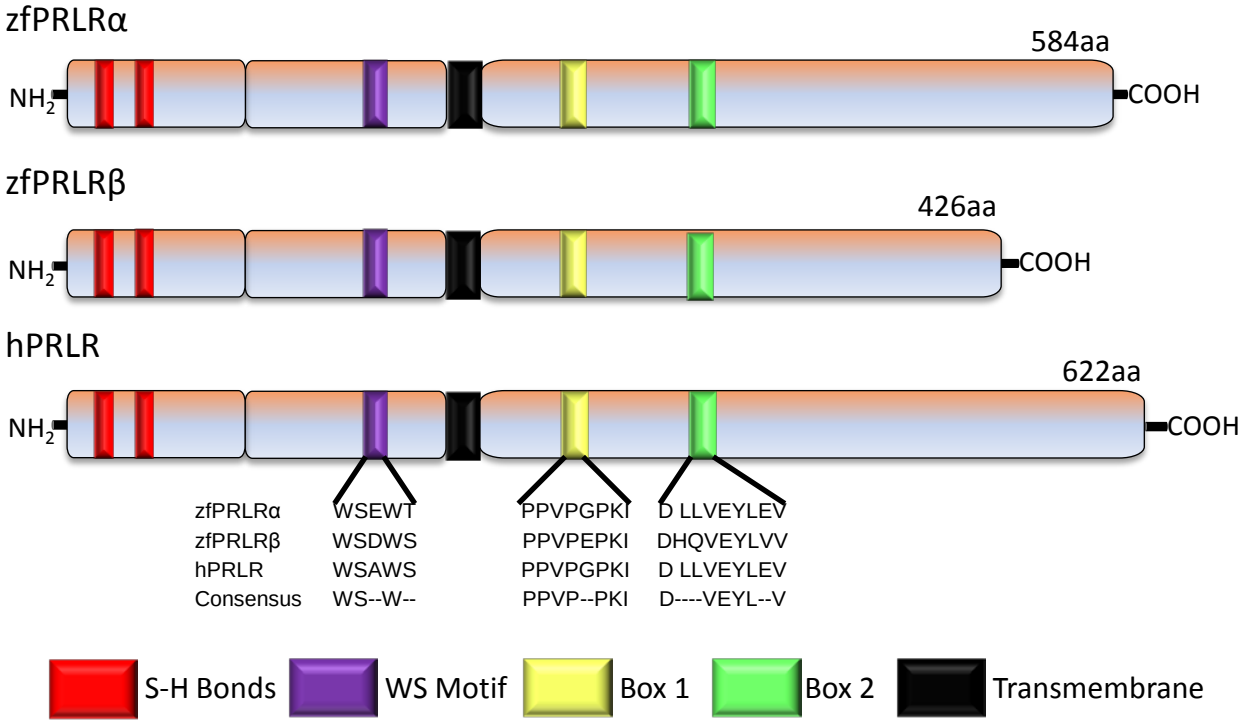


Fig. 2.1-7. Quantitative real-time PCR analyses of zebrafish GHR-I, GHR-II, PRLRα and PRLRβ at different developmental stages and times. (A) growth hormone receptor type I (GHR-I); (B) prolactin receptor alpha (PRLRα); (C) growth hormone receptor type II (GHR-II); and (D) prolactin receptor beta (PRLRβ) throughout early zebrafish development. Cell number (c), hour (h), day (d), ovary (ov). For each given gene, different letters denoted above the bars indicate statistically significant difference (P<0.05) between developmental stage, time, or from ovaries of the gene being measured.

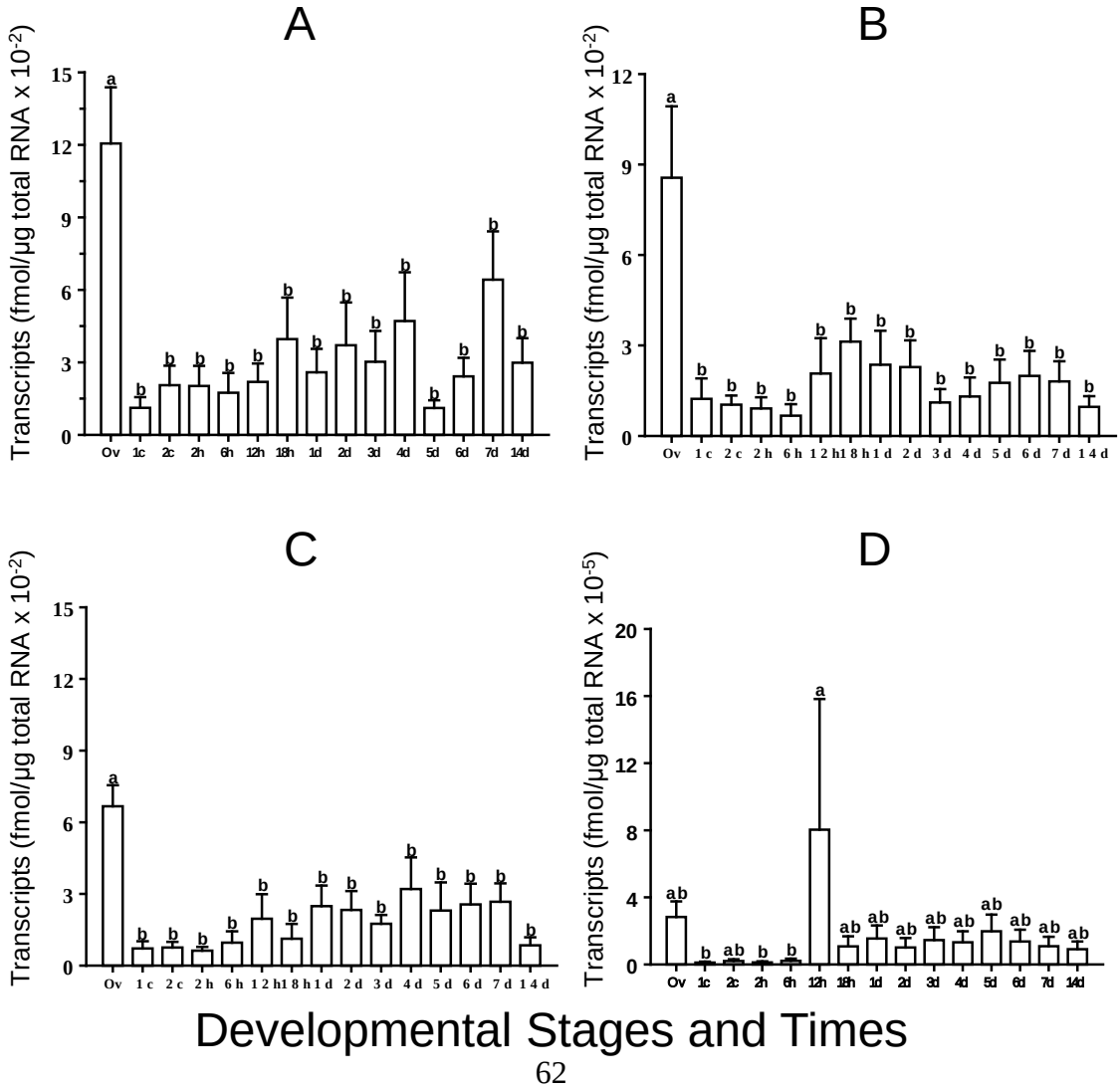


Fig. 2.1-8. Transcript and protein expression of GHR-I, GHR-II, PRLRα, and PRLRβ. (A) Detection of transcripts by means of reverse transcriptase PCR in selected adult zebrafish tissues. (B) Western blot detection of proteins for GHR-I, GHR-II and PRLRα in tissues isolated from adult zebrafish.

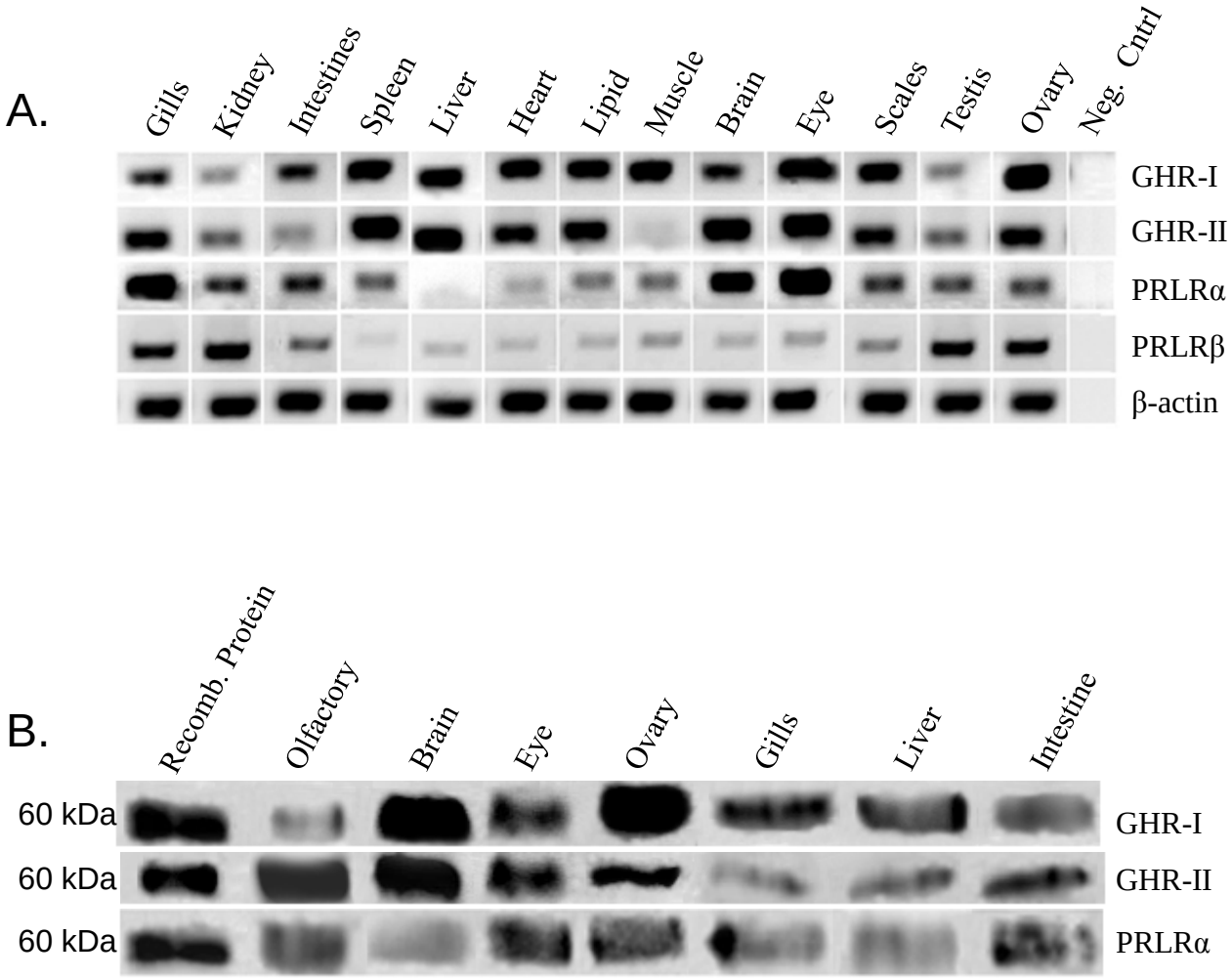


Fig. 2.1-9. Expression of PRLRα in 15 hpf to 24 hpf embryos. Expression of PRLRα was obtained by whole mount *in situ* hybridization using an antisense PRLRα riboprobe. Anterior is to the left (B, E, I, K-M); for all others anterior is up. The expression of PRLRα in the pancreas was indicated by arrowheads; in the kidney by arrows; and * indicates pituitary gland . Scale bar, 50 μm: A, C, D, E, K. Scale bar, 100μm: B, E-G, I-J, L-M. e: eye.

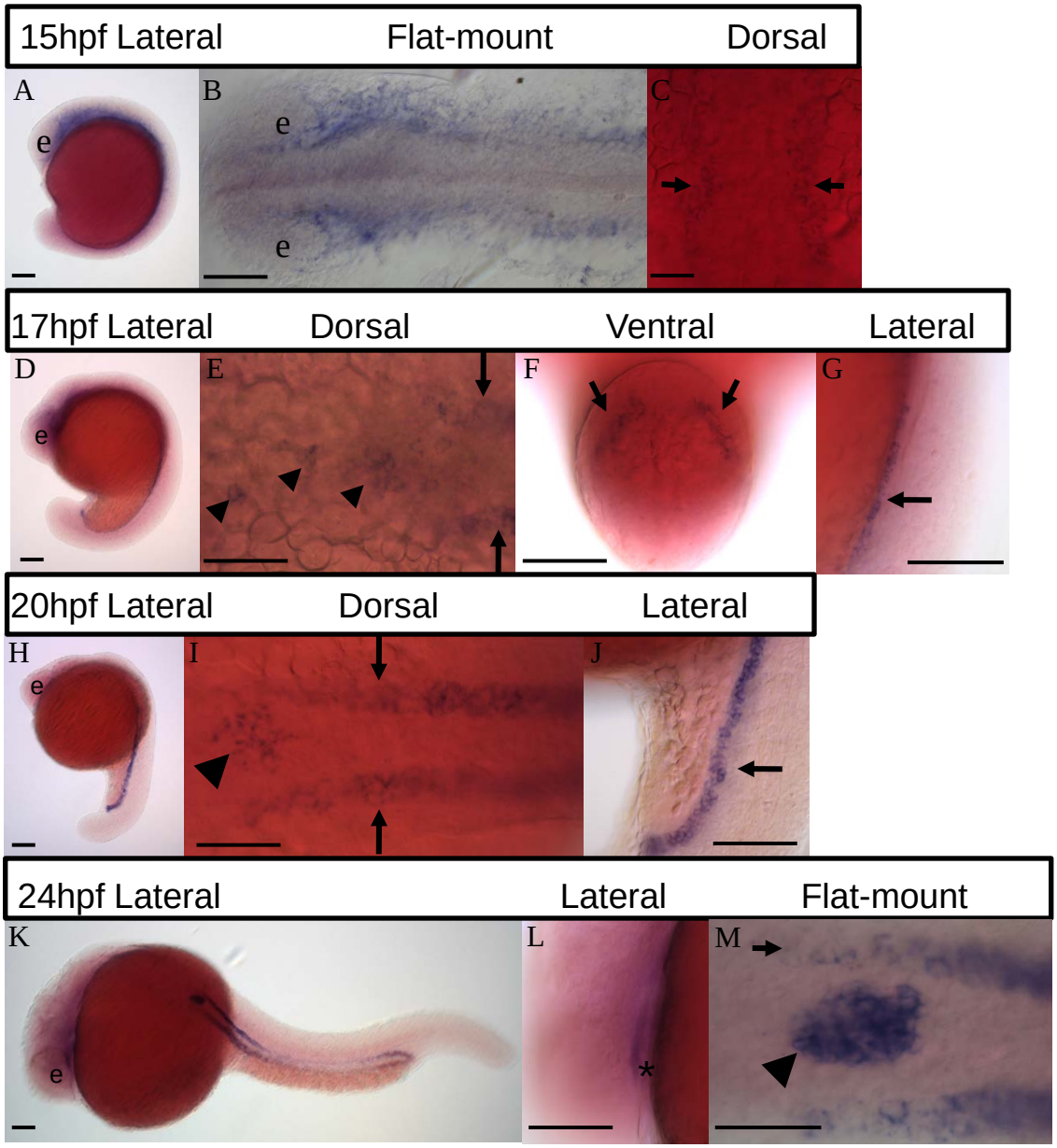


Fig. 2.1-10. Expression of PRLRα in 72 hpf to 5 dpf embryos. Expression of PRLRα was obtained by whole mount *in situ* hybridization using an antisense PRLRα riboprobe. Anterior is to the left. The expression of PRLRα in the pancreas was indicated by arrowheads; in the kidney by arrows; and * indicates expression in the eyes. Scale bar, 50 μm: C, G, J. Scale bar, 100 μm: A-B, D-G, H-I.

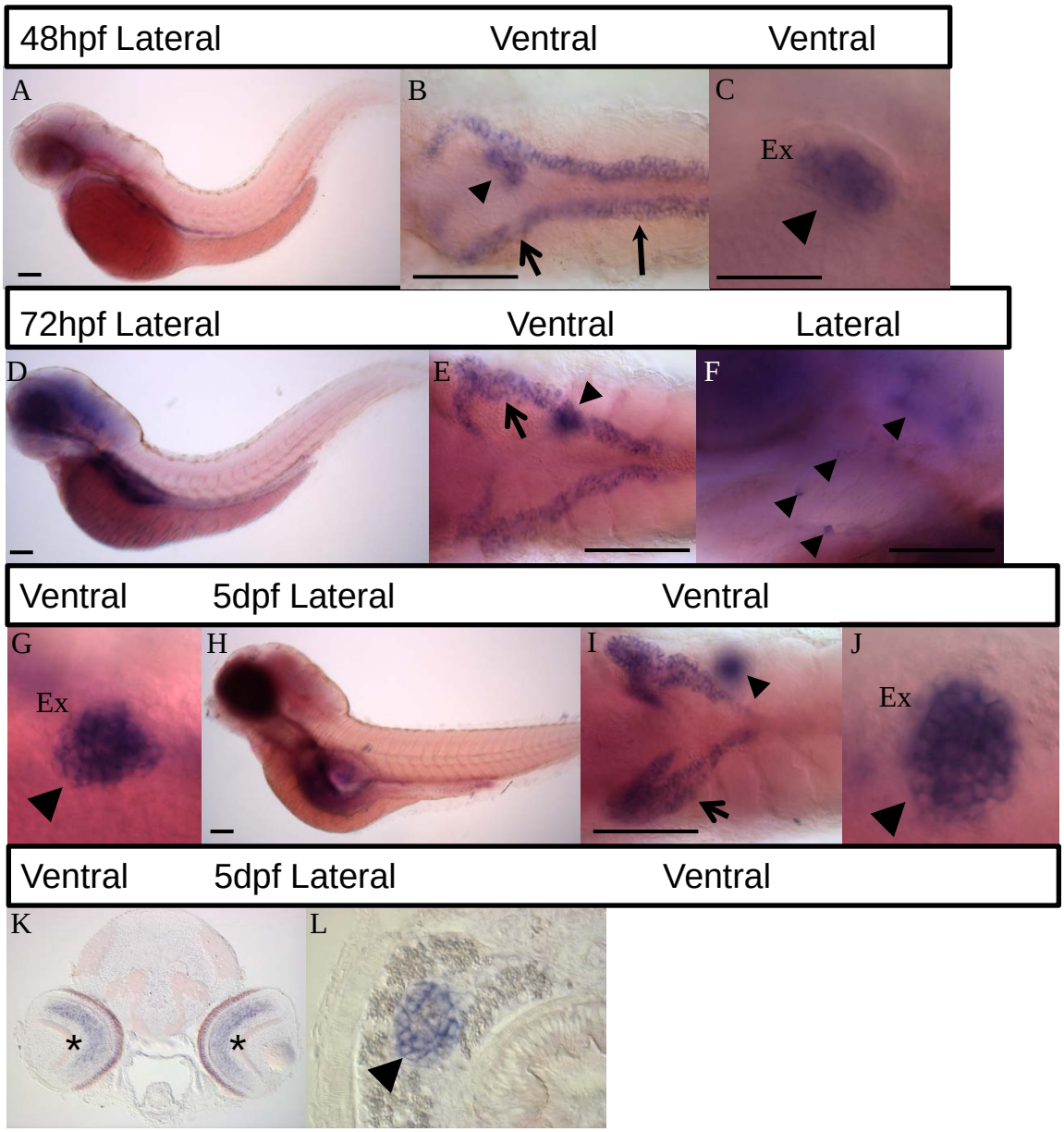


Fig. 2.1-11. Expression of PRLRβ in 17 hpf to 72 hpf embryos. The expression of PRLRβ was obtained by whole mount *in situ* hybridization using an antisense PRLRβ riboprobe. Anterior is to the left. The expression of PRLRβ in the kidney indicates by arrows and * represents expression along developing anterior structures. Scale bar, 100 μm. e: eye.

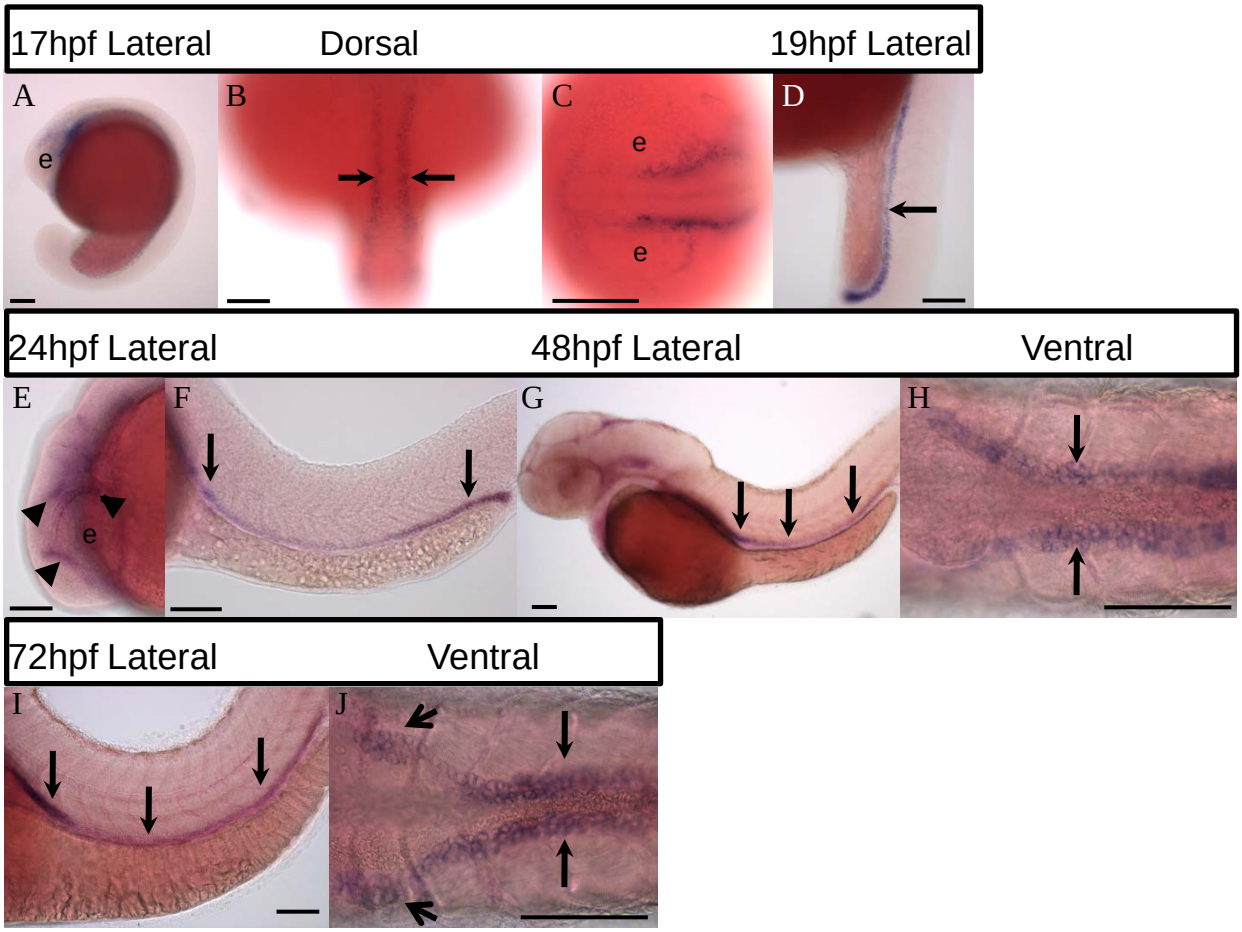
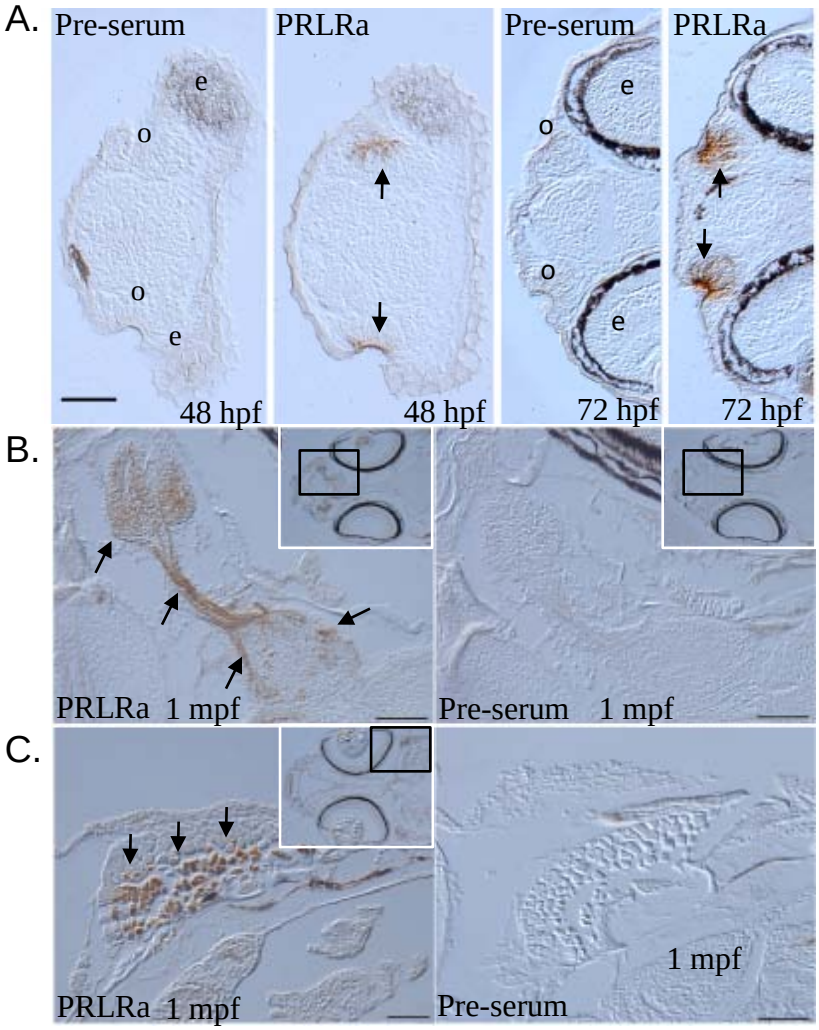


Fig. 2.1-12. Protein expression of PRLRα in the olfactory placode, olfactory bulb and levator arcus palatine. (A) and (B) show strong immunostaining with PRLRα antibody in 48-72 hpf embryos, and 1-month post-fertilization (mpf) juveniles within the olfactory placode and olfactory bulb, respectively. The pictures in (B) are high magnification (20X) of the boxed area of the inserts. (C) Immunohistochemical staining of the levator arcus palatine in 1 mpf juveniles. PRLRα: tissues were incubated with a zebrafish specific PRLRα antibody (anti-zfPRLRα, 1:1000); Pre-serum: control serum lacking anti-zfPRLRα antibody. Arrowheads indicate PRLRα protein positive regions detected by the presence of anti-zfPRLRα binding. Bar: 50 μm.



CHAPTER 2.2: FUNCTIONAL ANALYSIS OF PROLACTIN RECEPTORS

Chapter Summary

We recently demonstrated that *in vivo* inhibition of prolactin (PRL) translation produced embryos with multiple morphological abnormalities, but the molecular mechanisms responsible for these phenotypes are unknown. Zebrafish PRL appears to regulate its function through both prolactin receptor (PRLR) subtypes, PRLRα and PRLRβ, which are also expressed throughout zebrafish development. Our functional knockdown of PRLRα resulting from treatment with antisense morpholino (MO) failed to produce similar morphological defects to the previously described PRL morphant (Zhu et al., 2007), but two independent laboratories using different MOs indicated that the knockdown of PRLRα in zebrafish embryos exhibited many characteristics of PRL morphants. Knockdown of PRLRβ produced different effects on the eye size, but simultaneous knockdown of PRLRβ with PRL produced complementary effects on both eye size and body length, suggesting that PRL may function through PRLRβ. Furthermore, morphological abnormalities associated with PRL morphants were determined to be specific by: 1.) the observation of reduced PRL peptide hormone levels in PRL knockdown, 2.) phenotypes of PRL knockdown were independent of the off-target effect by activation of the p53 pathway, and 3.) multiple constitutively active signaling molecules of the PRLR signaling pathways rescued phenotypes of PRL morphants. We suggest that the JAK2/STAT5 and PI3K/AKT are important signaling pathways responsible for normal anterior structure development and body length during zebrafish embryogenesis.

Introduction

Prolactin (PRL) is a pleiotropic hormone produced and secreted by the anterior pituitary gland and in a variety of extra-pituitary tissues (Ben-Jonathan et al., 1996). The actions of PRL

can be mediated by an autocrine, paracrine or endocrine mechanism (Clevenger et al., 2003, 2009; Grattan and Kokay 2008). These diverse modes of action and sources of PRL contribute to the multifunctional nature of this hormone. PRL has been identified to modulate over three hundred different physiological processes, including effects on water and salt balance, growth and development, brain and behavior, and a critical role in reproduction (Bole-Feysot et al., 1998; Ben-Jonathan et al., 2008).

The biological effects of PRL are mediated by interaction with a membrane bound prolactin receptor (PRLR) belonging to the class 1 cytokine receptor superfamily (Huising et al., 2006). PRL signaling is initiated by hormone binding to two cell surface PRLR monomers, leading to their dimerization and subsequent activation of post-receptor signaling molecules (Rui et al., 1994). PRLR signaling can activate a diverse set of signaling transducers, including the JAK2 tyrosine kinase and the signaling transducers of transcription (STAT), phosphoinositol-3-kinase (PI3K), and AKT (Bole-Feysot et al., 1998). Mammalian PRLRs exists in multiple isoforms with identical extracellular domains (ECD) and varying composition of the intracellular domain (ICD) (Hu et al., 1991, 1996, 2001). Differences in the ICD have been found to mediate divergent signaling pathways (Hu et al., 2001).

Teleosts appear to also have a conserved mechanism of PRLR activation and signaling. The activation of PRLR in fish follows the same mechanism of PRL inducing PRLR dimerization (Le Rouzic et al., 2001). Zebrafish also possess multiple JAKs and STATs that share a high degree of amino acid similarity with their mammalian orthologs (Conway, et al., 1997; Oates et al., 1999a, b; Yamashita et al., 2002). Furthermore, inhibitors of PI3K and AKT in mammalian cell lines were similarly shown to inhibit the PI3K/AKT pathway in zebrafish embryos (Montero et al., 2003; Hong et al., 2006). In fish, the PRLR heterogeneity is a result of

PRLR duplication producing two distinct PRLR isoforms with several differences in the ICD that have been shown to regulate different signaling mechanisms (Huang et al., 2007). These data suggest that zebrafish may serve as a useful model to study the role of PRL and PRLR signaling *in vivo*.

Recently, we demonstrated that PRL is an important regulator for normal development of the eyes, brain, melanophores and body size in zebrafish (Zhu et al., 2007). Our studies also suggest that PRL functions in embryos by acting as an anti-apoptotic factor during zebrafish embryogenesis (Nguyen and Zhu, 2009) and probably holds many functions yet to be discovered. For the first time, PRL was demonstrated to be functional and important for the development of several organs/tissues during vertebrate embryogenesis. These novel results highlight the need to understand the mechanisms that may be responsible for the observed morphological defects due to the reduction of PRL. The focus of this study was to determine the biological significance of PRLRα and PRLRβ using antisense oligonucleotide-mediated knockdown and to examine the signal transducers that may be involved in PRLR signaling during zebrafish development.

Methods and Materials

Experimental Animal and Conditions

Zebrafish, *Danio rerio*, were maintained according to standard protocols (Westerfield et al., 1993). Zebrafish were purchased from a local pet store and maintained at 28.5°C on a 14-hour light and 10-hour dark cycle. Embryos were staged in hours post-fertilization (hpf) and days post-fertilization (dpf) with reference to morphological features as previously described (Kimmel et al., 1995).

Eight females and four males were placed into a 38-l spawning tank for 2 days before collecting the embryos. To prevent the fish from eating the embryos, two layers of marbles were placed in the bottom of the spawning tank the night before embryo collection. Approximately 15-20 min after the beginning of the light cycle, embryos were siphoned from the bottom of the tank.

Microinjection of Antisense Morpholino (MO)

Antisense morpholino (MO) oligomers specific for PRL^{ATGMO}, $PRLR\alpha^{ATGMO}$, $PRLR\alpha^{SpliceMO}$, $PRLR\beta^{ATGMO}$, and $p53^{ATGMO}$ were purchased from Gene Tools, LLC (Philomath, OR) and were microinjected as previously described (Zhu et al., 2007; Nguyen and Zhu, 2009). Sequences for MOs used in this study are listed in Table 2.2-1 and Fig. 2.2-1A.

Rescuing PRL Knockdown using Modified *Prl* mRNA and Constitutively Active JAK2a, STAT5.1, PI3K and AKT

Constitutively active JAK2a (CA-JAK2a) and STAT5.1 (CA-STAT5.1) constructs were kindly provided by Dr. Alister Ward while CA PI3K (CA-PI3K) and AKT (CA-AKT) constructs were a gift from Dr. Juan-Antonio Montero and Dr. Charles Hong, respectively. Capped mRNA for CA-JAK2a, CA-PI3K, CA-AKT were generated by transcription of XhoI (Invitrogen) linearized plasmid DNA (1µg/µl; pA301.CMV.Tel-Jak2a, pCS2-p110CAAX, and pAdTrack.CMV-myr-AKT) using the SP6 and T7 mMessage mMachine kit (Ambion, TX). CA-STAT5.1 (pBK.CMV-Stat5.1) was linearized with BamHI (Invitrogen) and transcribed with T3 mMessage mMachine kit (Ambion, TX). The mRNAs were diluted with nuclease-free water and phenol red dye to a concentration of 50 ng/nl for CA-JAK2a, CA-PI3K, and CA-AKT; and CA-STAT5.1 was diluted to 100 ng/nl. One nanoliter was microinjected into one- or two-cell stage

embryos independently or simultaneously with PRL-MO by the methods described previously (Zhu et al., 2007).

Immunohistochemistry (IHC) and Western Blotting

Three whole zebrafish brains were dissected and immediately fixed in 30 ml of 10% Bouin's fixative (Fisher Scientific). Zebrafish brains were then dehydrated using 10 ml of the following solutions for 30 min unless indicated otherwise at room temperature: washing 2X with 70% ethanol (EtOH), 1X with 95% EtOH, 1X with 100% EtOH, 1X with Xylene, 1X with Xylene:Methyl Salicylate (1:1), and finally 1X with Methyl Salicylate for 1 h. Then samples were mounted in paraffin using cassettes, sectioned at 8 μm thickness, and collected onto a pre-frosted glass microscope slide (Fisher Scientific, Pittsburgh, PA). The sections were then deparaffinized by washing 2X for 5 min with Xylene and rehydrated by washing the sections 1X with 100% ethanol (EtOH), 1X with 95% EtOH, and 1X with 70% EtOH for 5 min each and finally washed 3X with PBS for 5 min. Then, the sections were incubated in blocking solution (PBS with 3% BSA, and 1% Normal goat serum) for 1 h at room temperature. Finally the sections were incubated with anti-salmon PRL (1:10000 diluted in blocking solution) overnight at 4°C. Next, the primary antibody was washed away with PBS for 4X for 5 min and incubated with horseradish peroxidase conjugated to goat anti-rabbit (1:2000; Cell Signaling, Beverly, MA). The secondary antibody was detected using Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol.

Embryos at 24 hpf were dechorionated using 23-gauge needles (BD Biosciences) and transferred to cold Ringer's solution (116 mM NaCl, 2.9mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES) with EDTA (final conc. 1mM) and PMSF (final conc. 0.3mM; general protease inhibitor) (Westerfield et al., 2000). Embryos were de-yolked in deyolking buffer without

calcium (55mM NaCl, 1.8mM KCl, 1.25mM $NaHCO_3$) (Link et al., 2006) by repeated pipetting with a 200 µl pipette tip until the majority of the yolk cells dissolved into the solution. The extent of yolk removal with minimal disruption to the embryo tissue was monitored under a stereomicroscope. The embryos were shaken for 5 min at 1100 rpm (Thermomixer, Eppendorf) followed by centrifugation at 300 x g for 30 sec to pellet and collect the tissues. The supernatant was discarded and 1 µl of lysis buffer was added per embryo along with 20% 5X SDS loading buffer. An extract equivalent to approximately 10 embryos was loaded per lane i.e. 10 µl. Proteins were resolved on a 12% SDS/PAGE gel in a Bio-Rad apparatus (Bio-Rad Laboratories, CA) and electrophoresed at 200V for 1 h on ice. The protein was transferred to a nitrocellulose membrane (Whatman) in transfer buffer (25 mM Tris-base, 192 mM Glycine, 20% v/v Methanol, pH 8.3) at 100V for 1 hour. The membrane was blocked with 5% nonfat milk in TBST (50 mM Tris, 100 mM NaCl, 0.1% Tween 20, pH 7.4) for 30 min at RT and incubated with anti-salmon PRL at a dilution of 1:2000 in 10 ml of 5% nonfat milk in TBST overnight at 4°C. Subsequently, the membrane was washed with 15 ml of TBST five times for 5 min at RT, incubated for 1 h with horseradish peroxidase conjugated to goat anti-rabbit antibody (1:5000; Cell Signaling, Beverly, MA) at RT, and finally washed five times for 5 min each with 15 ml of TBST at RT to remove excess secondary antibody. The Western blots were then treated with a chemiluminescent substrate (Super Signal West Extended Dura Substrate, Pierce, Rockford, IL, USA) at room temperature for 5 min. The signals were digitally recorded using a chemiluminescence image system (FluorChem^{TM} 8800, Alpha Innotech, San Leandro, CA). Protein size was determined by comparing blotted protein size to a biotinylated protein ladder (Cell Signaling, Beverly, MA) following the manufacturer's instructions and β-actin served as the loading control.

Statistical analysis

The significance of the mean differences between various experimental groups was determined by one-way ANOVA followed by Tukey test analysis. A *P* value <0.05 was considered statistically significant.

Results

Functional Analysis of PRLRα and PRLRβ Gene Knockdown

Unexpectedly, microinjection of antisense morpholino (MO) into zebrafish zygotes with either PRLRα^{spliceMO} or PRLRα$^{ATG\text{-}MO}$ did not phenocopy our previous PRL knockdown (PRL-KD) results. Although, RT-PCR indicated an absence of PRLRα transcripts likely due to nonsense-mediated decay (NMD) within PRLRα^{spliceMO} (Fig. 2.2-1B), no morphological defects were observed in PRLRα morphants. Consistently, no complementary effect was observed when PRLRα-MO was co-injected with PRL-MO (Fig. 2.2-3). However, two independent research groups demonstrated that PRLRα knockdown produced several phenotypes similar to PRL-KD (Liu et al., 2006; Lewis et al., unpublished). In contrast, morpholino-mediated gene knockdown of PRLRβ increased eye size at 2.5 ng injection while administration of 1.25 ng or 5 ng of PRLRβ-MO did not affect eye size (Fig. 2.2-2). PRLRβ morphants also displayed shorter body length (Fig. 2.2-2). In addition, co-injection of PRL-MO with PRLRβ-MO produced a complementary reduction of both eye size and body length (Fig. 2.2-3).

Verification of Off-Target Effects

To provide further support that the observed phenotypes in PRL-KD embryos were not an off-target effect caused by activation of p53, embryos were co-injected with PRL-MO and a morpholino against p53 (p53-MO). The eye size, head area, and body length were significantly smaller/shorter compared to the controls; and were similar to microinjection of PRL-MO alone

(Fig. 2.2-4A). The embryos also lacked a swim bladder at 5 dpf (Fig. 2.2-4B). Furthermore, PRL protein was reduced in PRL knockdown embryos (Fig. 2.2-5).

Involvement of JAK2a, STAT5.1, PI3K and AKT in PRL Signaling during Zebrafish Embryonic Development

The involvement of the signaling pathways disrupted within PRL-KD embryos was examined using constitutively active (CA) signaling molecules known to be activated by PRLR, including JAK2a, STAT5.1, PI3K, and AKT. Under the scenario of PRL-KD, it is assumed that PRLR signaling would be reduced, thus leading to defective signal transduction for normal organ development. Co-injection of CA-JAK2a or CA-STAT5.1 with PRL-MO partially rescued eye size, head area, and body length (Fig. 2.2-6A-F). In contrast, injection of CA-JAK2a or CA-STAT5.1 alone did not induce additional defects independently of those shown following PRL-KD alone. Relatively normal development of these structures (i.e. eye size) in single injection of CA molecules indicated that co-injection with PRL-MO partially rescued PRLR signaling through the JAK2/STAT5 pathway. Furthermore, both JAK2a and STAT5.1 partially rescued swim bladder development (Fig. 2.2-6G).

The role of PI3K and AKT were also examined in PRL-KD embryos. Co-injection of CA-AKT with PRL-MO partially rescued body length of PRL-KD embryos, but PI3K did not show a significant rescue (Fig. 2.2-7B, D). In contrast, both PI3K and AKT partially rescued eye size (Fig. 2.2-7A, C). It is interesting to note that eye size was partially rescued to a similar degree when injected independently with either CA-PI3K or CA-AKT along with PRL-MO, suggesting the possibility that the PI3K/AKT pathway is one possible mechanism that operates to maintain normal eye size. PI3K was further shown to rescue head area (Fig. 2.2-7E).

Discussion

Contradictory results obtained for PRL function based on phenotypes induced by PRL-KD (Zhu et al., 2007) relative to those obtained from PRL and PRLR knockout mice (Horseman et al., 1997; Ormandy et al., 1997) has led us to explore the involvement of PRLRα and PRLRβ in early zebrafish development. Functional knockdown of both PRLR subtypes failed to phenocopy PRL morphants, probably due to the incomplete action of MOs used in this study. However, all of the morphological defects associated with PRL morphants were partially reversed by constitutively active signaling molecules known to mediate PRLR function, suggesting that the PRL/PRLR signal transduction is involved in regulating the normal development of the observed morphological abnormalities in PRL-KD embryos.

The failure of our two morpholinos targeted against different regions of the PRLRα gene to produce any phenotype would typically suggest that it is not functional during early development. This would render our previous observation of developmental defects in PRL morphants non-specific. Along the same line, many morpholinos exhibit off-target effects that are not displayed by characterized mutant genes and are represented by common neural cell death with a reduction in both eye and head size (Robu et al., 2007), consistent with our observation in PRL morphants (Zhu et al., 2007; Nguyen and Zhu, 2009). However, two independent groups have previously demonstrated that functional knockdown of PRLRα resulted in reduced eye and head size (Liu et al., 2006) with additional defects such as shortened fins, severe hydrocephaly, and neural mast abnormalities (Lewis et al., unpublished). It is interesting to note that the antisense morpholino used by Liu and colleagues overlapped with our translation blocking morpholino, yet we did not observe any phenotypes. In addition, verification of the effectiveness of our splice blocking morpholino targeted against the PRLRα gene showed a

complete absence of PRLRα transcripts (Fig. 2.2-1B) but no mistarget, off-target, or toxicity related phenotypes were observed. These differences may result from the use of wild-type strains with dissimilar genetic backgrounds. One notable example is the difference in MO activity against the one-eyed pinhead (*oep*) gene with a characterized mutant. In one wild-type strain, approximately 50% exhibited the expected loss-of-function phenotype following treatment with *oep*-MO, while a different wild-type strain failed to respond to the *oep*-MO (Nasevicius and Ekker, 2000). MOs directed against *nagie oko glass onion* and the CXCR4 genes were also observed to possess different MO activities due to strain-specific DNA polymorphism of different wild-type strains (Malicki et al., 2002).

Variation in MO activity resulting from differences in genetic background represents one limitation of the antisense morpholino technology and exemplifies the importance of multiple controls to determine the specificity of the morpholino or use of well established inbred lines, particularly if the gene function has not been characterized (Bill et al., 2009). We have previously demonstrated that *in vitro* transcribed *prl* mRNA with mutations in the PRL-MO binding site partially ameliorated the effects of PRL knockdown (Zhu et al., 2007). In addition, a second translation blocking morpholino and a splice blocking morpholino against the PRL gene produced the same phenotype described for the original PRL morphant (Table 2.2-1; Zhu et al., 2007; unpublished observation). One disadvantage of the translation blocking morpholino is the requirement of a specific antibody to verify the knockdown of the targeted gene. Using an anti-PRL antibody produced against salmon PRL (anti-sPRL), we demonstrated that anti-sPRL antibody specifically recognized the zebrafish PRL-producing lactotrophs of the anterior pituitary (Fig. 2.2-5A), and subsequently the knockdown of PRL proteins in PRL morphants (Fig 2.2-5B). Our PRL morphants exhibited phenotypes similar to those described for off-target

effects with other MOs that have been suggested to result from the activation of p53 (Robu et al., 2007). In this case, we verified the specificity of the PRL knockdown phenotypes by simultaneously co-injecting PRL-MO with a p53-MO (Fig. 2.2-4A-B). The results illustrated that the PRL-MO effects are p53-independent. Together, reduction in anterior structures (eyes and head), shorter body length, and absence of the swim bladder inflation are most likely specific phenotypes of PRL morphants.

The PRLR signal transduction mechanisms mediating various biological activities associated with PRL have been well characterized (Ben-Jonathan et al., 2008). To shed light on the importance of PRLRα and PRLRβ during embryonic development, we examined the JAK2a/STAT5.1 and PI3K/AKT signaling transduction pathways that are mediated by PRLR in various tissues (Freeman et al., 2000). By using constitutively active (CA) signaling molecules, we were able to bypass the requirement of PRLR activation by PRL binding, which is expected to be reduced in PRL knockdown embryos. The ability of CA-JAK2a and CA-STAT5.1 to partially rescue developmental defects in PRL morphants suggest that the JAK2/STAT5 pathway is also important in maintaining normal morphological development of anterior structures and overall body length in zebrafish. Moreover, knockdown of STAT5.1 in zebrafish recapitulates many of the phenotypes observed in PRL morphants (Lewis et al., unpublished), suggesting that STAT5.1 is an important downstream mediator of PRLR function. Although the JAK2/STAT5 mechanism is not unique to PRLRs, delivery of either CA-JAK2a or CA-STAT5.1 molecules alone did not interfere with normal zebrafish development. It is likely that the JAK2a/STAT5.1 pathway is involved in PRLR action during zebrafish development but is restricted to specific cell types. For example, we recently demonstrated that JAK2a rescued apoptosis in the eye and central nervous system of PRL knockdown embryos (Nguyen and Zhu, 2009); suggesting

JAK2a/STAT5.1 pathway potentially mediates the anti-apoptotic function of PRL during early zebrafish development. In fact, PRL has been found to activate JAK2/STAT5 signaling to induce the expression of the pro-survival regulator Bcl-xl (Fujinaka et al., 2007). In addition, JAK2/STAT5 signaling also targets the transcription of the cell cycle regulator c-Myc (Blakely et al., 2005) and cyclin D1 (Brockman et al., 2002; Brockman and Schuler 2005) which if disrupted in PRL-KD embryos could cause the reduction in growth seen in many tissues/organs. Similarly, since both the CA-PI3K and CA-AKT partially rescued eye size, they may also be activated by PRLR in zebrafish. PRL treated lymphoid cells were shown to induce c-Myc expression and promoted cell proliferation and survival by activation of a PI3K/AKT-dependent mechanism (Dominguez-Caceres et al., 2004). In contrast, CA-PI3K rescued head area but not AKT, while the reverse was true for body length in PRL-KD embryos. This observation may reflect the ability of PI3K and AKT to mediate functions independent of each other by activating other downstream signaling molecules. We hypothesize from these results that cell growth, proliferation, and/or apoptosis are general biological processes disrupted in PRL knockdown embryos, leading to the observed phenotypes.

The JAK2a/STAT5.1 and PI3K/AKT signaling pathways appears to be important mechanisms regulated by PRLR in zebrafish development, but our model does not allow us to distinguish which PRLR subtype is mediating these signaling pathways. The teleosts have been proposed to experience a fish-specific whole genome duplication event, resulting in the co-existence of two distinct PRLR subtypes (see Chapter2.1). The two PRLR isoforms in seabream were shown to differ in tissue distribution patterns, post-receptor signaling pathways, and different hormonal responses (Huang et al., 2007). In addition, stably transfected cell lines expressing tilapia PRLR1 and PRLR2 induced different gene expression patterns for *c-Fos*, *Bcl-*

xl, *c-Myc*, and *Spi2.1* (Fiol et al., 2009). In zebrafish, PRLR functions also appear to be controlled by two PRLR subtypes, PRLRα and PRLRβ. The PRLRβ subtype is distinct from PRLRα because of a lack of the conserved Box 2 functional domain and the absence of many tyrosine residues within the intracellular domain (ICD). The differences within the ICD of zebrafish PRLRβ compared to PRLRα likely reduces potential sites for signaling molecule phosphorylation that may lead to activation of different signaling pathways as previously observed in fish species possessing two PRLRs (Huang et al., 2007; Fiol et al., 2009). Recently, it was demonstrated that PRL, had the capacity to trigger downstream post-receptor events by interacting with both PRLRα and PRLRβ in zebrafish, and not with the two GHR-like receptors, confirming that PRL can mediate its specific function though both PRLRα and PRLRβ (Huang et al., 2009). In addition, simultaneous functional knockdown of PRL and PRLRβ resulted in a complementary reduction of eye size and body length, providing support for the notion that PRLRβ is a functional receptor for PRL during zebrafish embryogenesis (Fig. 2.2-3). However, these results do not provide information on the specific signaling molecules responsible for the observed phenotype in PRL and PRLRβ knockdown embryos. On the other hand, Lewis and colleagues demonstrated that PRLRα possessed conserved STAT5 binding sites, suggesting that at least PRLRα is capable of inducing the JAK2/STAT5 pathway. Recently, a new PRL isoform, PRL2, was shown to have a strict binding affinity for PRLRα (Huang et al., 2009). Analysis of PRL2 indicated that it was expressed in extra-pituitary tissues including the eyes and brain and knockdown of PRL2 affected neuron differentiation in retina development (Huang et al., 2009), further complicating the distinction between PRLRα and PRLRβ signaling in our model. Whether PRL and PRL2 have overlapping or unique signaling pathways mediated by PRLRα and PRLRβ, and the significance of potential divergent signaling mechanisms between the two

PRLR subtypes awaits further experimentation.

Although we have proposed that PRLRs likely regulate the normal development of several organs/tissues during zebrafish development, we could not demonstrate a direct function of PRLRs on eye and brain development with our current data. Whole mount *in situ* hybridization (WISH) failed to detect transcripts for either PRLRα or PRLRβ within the eyes or the central nervous system. This may reflect the detection limitation with WISH compared to real-time PCR which is more sensitive at detecting transcripts for PRLRα and PRLRβ (Fig. 2.1-7). The detection limitations between the two techniques may also explain why Huang et al (2009) did not provide evidence for local expression of PRL2 by *in situ* hybridization in zebrafish embryos, but instead used reverse transcriptase PCR to demonstrate the existence of PRL2 during embryonic stages. Alternatively, PRL could mediate an indirect function on eye and brain development by regulating other growth factors. The insulin-like growth factors (IGFs) and their cognate receptors, insulin-like growth factor receptors (IGFRs), have been demonstrated to be essential for normal growth and development, particularly within the central nervous system (Anlar et al., 1999; Russo et al., 2005). Several lines of evidence indicate that lactogenic hormones increase IGFs serum levels both *in vitro* and *in vivo* (Murphy et al., 1988; Hill et al., 1989; Lassare et al., et al., 1991; Karabulut and Pratten, 1998; Karabulut et al., 1999, 2000). More recently, it was demonstrated that PRL increased IGF-2 mRNA in mammary epithelial cells (Brisken et al., 2002). Both IGFs and IGF-1R are expressed in pancreatic β-cells (Fehmann et al., 1996; Hill et al., 1999). The existence of PRLRα within the pancreatic β-cells provides a plausible target for PRL signaling to stimulate production and secretion of IGFs into the circulatory system where they can interact with IGFRs in the brain and eyes. This idea is in line with a previous report indicating that newborn PRLR KO mice had a 70% reduction in IGF-

2 mRNA in brown adipocyte tissues (BAT) and a 35% reduction in plasma IGF-2 levels (Viengchareun et al., 2008). Furthermore, Viengchareun et al (2008) also suggested that the mechanism by which PRLR induced IGF-2 transcription in BAT was mediated by the JAK2/STAT5 pathway. It is possible that in zebrafish, PRL may also activate the JAK2/STAT5 pathway via PRLRα to stimulate the production of IGF-2 that would be secreted and affect other target tissues such as the head and eyes. Knockdown of the IGFRs with antisense morpholino or dominant negative IGFRs in zebrafish resulted in severe developmental retardation with shorter body length and defects in the eyes, head, other parts of the central nervous system (Eivers et al., 2004; Schlueter et al., 2006). Analysis of the IGFs and IGFRs expression level in PRL morphants would lend support for an indirect function of PRL on anterior structure development.

In summary, we provide additional support for the specificity of the previously identified phenotypes for PRL morphants. Although antisense morpholinos against PRLRα appears to be ineffective in our wild-type strain, other laboratories demonstrate phenotypic abnormalities consistent with our PRL morphants (Liu et al., 2006; Lewis et al., unpublished). We further show that PRL proteins are decreased in PRL morphants and that the effects observed in PRL knockdown embryos are independent of the off-target effects activated by p53. Successful rescue of PRL morphants by constitutively active JAK2a, STAT5.1, PI3K, and AKT *in vivo* indicate they are important mediators of PRLR signaling for regulating normal development of the eyes, head, body length and swim bladder. Furthermore, the involvement of these signaling molecules also provide plausible mechanisms (JAK2/STAT5.1 and the PI3K/AKT) that may explain for the observed morphological abnormalities observed in PRL-KD embryos. Finally, we have established the zebrafish as an appropriate model to study PRL functions *in vivo*. Although the PRLRα MOs used in this study were not functional in our pet store bought zebrafish, it

highlights the importance of using properly maintained inbred wild-type zebrafish strains. The combined results from this study provide support for the specificity of the previous phenotypes found in PRL morphants. With the additional confidence that phenotypes of the PRL morphants are specific and the effective use of CA signaling molecules *in vivo*, the zebrafish will be a useful model to identify tissue- and cell-specific actions of these molecules in PRLR targets to further our understanding of PRL/PRLR signaling in zebrafish embryogenesis in the future.

Table 2.2-1. Sequences of antisense oligonucleotide morpholinos used in the study to target *prl*, *prlrα*, *prlrβ*.

Gene Name	**MO Name**	**Target Sequence**	**Target Region**
prl	**ATG-*prl*-MO1**	TAGACCCTTGAGCCATTACTAGAAC	**ATG**
prl	**ATG-*prl*-MO2**	TATTTTCTTGCGTGAATCTGTGTGG	**5’ UTR**
prl	**Spl-*prl*-MO**	GCCGGtaagagtgtactttattacat	**Exon 3**
prlrα	**ATG-*prlrα*-MO**	CATTAGGTGATGATGAGGATTTCCG	**ATG**
prlrα	**Spl-*prlrα*-MO**	GTGGtgggttcaaaataaatacgat	**Exon 4**
prlrβ	**ATG-*prlrβ*-MO**	TCCAGGACACAATGAGAGATGCAGA	**5’ UTR**

Fig. 2.2-1. Positions of morpholinos within the PRLRα gene and verification of PRLRα knockdown. A: Two types of morpholinos are indicated: ATG MO and Splice MO. Bolded and underlined sequences represent primers used for RT-PCR to verify efficiency of PRLRα splice morpholino knockdown. Sequences highlighted in yellow and green represent different exons. B: RT-PCR products using primers specific for PRLRα. Open arrowheads indicate expected size of PRLRα transcripts in the absence of Splice MO and closed arrowheads represent expected truncated PRLRα transcripts in the presence of effective Splice MO.

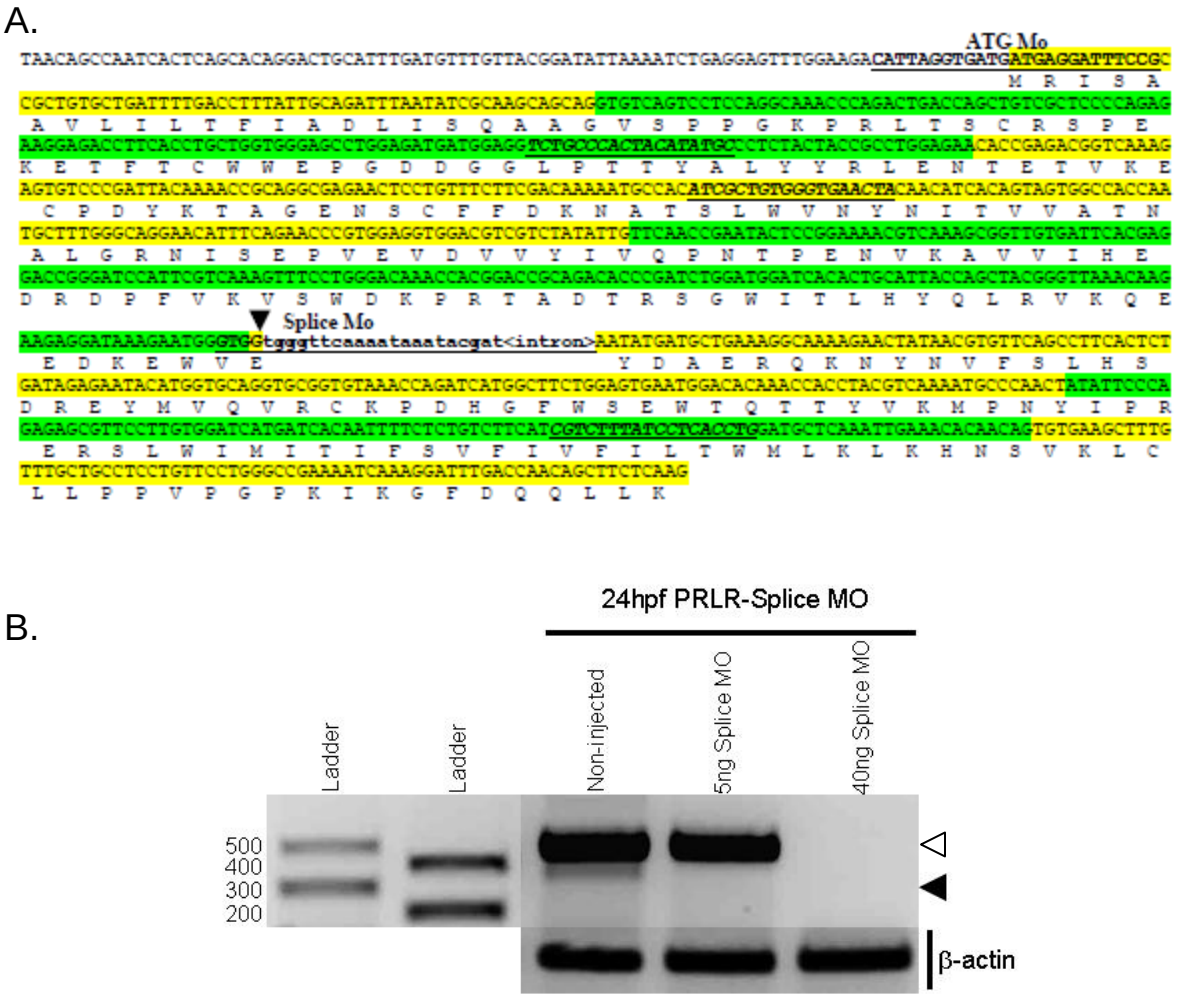


Fig. 2.2-2. Effects of PRLRβ knockdown on body length and eye size at 3 days post-fertilization (dpf) in zebrafish larvae. Results shown as average (mean±SEM) of twenty individuals from one representative experiment. Each experiment consists of a non-injected control group (non-injected), a standard morpholino control group (MO-control, 2.5ng per embryos), a PRL-MO group and three PRLRβ–MO groups (1.25, 2.5 and 5 ng per embryo). Means with different letters indicated statistically significant differences between each treatment group $P<0.05$. Similar results were obtained at least three times from independent experiments.

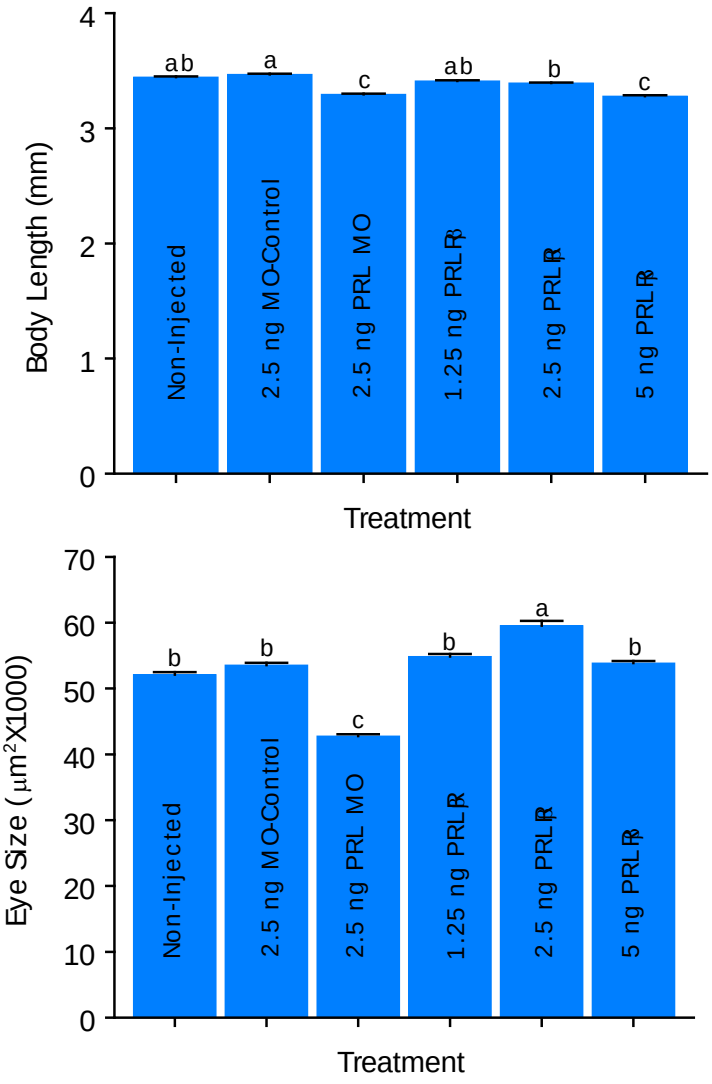


Fig. 2.2-3. Complementary knockdown effects of prolactin and its receptors on body length and eye size at 3 days post-fertilization (dpf) in zebrafish larvae. Results are shown as average (mean±SEM) of twenty individuals from one representative experiment. Each experiment consists of a non-injected control group (non-injected), a standard morpholino control group (MO-control, 2.5ng per embryos), a PRL-MO group and a combination of PRL-MO with either PRLRα-MO or PRLRβ-MO group (2.5 ng per embryo). Means with different letters indicate statistically significant differences between each treatment group with $P< 0.05$. Similar results were obtained at least three times from independent experiments.

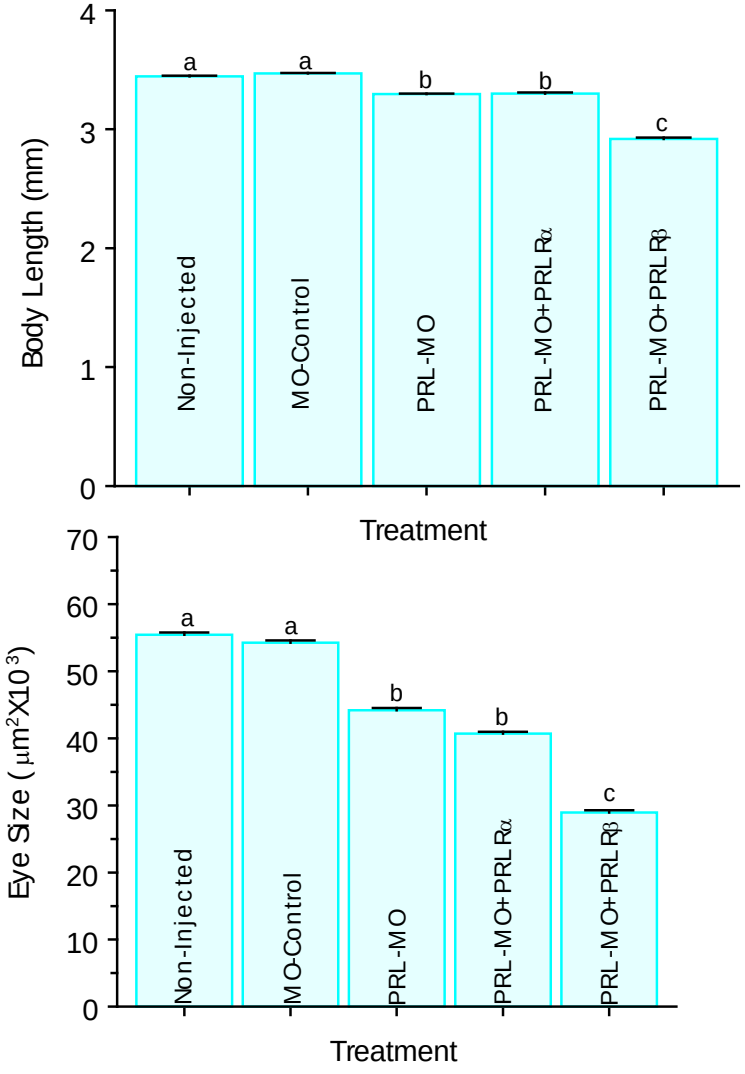


Fig. 2.2-4. Effects of co-injection of p53 morpholino (p53-MO, 2.5 ng/embryo) and prolactin antisense morpholino (PRL-MO, 2.5 ng/embryo) on the eye size, head size, body length and gas bladder of 3 days post-fertilization (dpf) zebrafish larvae. A: Approximately 100 embryos for each treatment group were microinjected at the one-cell stage, and the data were collected at 3 dpf. Similar results were obtained from at 3 independent experiments. (*): show statistically significant differences (P<0.05). from non-injected and morpholino controls (MO-control). B: Representative images of 3 dpf zebrafish co-injected with PRL-MO and p53-MO effect on eye size (lateral) and head size (dorsal). The third panel to the far right represents a lateral view of the gas bladder of zebrafish larvae at 5 dpf.

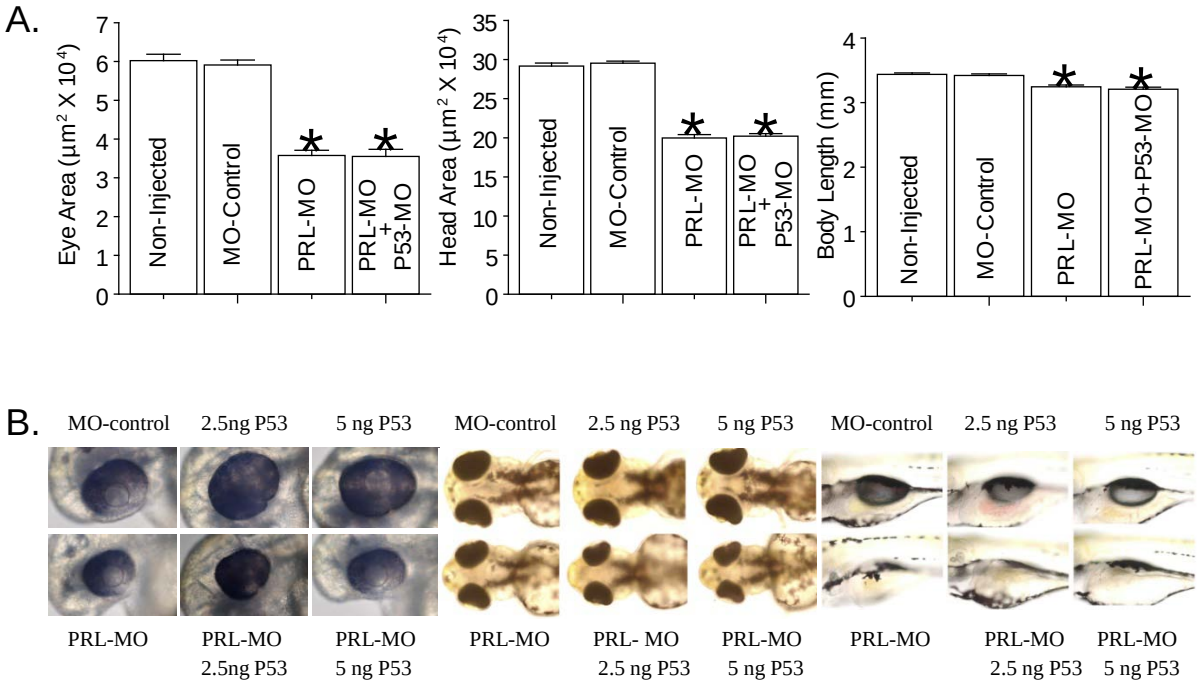


Fig. 2.2-5. PRL morphant effects on PRL protein in zebrafish embryos at 24 hpf. A: Section immunohistochemistry on an adult zebrafish gland using an anti-salmon PRL (sPRL) antibody (1:10000). Closed arrowheads indicate PRL-producing cells. RPD, rostal pars distalis; PPD, proximal pars distalis; PI, pars intermedia. B: Western blot analysis of 24 hpf zebrafish embryos using an anti-sPRL antibody (1:2000) comparing the levels of PRL between non-injected (NI), morpholino control (MO-control), and prolactin morpholino (PRL-MO) embryos. Anti-β-actin was used as a loading control.

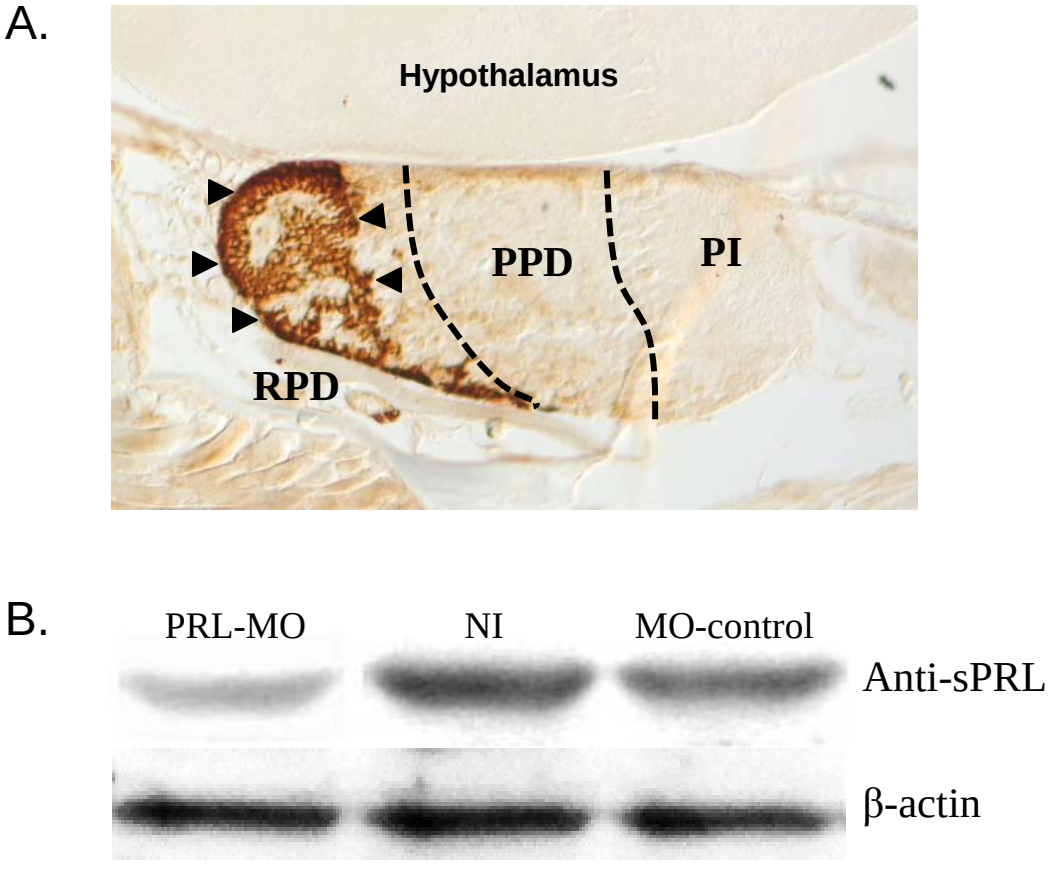


Fig. 2.2-6. The effects of constitutively active JAK2a and STAT5.1 on the eye size, body length, head area, and gas bladder development in PRL-MO embryos. Each bar represents average data of approximately 80 individuals (mean±SEM). Different letters denoted above the bars indicate statistically significant difference (P<0.05) between the treatment groups. Similar results were obtained from 3 independent experiments.

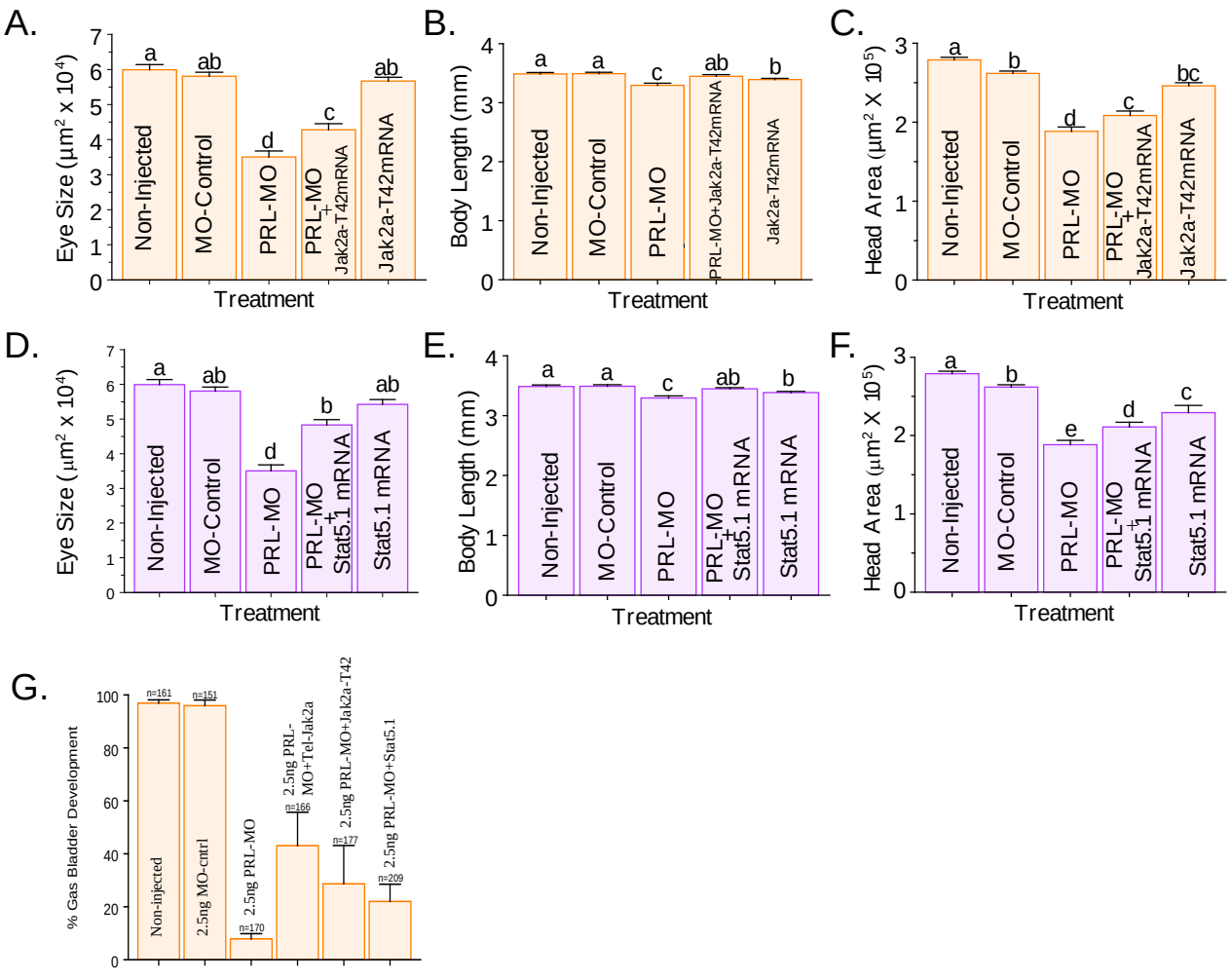
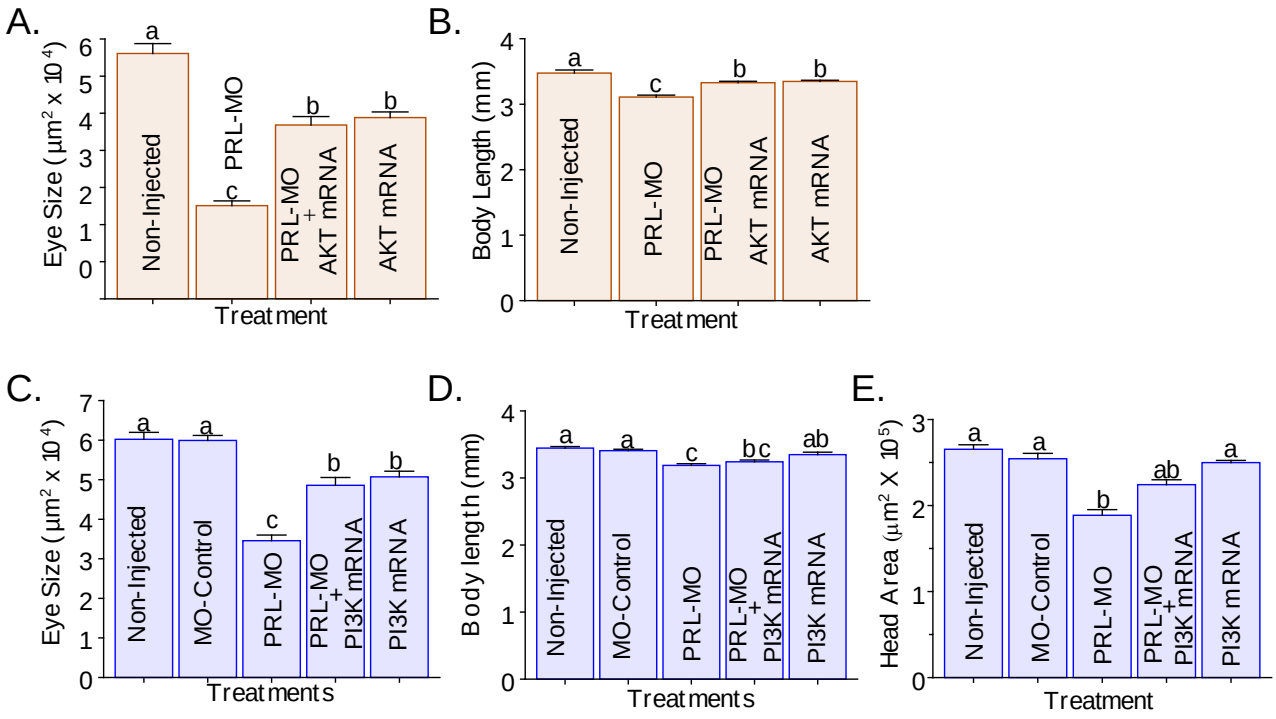


Fig. 2.2-7. The effects of constitutively active PI3K and AKT on the eye size, body length, and head area on PRL-MO embryos. Each bar represents average data of approximately 80 individuals (mean±SEM). Different letters denoted above the bars indicate statistically significant difference (P<0.05) between the treatment groups. Similar results were obtained from 3 independent experiments.



CHAPTER 3: PROLACTIN FUNCTIONS AS AN ANTI-APOPTOTIC FACTOR DURING ZEBRAFISH EMBRYOGENESIS

Chapter Summary

Prolactin (PRL) is a multifaceted hormone that is capable of modulating hundreds of physiological processes in adult vertebrates. However, the physiological functions of PRL in embryonic development are still controversial. One of the biological actions of PRL is to promote survival of cells. Almost all studies on the anti-apoptotic action of PRL have involved the use of mammalian cell lines and tissues, rather than *in vivo*. In order to determine whether PRL acts as a survival factor for embryonic cells during development, PRL protein was knocked-down in zebrafish embryo by the microinjection of PRL antisense morpholino (PRL-MO) to inhibit the translation of the PRL transcript. A significant increase in the number of apoptotic cells was observed in embryos treated with PRL-MO compared to control embryos injected with control morpholino or non-injected controls. The number of apoptotic cells increased more significantly between 15 and 35 hours post-fertilization (hpf). Interestingly, apoptotic cells were restricted to the central nervous system, particularly in the eyes and brain. Apoptosis of these cells was further demonstrated using the Neutral Comet assay to detect DNA damage, a hallmark of apoptosis. It was found that the level of DNA damage was dependent on the dose of PRL-MO injected and consistent with higher levels of nick ends detected by the TUNEL assay in PRL-MO embryos. An examination of genes linked to the apoptotic pathway indicated the transcript of caspase-8, a representative caspase gene of the extrinsic pathway, was significantly higher in PRL knockdown embryos than the non-injected control or control morpholino. Together, these results suggest that PRL acts as a survival factor during zebrafish embryogenesis.

Introduction

Apoptosis or programmed cell death (PCD) is an important biological process involved in the maintenance of tissue homeostasis in adult multicellular organisms and during normal development of vertebrates that eliminates extraneous or damaged cells (Ellis et al., 1991; Jacobson et al., 1997). In adult animals, the primary role of PCD is to restrain the proliferation of abnormal cells. The mechanism by which PCD is induced or inhibited is dependent on cell types and their capacity for self-renewal (Solary et al., 1996), but the common function of PCD is to prevent the progression of disease resulting from proliferation of damaged or abnormal cells. One of the major roles of apoptosis during early embryonic development is to sculpt and shape organ and tissue development (Milligan and Schwartz, 1997). In mammals, PCD has been observed throughout embryogenesis, during the blastocyst stage for cavitation and formation of the inner cell mass (Hardy et al., 1989). PCD has also been demonstrated during gastrulation in mouse, chick, and frogs (Coucouvanis and Martin, 1995; Alnemri et al., 1996; Plosazj et al., 1998,). Similarly, apoptosis is a normal process throughout zebrafish embryogenesis after the mid-blastula transition state and affecting all tissues (Cole and Ross, 2001; Negron and Lockshin, 2004). Despite the different onset of apoptosis among different species of vertebrates, the biochemical and molecular mechanisms of apoptosis appears to be conserved between zebrafish and other vertebrates. A number of zebrafish apoptotic apoptotic regulators such as the bcl-2 family and a variety of caspases are highly homologous to mammalian apoptotic related genes (Inohara and Nunez, 2000). Characterization of the zebrafish bcl-2 gene family with the mammalian bcl-2 family indicates a substantial functional similarity between these vertebrate species (Kratz et al., 2006). Furthermore, examination of the major effector caspase, caspase-3, in zebrafish demonstrates similar characteristics in structure, functions, and substrate specificity

to that of human caspase-3 (Yabu et al., 2001a, 2001b; Yamashita, 2003), indicating functional conservation of the apoptotic pathway.

Recently, we demonstrated that prolactin (PRL) is an important hormone responsible for the proper development of several tissues/cells in zebrafish (Zhu et al., 2007). The temporary knockdown of PRL protein resulted in abnormal development of several tissues resulting in smaller head, absence of swim bladder, smaller eyes, and reduced melanophore differentiation. Some of the physiological processes deviating from normal functioning as a result of PRL knockdown (PRL-KD) may include growth (Shepard et al., 1997), cell proliferation (Bole-Feysot et al., 1998) and neurogenesis (Shingo et al., 2003). All three biological processes may be affected by programmed cell death as a result of abnormal cell growth, inhibition of proliferation, and improper connections among the neuron cells in PRL-KD embryos. Experimental studies over the past decade have provided evidence to support the role of PRL in the suppression of PCD. The anti-apoptotic effect of PRL was first suggested in amphibians as PRL was demonstrated to inhibit thyroid hormone-induced metamorphosis, a process characterized by extensive apoptosis (White and Nicoll, 1981; Ray and Dent, 1986). Subsequently, numerous studies in the Nb2 lymphoma cell lines suggested an anti-apoptotic role for PRL (Buckley et al., 1995; Leff et al., 1996; Krishan et al., 2001).

Since nearly all studies on anti-apoptotic role of prolactin have been conducted *in vitro* by using cell lines, we examined the role of prolactin on cell death *in vivo* in zebrafish embryos in the current study. Furthermore, we investigated the effect of prolactin knockdown on the transcriptional activity of embryonic cells during embryogenesis. These results provide the first functional study on PRL's role in suppressing apoptosis in an *in vivo* system and provide insight into potential physiological roles of prolactin during early embryonic development in vertebrates.

Methods and Materials

Maintenance of Fish, Embryo Collection and Staging

Zebrafish, *Danio rerio*, were bred and reared in multiple 38-l holding tanks. The water temperature was maintained at 28-29°C. The photoperiod was 14 hours light (8:00 am-10:00 pm) and 10 hours dark (10:00 pm-8:00 am). Fish were fed with a high protein food (Fry feed Kyowa B, Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan), three times daily. The ratio of female fish to male fish was kept at 2:1 in 38-l spawning tanks. Two layers of marbles were laid across the bottom of the spawning tanks to prevent fish from eating their embryos the night before embryo collection. Embryos were siphoned from the bottom of the marbles within 15 minutes of spawning after the start of the light cycle in the following morning. Embryos were washed several times with 10% Hank's solution (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$) prior to being subjected to various treatments including microinjection. Then, embryos were transferred to 100 X 15 mm Petri dishes and incubated at 28.5°C. Embryos were staged according to the time post-fertilization and morphological criteria described previously (Kimmel et al., 1995).

Microinjection of Zebrafish Embryos

All chemicals and reagents were purchased from Sigma unless indicated otherwise. Morpholino antisense oligomers for prolactin were purchased from Gene Tools (Philomath, Oregon) and microinjected as described previously (Zhu et al., 2007). Briefly, prolactin morpholino (PRL-MO) was resuspended in nuclease-free sterile water to a concentration of 10 ng/nl (1.25 mM), which was then further diluted to a series of working concentrations of 1.25, 2.5 and 5 ng/nl immediately before the injection using nuclease-free sterile water and phenol red dye. These concentrations of PRL-MO showed specific effect of prolactin knockdown and did

not show any non-specific effect of morpholino (Zhu et al., 2007). The survival rates of morpholino injected zebrafish embryos were generally above 85%, which were comparable to control morpholino and non-injected embryos. The prolactin antisense morpholino was microinjected into embryos at the 1-2 cell stages using glass microcapillary pipettes attached to a micro-manipulator, under a Leica MZ6 microscope (Leica, Germany). Injection was driven by compressed N_2 gas, under the control of a PV820 Pneumatic PicoPump (World Precision 1 Instruments, Sarasota, FL, USA). Microinjection volume was estimated at 1 nl/embryo. Non-injected (NI, physiological state) and morpholino control (Cntrl-MO) injected embryos with no known target transcript in the zebrafish (which takes into consideration the effects of injection) served as control groups for the comparison of PRL-MO injected embryos.

Neutral Comet Assay for Detection of DNA Damage

For analyses of DNA damage, neutral comet assay rather than Alkaline Comet assay was used since the latter typically detects single strand breaks compared to Neutral Comet assay that detect double stranded breaks. Apoptosis is commonly associated with double stranded DNA fragmentation. Furthermore, alkaline conditions can also detect AP labile sites and excision/repair or single stranded DNA damage in the process of the repair mechanism that does not represent DNA damage associated with apoptosis. Dechorionated embryos were macerated and suspended in 50 µl of phosphate buffered saline by repeated pippetting. Low melting agarose (0.8%, 250 µl) was added to the mixture (Sigma-Aldrich). The entire mixture was then spread on a microscope slide pre-frosted with a thin layer of low melting agarose (GibcoBRL, San Francisco, CA, USA) and incubated at 4°C for 15 min. Solidified slides were then incubated in lysis buffer (10 mM Tris HCl (pH 10), 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 1% sarcosyl) for 1 h at 4°C, followed by equilibration in 50 ml of 1X TBE for 45 min (using fresh

1X TBE every 15 min). Next, slides were placed on the base of an electrophoresis apparatus and electrophoresed at 0.7 V/cm and 300 mA for 15 min. Slides were then neutralized by washing three times (5 min each wash) with 0.4 M Tris-HCl (pH 7.5), followed by fixation for 5 min in 100% methanol. Each slide was re-hydrated in ultrapure water for 10 min and stained with ethidium bromide (20 μg/ml). Pictures of "comets" (damaged DNA migrates out from the condensed nucleus, forming structure that resembles a comet) were taken with a fluorescent microscope (Olympus BX-40) and a spot digital camera at 510-560 nm in a horizontal sweep to prevent analysis of the same comets. Three slides were produced for each time point, and fifty individual comets were randomly photographed per slide. Comets were analyzed using Comet Score (AutoComet.com) for the tail moment, taking into consideration both the tail length and tail intensity migrating away from the condensed nucleus. Each treated or control group consisted of three zebrafish embryos with fifty individual comets assessed from each embryo. A mean value of tail moment was obtained for each embryo, and the average of these mean values for all embryos in a group was obtained (Jarvis and Knowles, 2003).

Quantitative Real-Time PCR (qRT-PCR) of RNA for Caspase-8 Gene

Total RNA was obtained from approximately 100 embryos by adding 1 ml TRIzol reagent (Invitrogen), then homogenized using a sonicator (Sonic Dismembrator Model 100, Fisher), and purified following the manufacturer's instructions. Following the manufacturer's (Invitrogen) instructions, first-strand cDNA was synthesized in a 10 μl reaction including 4 μl total RNA (1 μg), 0.5 μl oligo dT primer (0.5 g/L), 0.5 μl 10 mM dNTP, 1 μl 10X RT buffer, 2 μl 25 mM $MgCl_2$, 1 μl 0.1 M DTT, 0.5 μl RNase out and 0.5 μL (25 units) Superscript III reverse transcriptase. Quantitative real-time PCR (qRT-PCR) was performed with SYBR green dye (Stragtagene, La Jolla, CA, USA) in a Cepheid Smart Cycler MX4000 (Cepheid, Sunnyvale, CA,

USA). The PCR mixture (25 µl) consisted of a 1X Cepeid enhancer additive (1 mM Tris, pH 8.0; 0.1 mg/ml bovine serum albumin, non-acetylated; 0.75 M trehalose; 1% Tween-20), 10 µl Master Mix (2.5X) (Eppendorf), 500 nM forward and reverse primers, and 0.25X SYBR green dye. The amplification protocol consisted of an initial denaturation of 95°C for 2 min, followed by 40 cycles of 95°C denaturation for 15 sec, 60°C annealing for 30 sec, and 72°C extension for 30 sec using zfcaspase 8 F3 and zfcaspase 8R3 (Table 3-1).

The same cDNA was used for reverse transcriptase PCR and performed according to the protocol outlined in Chapter 2.1 with the appropriate primers for the candidate apoptosis-related genes in Table 3-1.

Apoptotic Assays

Apoptotic cells of the embryos were determined by TUNEL assay, caspase-3 immunostaining, or acridine orange staining. Embryos were collected at 24 hours post-fertilization (hpf), dechorionated using watchmaker forceps, and fixed in fresh 4% paraformaldehyde at 4°C. After 24 h fixation, the embryos were dehydrated through a series of methanol solutions (30%, 50%, 70%, 90%, 5 min in each solution) and finally preserved in 100% methanol at -20°C for at least overnight. Embryos were then rehydrated in phosphate buffered saline containing 0.1% Triton X-100 (PBST). Following rehydration, embryos were permeabilized with pre-cooled acetone for 15 min at -20°C and washed twice in PBST for 5 min each wash at room temperature (RT). Embryos were then blocked in 1 ml of 2% goat serum for 3 h at RT. Goat serum was removed with two 1 ml washes for 5 min with PBST. PBST was removed and embryos were then incubated in 50 µl of terminal deoxnucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture, 25 µl of Enzyme solution and 225 ul of Labeling solution according to the manufacturer's instructions (Roche Applied Science

Cat#2156792), for 1.5 h in the dark at 37°C. After 5X washes with PBST for 5 min each, embryos were incubated in horseradish peroxidase converter (identifies dUTP) for 30 min at 37°C in a water bath. Excess converter-POD was removed by rinsing 4X for 5 min with 1 ml PBS. Embryos were then developed in 1 ml of metal enhanced diaminobenzidine (for 5 ml solution: used 100 µl of 40 mg/ml DAB in 50 mM Tris, 25 µl NiCl, 5 ml 100 mM Tris, 1.5 µl of 30% hydrogen peroxide).

An anti-active caspase-3 antibody (BD Biosciences #559565, San Jose, CA, USA) generated against conserved region of the active form of human caspase-3 (aa 163-175, CRGTELDCGIETD) shared 85% identity with zebrafish caspase-3 at amino acid 166-177 (CRGTELDPGVETD). Several groups have used the antibody to determine apoptosis in zebrafish (Kratz et al., 2006). Embryos were processed using the same protocol described in the previous paragraph and blocked in 5% goat serum and 2 mg/ml bovine serum albumin for 2 h before addition of anti-active caspase-3 (1:500). Embryos were incubated in primary antibody at 4°C overnight on a shaker. Following three 20 min washes with PBST, embryos were incubated with 1:1000 biotinylated antibody (ABC Vectastain Kit) for 2 h at room temperature. Embryos were washed again with PBST to remove excess biotinylated antibody and incubated with AB reagent (5 µL reagent A+5 µL reagent B/mL in PBST) for 45 min. After several washes with PBST, embryos containing cells possessing active caspase-3 were visualized using diaminobenzidine (20 mg/mL in 50 mM Tris).

Acridine orange is a nucleic acid selective metachromatic vital dye that is a useful and cost effective method for measuring apoptosis. For acridine orange staining, live embryos were dechorionated and submerged in a 1.5 ml microcentrifuge tube containing a final concentration of 5 µg/ml of acridine orange in 10% Hanks' solution. Embryos were incubated at 28.5°C for 30

min in the dark. Prior to fluorescent microscopic image acquisition, 20 embryos were washed 5X with 1 ml of 10% Hanks' solution and mounted in low melting agarose for positioning.

Rescuing PRL Knockdown using Modified *Prl* mRNA and Constitutively Active JAK2a

Construction of modified PRL cDNA for rescue was described previously (Zhu et al., 2007). Capped mRNA from the mutated *prl* cDNA was generated by transcription of Not I (Invitrogen) linearized plasmid DNA (1μg/μl; pCS2^{+}.EGFP-MutatedPRL) using the SP6 mMessage mMachine kit (Ambion, TX). Constitutively active JAK2a (CA-JAK2a) construct was kindly provided by Dr. Alister Ward. Capped mRNA for CA-JAK2a was generated by transcription of Xho I (Invitrogen) linearized plasmid DNA (1μg/μl; pA301.CMV.Tel-Jak2a) using the SP6 mMessage mMachine kit (Ambion, TX). Both mRNAs were diluted with nuclease-free water and phenol red dye to a concentration of 0.3-0.45 ng/ml for *prl* mRNA and 50 ng/nl for CA-JAK2a. One nanoliter was microinjected into one or two-cell stage embryos by the methods described previously (Zhu et al., 2007).

Statistical Analysis

The significance of the mean differences between various experimental groups was determined by one-way ANOVA followed by Tukey test analyses. A *P* value <0.05 was considered statistical significant.

Results

Inhibition of PRL Translation Results in Increased Apoptosis in the Central Nervous System

A dose-dependent increase in DNA damage, assessed by the Neutral Comet assay, was observed in embryos treated with increasing concentrations of prolactin morpholino. Significantly higher levels of DNA damage were observed in the prolactin knockdown embryos

treated with 2.5 ng or 5 ng prolactin morpholino compared to both the non-injected and control morpholino-injected embryos at all time points examined (Fig. 3-1). Although there was an apparent increase in DNA damage between 16 to 20 hours post-fertilization (hpf) in control embryos, the changes were not statistically significant (Fig. 3-1).

There were a larger number of apoptotic cells observed in prolactin knockdown embryos than in control morpholino injected embryos during embryonic development (Fig. 3-2). Most of the apoptotic cells were localized within the eyes and throughout the brain. The result was further confirmed by the TUNEL assay (Fig. 3-3). The effect of prolactin knockdown on apoptotic cells was reduced by co-injection of prolactin morpholino with either mutated *prl* mRNA that has low binding affinity with prolactin morpholino or constitutively active JAK2a (Fig. 3-3). Similar results were obtained using TUNEL assay, whole mount immunostaining with anti-active caspase-3 (Fig. 3-3A), or acridine orange staining (3-4).

Increased apoptosis in PRL-KD embryos involves caspase-8 and caspase-3

Several apoptotic-related genes, Bax, bad, caspy, caspy-2, caspase-3, and p53, were all present between 14 and 30 hpf, but the levels of these transcripts between the controls and prolactin knockdown embryos were not significantly different (Fig. 3-5). Interestingly, caspase-8 transcript, an essential component of the extrinsic apoptotic pathway, increased significantly in prolactin knockdown embryos compared to control morpholino or non-injected controls (Fig. 3-6).

Discussion

In this study, we were the first to demonstrate an anti-apoptotic role of prolactin in zebrafish embryos *in vivo*, although the anti-apoptotic role of prolactin has already been reported in cell lines and carcinoma tissues. Our results suggest that prolactin suppresses cell death in

zebrafish embryos at least partly by inhibiting DNA damage and the expression of the initiator caspase-8. In addition, prolactin-dependent apoptosis in the central nervous system in prolactin knockdown embryos provides a plausible explanation for the observed reduction in the eye size, head size, and melanophore differentiation of prolactin knockdown embryos reported previously (Zhu et al., 2007).

During segmentation at 10-24 hpf in zebrafish, many organs begin to form, including the brain, eyes, and melanophores. This period coincides with a progressive increase in programmed cell death associated with normal development (Furutani-Seiki et al., 1996; Cole and Ross, 2001). The reduction in the size of the brain and eyes or in the number of melanophores in prolactin knockdown embryos may be partially attributed to increased cell death as a result of decreasing prolactin levels. We have recently shown that both the transcripts for prolactin and its associated prolactin receptor (PRLR) are present throughout zebrafish development (Nguyen et al., 2008). Knocking down the levels of both the prolactin hormone and the receptor increased apoptosis during early zebrafish embryogenesis (unpublished data). In fact, prolactin has been implicated to function as an anti-apoptotic factor in a number of cell lines and/or tissues derived from the mammary gland, prostate, and ovaries (Ahonen et al., 1999; Tessier et al., 2001; Ruffion et al., 2003; Asai-Sato et al., 2005) along with other specific cell types. These results suggest that the anti-apoptotic role of prolactin is conserved in vertebrates and that prolactin plays an important role in not only survival of carcinogenic tissues or cells in adults (Yamashita, 2003) but also in normal development of vertebrates.

Apoptosis during normal zebrafish development is initially observed at the onset of the tail bud stage (Yabu et al., 2001b). As embryogenesis progresses, there is an increase in apoptosis until the formation of the neural tube (~24 hpf). Knockdown of prolactin further

increased apoptosis between 14-24 hpf compared to controls. Consistent with the increase in the level of apoptosis, embryos treated with prolactin morpholino also suffered from increased DNA damage which is an indication of apoptosis. Prolactin has been demonstrated to suppress DNA fragmentation in Nb2 lymphoma cells (LaVoie and Witorsch, 1995). Increased DNA degradation was also reported in mammary glands with a deficiency in growth hormone and prolactin (Travers et al., 1996). However, further studies are required to delineate the specific mechanism responsible for the inhibitory effects of prolactin on DNA fragmentation leading to apoptosis.

Interestingly, these PRL-KD-induced apoptotic cells appear to be localized mainly in the central nervous system, around the eyes, surrounding the ventricles, and the optic tectum. Apoptotic cells restricted to the central nervous system suggests that the majority of the dying cells are neuronal derived, including the melanophore cells which are neural crest derivatives. Shingo and colleagues recently demonstrated that prolactin stimulates neurogenesis in the maternal brain during pregnancy (Shingo et al., 2003). Reduced prolactin may be accompanied by insufficient growth factors that would eventually lead to apoptosis of these neuron cells. The rescue effect resulting from using constitutively active JAK2a mRNA or mutated *prl* mRNA further supports the anti-apoptotic role of prolactin in zebrafish embryos.

Prolactin-treated cells have been found to promote cell survival by mediating the up-regulation of a number of pro-survival members of the bcl-2 family in mouse mammary epithelial cells, human breast cancer cells, and rat prostate (Leff et al., 1996; Coppenolle et al., 2001; Peirce and Chen, 2003). However, we found that none of the bcl-2 family of genes examined was differentially expressed during early zebrafish development. One of the reasons for the discrepancy may be due to the use of whole zebrafish embryos for cDNA synthesis. The apoptotic cells were restricted to specific regions of the eyes and central nervous system. First

strand cDNA synthesis using whole embryos that include the yolk and other regions that do not harbor apoptotic cells could greatly dilute the actual levels of transcripts being detected. Alternatively, the levels of these transcripts may not differ, but the functional protein concentrations may be altered.

We have demonstrated for the first time, a physiological role of prolactin during embryonic development and provided further evidence for the anti-apoptotic role of prolactin, indicating another functional conservation between zebrafish and mammalian models. It is known that more than half of neuronal cells undergo programmed cell death during the formation of the central nervous system. Although prolactin knockdown embryos appear to have a proportionate reduction in some physical structures, continued cell death in the central nervous system in prolactin knockdown embryos can potentially cause neurodegenerative diseases or decrease the cognitive ability of the embryo in the long term. One of the future goals is to determine the long-term effects of the morphological abnormalities (Zhu et al., 2007) and apoptosis (current study) in prolactin knockdown fish. In addition, it will be interesting to determine the relationship between prolactin and the apoptotic signaling pathway in zebrafish embryogenesis and the possible cross-talk between the two disparate signaling pathways.

Table 3-1 Primers used for RT-PCR of genes involved in the apoptotic pathways.

Gene Name	**Primer Name**	**Direction**	**Target Sequence**
Caspase-8	zfcaspase 8 F3	Forward	GCCTCTTGGATACTGTCT
	zfcaspase 8 R3	Reverse	CCAAAACTGTGCCCTTCT
Bad	zfbad F1	Forward	GACTTGCTGGAAACTGGA
	zfbad R1	Reverse	AGAAATGCCAACCAGCTG
Bax	zfbax F1	Forward	GCTGCACTTCTCAACAAC
	zfbaxR1	Reverse	GTCGGCTGAAGATTAGAG
Caspase-3	zfcaspase 3 F1	Forward	AATGACCAGACAGTTGCG
	zfcaspase 3 R1	Reverse	GAGCCGGTCATTGTGTTT
Caspy-2	zfcaspy2 F1	Forward	CTGGAGAATAAGGACCGT
	zfcaspy2 R1	Reverse	TTTCCTTTGAGTCCCGCT
Caspy	zfcaspy F1	Forward	CGCGTCCGAAAATCTACA
	zfcaspyR1	Reverse	AGCAAGGCCAGTCGTTTT
p53	zfp53 F1	Forward	GATGGAGATAACTTGGCG
	zfp53 R1	Reverse	GGTTTTGGTCTCTTGGTC
β-actin	zfβ-actin	Forward	TTCGAGACCTTCAACACCC
	zfβ-actin	Reverse	TGGTGGTGAAGCTGTAGCC

Fig. 3-1. Neutral comet assay for DNA damage. The insert pictures are representative of no DNA damage (left) vs. a cell undergoing DNA damage (right). Migration of damaged DNA proceeds to the right toward the anode in the electrophoretic field, while non-damaged but loosely compacted DNA surrounds the "head" (nucleus). Three embryos were analyzed per treatment and fifty individual comets were examined for each embryo. The parameter used to measure the extent of DNA damage in each treatment was the tail moment, which is the product of the tail length and tail intensity migrating away from the condensed nucleus. (*) indicates statistically significant difference (P<0.05) between treatments in a given time point.

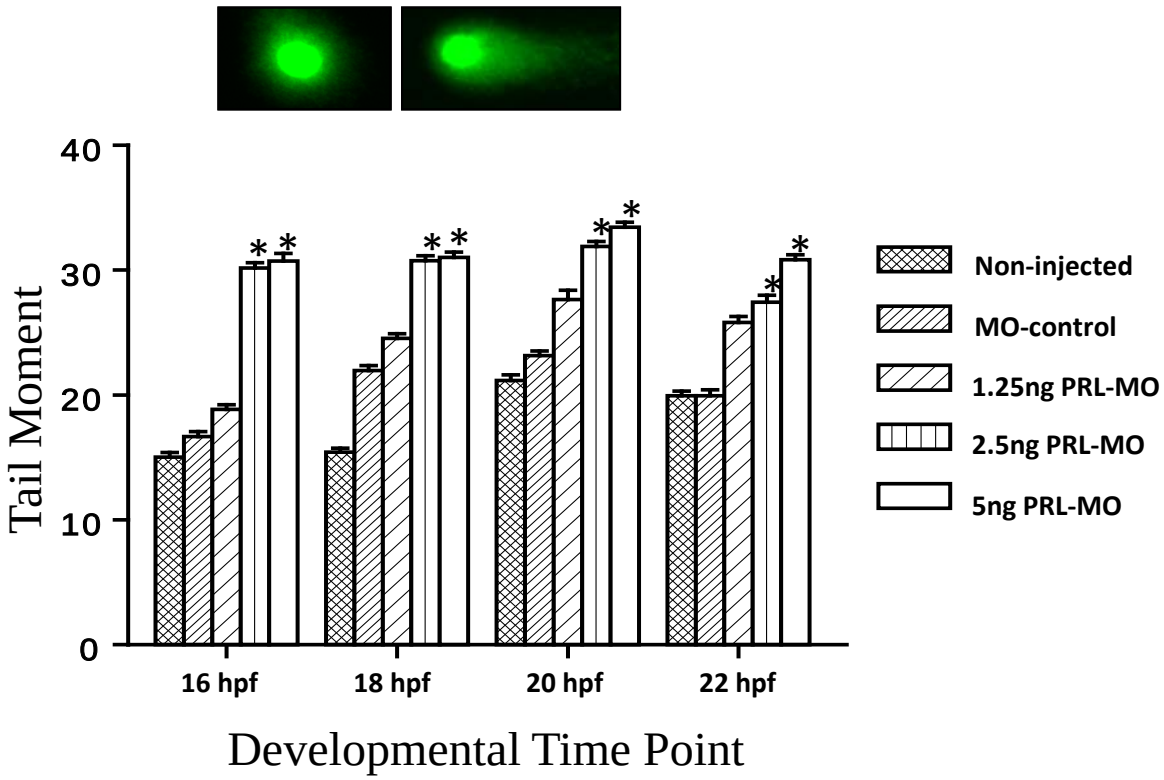


Fig. 3-2. Whole-mount immunostaining of PRL-MO-treated embryos using an anti-active caspase-3 antibody. Knockdown embryos were sampled from four time points between 18 hours and 24 hours post-fertilization (hpf). Control embryos were injected with morpholino control (MO-control) with no known sequence specificity to any zebrafish gene. Arrows represent sites of apoptosis; arrowheads point to regions undergoing apoptosis off the body axis of the embryo.

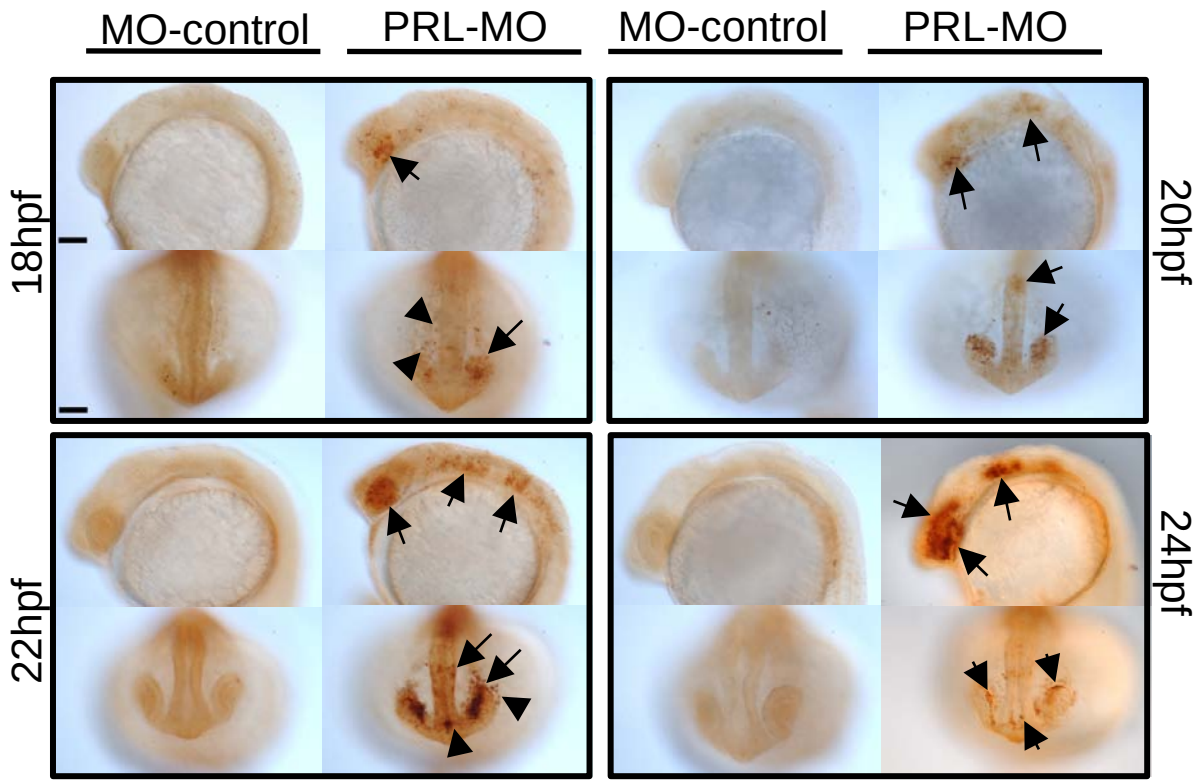


Fig. 3-3. Comparison of apoptotic cells in zebrafish embryos at 24 hpf with different treatments. Embryos were treated with prolactin morpholino (PRL-MO), PRL-MO co-injected with either mutated prolactin transcript (PRL mRNA) or constitutively active JAK2a transcript (CA-JAK2a), and controls (growth hormone morpholino, GH-MO; non-injected; or MO-control). A). Representative images of zebrafish embryos analyzed by anti-active caspase-3 immunostaining (top panel) and TUNEL assay (bottom panel). Apoptotic regions are indicated by arrows in both panels. B). Results shown as average number (mean±SEM) of apoptotic cells in individual larvae (n=3) stained with TUNEL assay from a representative experiment. (*) indicated statistically significant differences from control. The experiment was repeated at least three times.

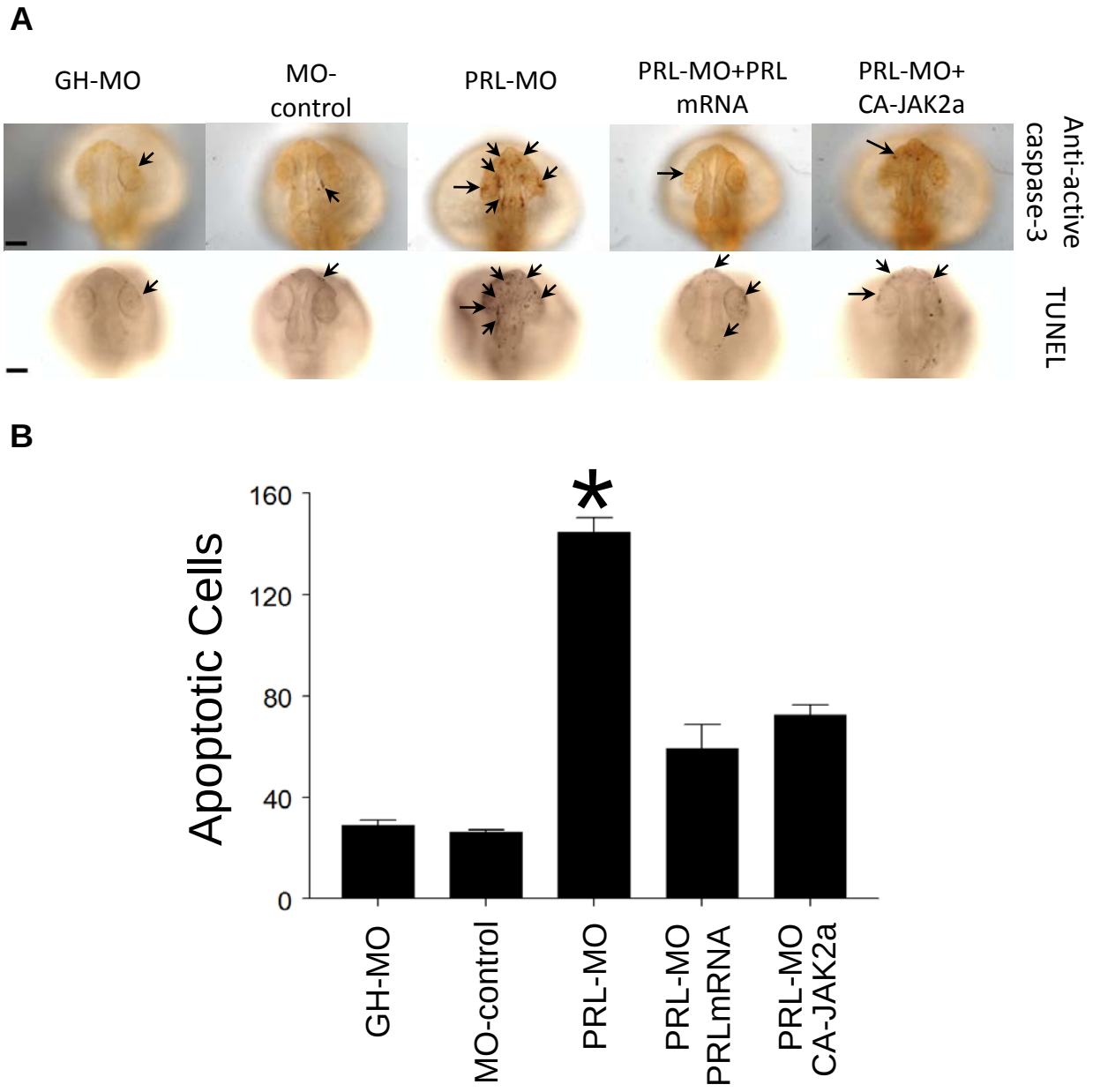

A
GH-MO
MO-control
PRL-MO
PRL-MO+PRL mRNA
PRL-MO+ CA-JAK2a
Anti-active caspase-3
TUNEL
B
Apoptotic Cells
160
120
80
40
0
*
GH-MO
MO-control
PRL-MO
PRL-MO PRLmRNA
PRL-MO CA-JAK2a

Fig. 3-4. Comparison of AO staining between non-injected embryos, PRL morphants, and PRL mRNA rescued embryos. Embryos were treated with prolactin morpholino (PRL-MO) or PRL-MO co-injected with PRL mRNA at 24 hours post-fertilization (hpf). Fluorescent microscopic images of representative were larvae viewed from three different positions (lateral, dorsal/lateral, dorsal) stained with acridine orange (AO) with superimposed white dots representing apoptotic cells. The experiment was repeated at least 3 times. (e, eye; n, notochord; Y, yolk; F, forebrain ventricle; M, midbrain ventricle; H, hindbrain ventricle.

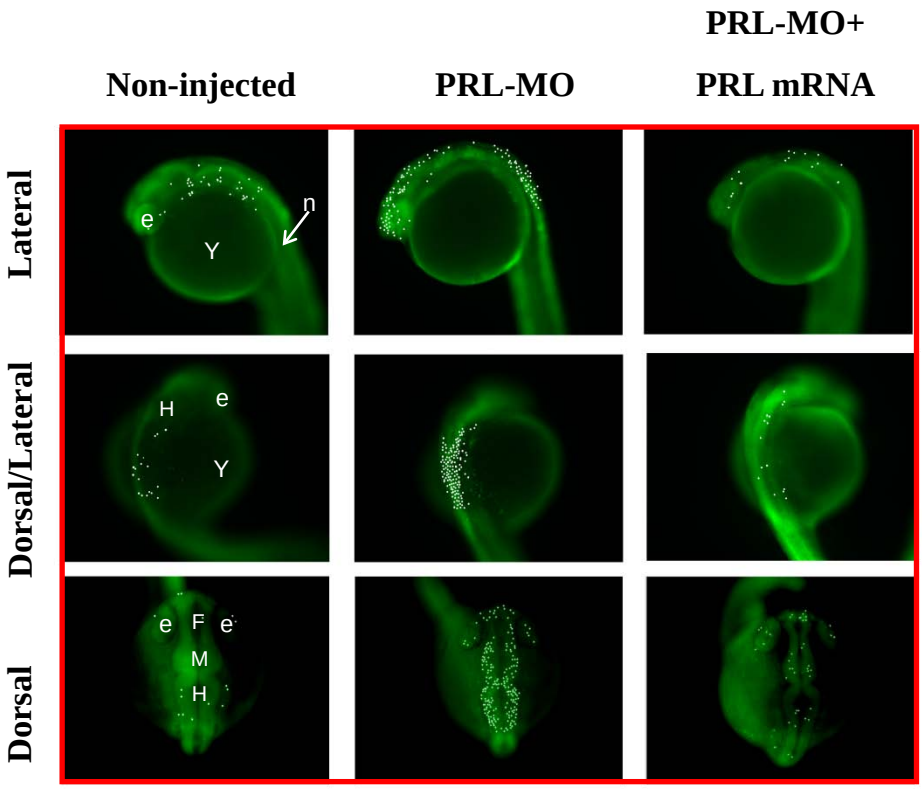


Fig. 3-5. Differential expression of apoptotic related genes analyzed by RT-PCR in PRL-MO-treated 18-30 hpf zebrafish embryos. Expression of each transcript was compared between (1) non-injected, (2) MO-control and (3) 2.5ng PRL-MO. RT-PCR products were electrophoresed on a 2% agarose gel, detected by staining with ethidium bromide, and photographed. β-actin was used as a loading control.

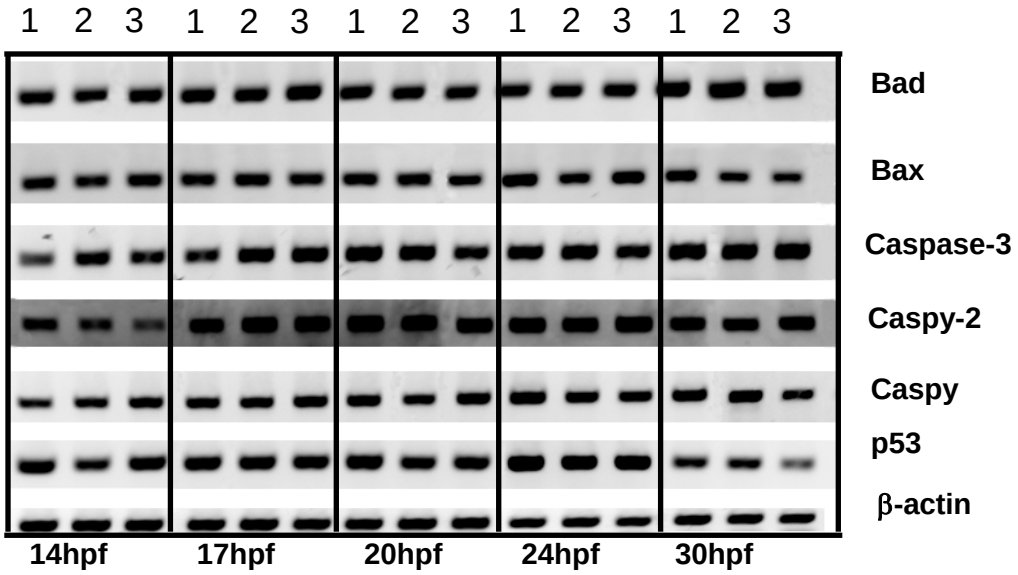
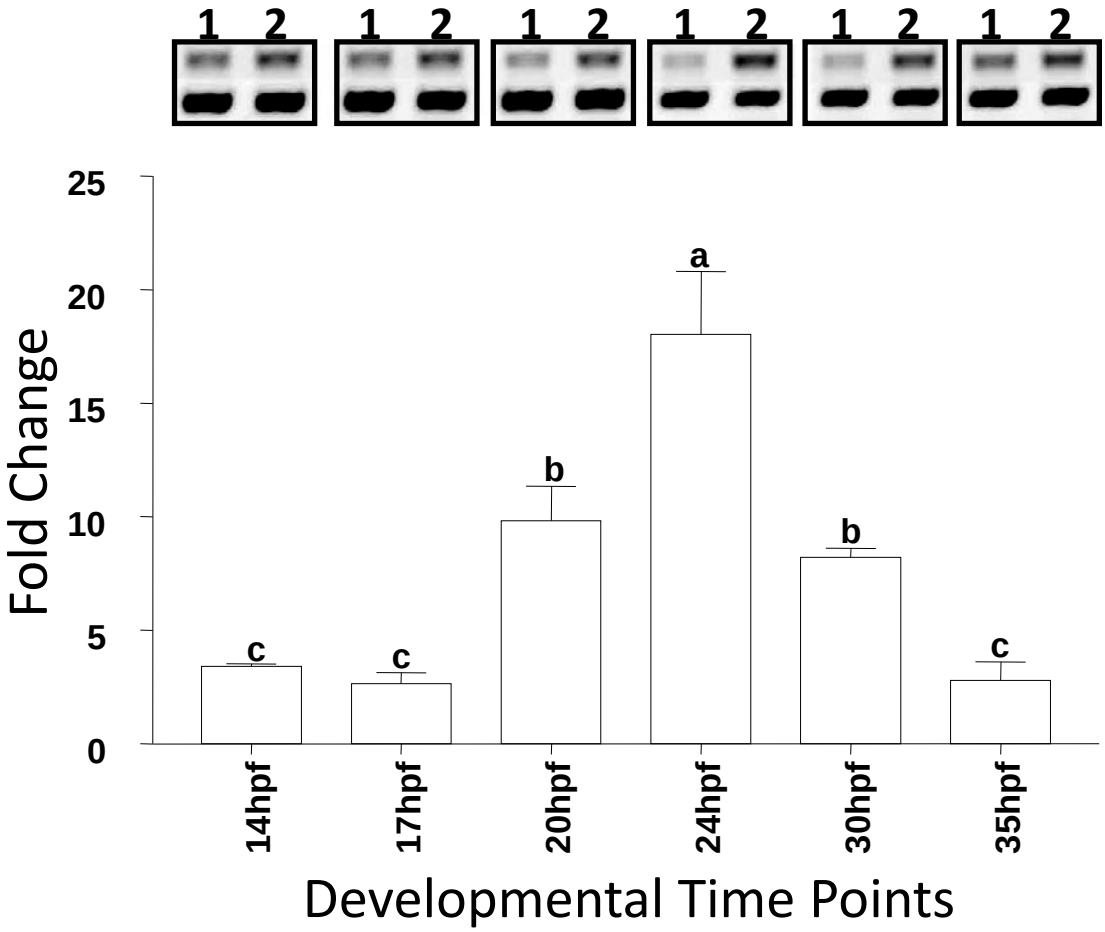


Fig. 3-6. Comparison of caspase-8 transcript in prolactin morpholino and control embryos analyzed by real-time PCR. The results were expressed as a ratio of caspase-8 in embryos injected with 2.5ng PRL-MO to MO-control embryos. The expression level was normalized with β-actin. Values were average of three independent experiments. Significance ($P<0.05$) is denoted by different letters. Insert panel: representative results analyzed from one experiment. Expression of each transcript was compared between (1) MO-control and (2) 2.5ng PRL-MO at each of the developmental time points sampled.



CHAPTER 4: PRLRα MAINTAINS NORMAL β-CELL POPULATIONS DURING PANCREAS DEVELOPMENT IN ZEBRAFISH

Chapter Summary

Previous studies have indicated that prolactin (PRL) plays a major role in the up-regulation of maternal insulin levels and β-cell population in response to the increase metabolic demand of the fetus during pregnancy. However, little is known about the roles of PRL on β-cells in early pancreatic development. We examined the roles of PRL and PRL receptor alpha (PRLRα) by establishing a transgenic line that conditionally expresses a non-functional PRLRα (*nPrlrα*) in β-cells under the regulation of the zebrafish insulin promoter. Antisense oligonucleotide morpholino-mediated PRL knockdown in whole embryos unexpectedly increased the β-cell population and insulin transcripts, but it was accompanied by an increase in endogenous PRLRα mRNA within the endocrine pancreas. The *nPrlrα* transgenic line was produced to express a hybrid transcription factor (LexPR transactivator) that functions in a ligand-dependent manner to induce expression of *nPrlrα* under the control of the LexOP. Addition of the ligand, mifepristone (RU486), binds to the LexPR transactivator which activates the LexOP to provide a spatio-temporally controlled expression of *nPrlrα* on the cell surface of insulin-expressing β-cells, providing a model of reduced PRLRα signaling. Consistent with results from mammalian models, this loss-of-function of PRLRα led to a reduction in the β-cell number. We further demonstrated that the reduced β-cell population and insulin production was accompanied by a reduction of known endocrine cell-specific transcription factors, *hb9* and *neuroD*, which have been shown to be essential for the differentiation and maintenance of β-cell progenitors. Our results suggest that PRL and PRLRα functions to maintain β-cell populations by regulating β-cell differentiation during zebrafish pancreas development. Importantly, we have

established the first cell-specific *in vivo* system to study the role of PRLRα in β-cell development. It is possible that this system can provide useful information with potential applications in diabetes research.

Introduction

Increasingly prevalent cases of diabetes that are characterized by autoimmune destruction of β-cells and reduced insulin sensitivity are prompting an increase in investigations into the mechanisms responsible for pancreas development and maintenance. Extensive studies have implicated a variety of growth factors responsible for β-cell size, number and function, including growth hormone (GH) and the lactogenic hormones, prolactin (PRL) and placental lactogen (PL) (Vasavada et al., 2006). PRL and PL are both ligands for prolactin receptor (PRLR) which are actively expressed and maintained in β-cells throughout vertebrate development (Sorenson and Stout, 1995; Nielsen et al., 1999). These lactogenic hormones were demonstrated to act as potent mitogenic factors on isolated islets and in insulin-secreting cell lines (Brelje and Sorenson, 1991; Brelje et al., 1993). Ectopic *in vivo* over-expression of PL in β-cells of mice elevated plasma insulin level with increased β-cell insulin content, proliferation, size, and mass (Vasavada et al., 2000). Collectively, lactogenic hormone-mediated changes in β-cell mass and function in isolated islets is reminiscent of the physiological changes in β-cell structure and function associated with pregnancy (Sorenson and Brelje, 1997). Indeed, heterozygous PRLR-/+ pregnant mice were recently demonstrated to suffer from a reduced serum insulin level, β-cell mass and number compared to homozygous PRLR+/+ mothers, indicating a critical role for lactogenic ligand-induced PRLR activation to maintain glucose homeostasis in pregnancy (Huang et al., 2009).

The changes in β-cell physiology have been attributed to an up-regulation of PRLR (Moldrup et al., 1993; Sorenson and Stout, 1995). Activation of PRLR stimulated JAK2 which subsequently phosphorylated the signal transducers and activators of transcription (STAT) and resulted in the rapid translocation of STAT5 specifically in the nucleus of β-cells (Brelje et al., 2002, 2004). PRL treatment of *in vitro* cell lines and isolated islets also demonstrated the requirement of the JAK2/STAT5 pathway in regulating insulin and cyclin D2 transcription as a mechanism leading to the increased insulin production and β-cell proliferation (Galsgaard et al., 1999; Friedrichsen et al., 2001, 2003).

Less is known about the PRL/PRLR-mediated effects on early β-cell development. PRLR-deficient mice exhibited a reduction of β-cell mass, islet density, and insulin mRNA (Freemark et al., 2002). The decrease in β-cell mass was independent of apoptosis and was interpreted to reflect a defect in β-cell neogenesis. It is now generally accepted that normal adult β-cell mass expansion is primarily mediated by β-cell replication (Dor et al., 2004; Teta et al., 2007), while increases in fetal β-cell population are regulated by β-cell differentiation from pancreatic precursor cells (Bowens and Rooman, 2005). In the fetal pancreas, the key transcriptional factors required for β-cell differentiation from stem cells or endocrine pancreas precursors has been well documented (Edlund, 2002). *Pdx-1* is cell-autonomously required for pancreas development and functions to determine the pancreatic identity of common precursors, including β-cells (Jonsson et al., 1994). Although PRL-mediated β-cell hyperplasia increased *pdx-1 in vitro,* no changes of *pdx-1* were observed *in vivo* during pregnancy or in virgin mice infused with PRL (Nasir et al., 2005). It has been shown that *pdx-1* alone is insufficient to induce endocrine cell fate as demonstrated by ectopic expression targeting different endodermal domains by *in ovo* electroporation (Grapin-Botton et al., 2001) and suggests the involvement of

other PRL-responsive transcription factors in the regulation of β-cell number. One such factor is *hb9* which is essential for the development of the endocrine pancreas (Harrison et al., 1999). Deletion in the *hb9* gene in mice leads to a significant reduction in β-cell population and down regulation of several other transcription factors important for β-cell differentiation, including *pdx-1* (Harrison et al., 1999; Li et al., 1999). In addition, targeted gene disruption of *BETA2/neuroD* in mice also leads to a reduction of β-cell mass, and has been suggested to be an important transcription factor required for islet cell differentiation and survival (Naya et al., 1997). *NeuroD* is also a target of *neurogenin-3* (*ngn3*), a marker for the precursors of all endocrine cell lineages (Gradwohl et al., 2000; Huang et al., 2000). Involvement of PRL in the regulation of these transcription factors have not been examined or demonstrated during embryonic development.

Despite an accumulation of evidence to support roles for lactogenic hormones in β-cell mass regulation during pregnancy, little is known about the function of PRL during early islet development. This is partly because rodent fetuses are inaccessible due to *in utero* development. Using the zebrafish as a model, we took advantage of their external development to explore the different facets of PRL function on islet development. We show that PRL and its cognate receptor PRLRα are involved in the regulation of β-cell number during zebrafish pancreas development. In contrast to the defined role of PRL/PRLR on β-cell mass expansion through proliferation in pregnant rodents, our results suggest the importance of endocrine cell-specific transcription factors, *hb9* and *neuroD*, for maintaining normal β-cell number and insulin production in a PRL-dependent manner during embryogenesis. Furthermore, we established the first conditional transgenic line that expresses non-functional PRLRα (*nPrlrα*) within β-cells.

Methods and Materials

Fish Maintenance and Microinjection

AB wild-type zebrafish, *Danio rerio*, were maintained according to standard protocols (Westerfield et al., 1993) and IACUC regulation and rules of the Institute of Molecular and Cellular Biology (Singapore). Embryos were staged in hours post-fertilization (hpf) and days post-fertilization (dpf) with reference to morphological features as previously described (Kimmel et al., 1995).

Morpholino antisense (MO) oligomers specific for PRL (PRL-MO) and a 5-mismatch MO for PRL (mismatch MO; 5' TAGACCCTTGAGCCATTACTAGAAC 3') were purchased from Gene Tools, LLC (Philomath, OR). 5' capped *prl* mRNA was synthesized from Not I (Invitrogen) linearized $pCS2^{+}$.EGFP-MutatedPRL using the SP6 mMessage mMachine kit (Ambion, TX) following the manufacturer's instructions. PRL-MO, mismatch-MO, and *prl* mRNA were microinjected into zebrafish zygotes with modifications as previously described (Zhu et al., 2007). Briefly, PRL-MO were resuspended in nuclease-free sterile water to a concentration of 10 ng/nl (1.25 mM), which was then further diluted to a working concentration of 2.5 ng/nl immediately before injection with 1X Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES at pH 7.6); similarly, 2.5 ng/nl of MO-control was used as an injection control. The PRL-MO solutions were microinjected into embryos at the 1-2 cell stage using borosilicate glass microcapillaries attached to a micromanipulator, under a Leica MZ6 microscope (Leica, Germany). Injection was driven by compressed N_2 gas, under the control of a PLI-100 (Harvard Apparatus, Medical Systems Corp., USA). Microinjection was performed with a MPPI-2 pressure injection system (Applied Scientific Instrumentation, USA) with volumes estimated at 1 nl per embryo. Noninjected

zygotes (physiological state) served as the control group for the comparison of PRL-MO and *prl* mRNA injected embryos.

Generation of *Tg(ins:nPrlrα:gfp)* Zebrafish Line

Transgenic fish expressing non-functional PRLRα (*nPrlrα*) were developed by modification of the LexPR:LexOP system (Emelyanov and Parinov, 2008). Briefly, the LexPR:LexOP system consists of two elements: a chimeric transcription factor (LexPR transactivator) and a cis-acting operator-promoter sequence (LexOP) containing binding sites for this transcription factor. The LexPR transactivator binds to the operator and activates transcription of the gene of interest placed under the control of the LexOP only upon the binding of the progesterone antagonist, mifepristone (RU486), thus conditionally regulating the transcription of the target gene (Fig. 4-1). The extracellular domain (ECD) of PRLRα was amplified with primers containing Sac II restriction sites at both ends and was subsequently ligated into a pDS-04GLP4 plasmid downstream of LexOP to form a fusion protein with GFP, removing all sequences encoding intracellular components of PRLRα required for proper signaling, and creating the *nPrlrα*. A 900 bp zebrafish insulin promoter was inserted into the pDS-04GLP4 vector upstream of LexPR at the Xho I and Asc I positions to specifically drive expression of *nPrlrα* in insulin-producing β-cells. Modifications to the construct were verified for correct insertion by standard sequencing methods. Ten pg of plasmid DNA was co-injected into zebrafish embryos directly into the one-cell with 50 pg of *in vitro* synthesized transposase mRNA. The injected fish were raised and out-crossed to the AB wild-type zebrafish, and the resulting embryos were screened for mifepristone (RU486)-induced GFP expression. For screening, RU486 was added to the egg water at a final concentration of 1 μM at 12 hpf and GFP

expressing F_1 siblings were selected to establish stable transgenic lines: *tg(ins:nPrlrα:gfp).* F_2 offspring maintained GFP expression (Table 4-1).

Whole Mount *In Situ* Hybridization (WISH) with the Insulin and PRLRα Probes

Whole mount *in situ* hybridization (WISH) using digoxigenin (DIG)-labeled riboprobes was carried out as previously described (Korzh et al., 1998). Recombinant DNA clones containing gene encoding PRLRα, *insulin*, *hb9*, and *neuroD* were linearized with specific restriction enzymes, followed by *in vitro* transcription reaction with T7 or SP6 RNA polymerase (Ambion, TX, USA) for synthesis of the anti-sense RNA probe. The embryos were fixed with 4% paraformaldehyde (PFA) overnight (O/N), washed 4X for 20 min with PBST, and pre-hybridized O/N at 68°C. Next, the embryos were hybridized with the DIG-labeled probe in hybridization buffer [50% formamide, 5X standard saline citrate (SSC; 0.75 M NaCl, 0.075 M sodium citrate), 50 μg/ml heparin, 500 μg/ml yeast tRNA, and 0.1% Tween-20] at 68°C O/N and excess probes were removed with the following washes: 100%-, 75%-, 50%-, 25%-hybridization buffer, and 2X SSCT for 15 mins and ending with 2X washes with 0.2X SSCT at 68°C. Finally, embryos were blocked at room temperature for two hours with blocking buffer (Roche) and incubated with 1:2000 anti-DIG antibody conjugated with alkaline phosphatase at 4°C overnight. Hybridization of the probe was detected by incubating with NBT (nitroblue tetrazolium; 0.03%) and BCIP (5-bromo, 4-chloro, 3-indolyl phosphate; 0.02%) in 0.1 M TBS at a pH 9.5 until desired color development occurred (30 min to 1 hr) at room temperature. For sectioning, stained embryos were embedded in 1.5% bacto-agar and incubated in 30% sucrose at 4°C overnight. The embedded embryos were sectioned with a cryostat microtome (Microm HM 505E, Zeiss) in cross section orientation at 12 μm thickness and collected on polysine microscope slides (Thermo Scientific). Sections were fixed with 4% PFA in phosphate buffered saline (PBS) for 10

min, washed with PBS, and preserved with 1:1 PBS:glycerol under a glass cover slip and sealed with nail polish to prevent drying. Photographs were taken using a camera mounted to an Olympus AX-70 microscope (Olympus, Japan) using the 20X or 40X objectives with bright field illumination.

The numbers of β-cells were determined by counting DAPI positive nuclei in the areas of insulin positive cells of the embryos younger than 24 hpf as the pancreatic β-cells exist in a single layer. For embryos that were older than 24 hpf, embryos were sectioned as described above at 12 micron thickness following WISH with insulin antisense probe. Sections were then stained with DAPI, and the numbers of pancreatic β-cells were determined by counting the DAPI stained nuclei within the area corresponding to insulin-positive cells in serial sections (Fig. 4-3A).

Quantitative Real-Time PCR (qRT-PCR) for Insulin and PRLRα Genes

Total RNA was obtained from approximately 100 embryos using the RNeasy Mini Kit (Qiagen, Germany), and purified following the manufacturer's instructions. The cDNA synthesis and subsequent quantitative real-time PCR (qRT-PCR) were carried out in a single reaction mixture using One-Step RT PCR kit (Qiagen, Germany) in a DNA Engine Opticon System (MJ Research, USA), with SYB Green used as the reporter. The amplification protocol for *prlrα* consisted of 50°C for 30 min for cDNA synthesis, followed by an initial denaturation of 95°C for 5 mins, followed by forty cycles of 95°C denaturation for 15 secs, 60°C annealing for 30 secs, and 72°C extension for 30 secs with gene specific primers: forward 5'-TCTGCCCACTACATATGC-3', and reverse 5'-ACCGCTTTGACGTTTTCC-3'. For quantitation of insulin transcripts, *insa* was measured with the same protocol and primers described by Papasani et al., 2006. Quantitation of *prlrα* and *insa* gene expression was

normalized using amplification of EF1α (forward, 5'-AGACTGGTGTCCTCAAGCCTG-3'; reverse, 5'-TGAAGTTGGCAGCCTCCATGG-3') with established protocol (Fong et al., 2005) in each sample in order to standardize the results by eliminating variation in mRNA quality.

Western Blot Analysis

Embryos at 24, 36, 48, and 72 hpf were dechorionated using 23-gauge needles (BD Biosciences) and transferred to cold Ringer's solution (116 mM NaCl, 2.9mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES) with EDTA (final conc. 1mM) and PMSF (final conc. 0.3mM; general protease inhibitor) (Westerfield et al., 2000). Embryos were de-yolked in deyolking buffer without calcium (55mM NaCl, 1.8mM KCl, 1.25mM $NaHCO_3$) (Link et al., 2006) by repeated pipetting with a 200 µl pipette tip until the majority of the yolk cells dissolved into the solution. The extent of yolk removal with minimal disruption to the embryo tissue was monitored under a stereomicroscope. The embryos were shaken for 5 minutes at 1100 rpm (Thermomixer, Eppendorf) followed by centrifugation at 300 x g for 30 sec to pellet and collect the tissues. The supernatant was discarded and 1 µl of lysis buffer was added per embryo along with 20% 5X SDS loading buffer. An extract equivalent to approximately 10 embryos was loaded per lane i.e. 10 µl. Proteins were resolved by electrophoresis of the extracts through 12 percent SDS polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to a nitrocellulose membrane using a semi-dry blotting apparatus (Liu and Londraville, 2003) for 38 min at 20V (Polvino et al., 1983). The membrane was blocked with 5% nonfat milk in TBST (50 mM Tris, 100mM NaCl, 0.1% Tween 20, pH 7.4) for 1 h and incubated with anti-GFP (1:1000; Santa Cruz Biotechnology) or anti-β-tubulin antibody (1:2000) at 4°C overnight. Excess primary antibody was removed by washing the membrane four times for 5 min with TBST, followed by incubation for 1 h at room temperature with horseradish peroxidase

conjugated to goat anti-mouse antibody (GE Healthcare, Selangor, Malaysia), and finally washed (3 times for 15 min) with TBST. Thereafter, blots were treated with SuperSignal West Dura Subsrate (Thermo Scientific) according to manufacturer's instructions. The amount of GFP and β-tubulin proteins between the different treatments were recorded by autoradiography Hyper film (Amersham Biosciences, UK) using an auto-developing machine (Kodak X-OMAX 2000 processor; Carestream Health). Detection of GFP in *tg(ins:gfp)* between NI and PRL-MO embryos served as an indirect method to determine the approximate insulin protein level, while β-tubulin served as the loading control. Similar results were obtained at least three different times from independent experiments.

Tissue Mounting and Photography

Embryos treated by WISH for PRLRα or *insulin* and/or in combination with whole-mount immunohistochemistry with an anti-GFP antibody to detect *insulin* or *nPrlrα* expression were washed with PBST twice for 10 minutes each and transferred to 50% glycerol/PBS, equilibrated at room temperature for one hour. For whole mounts, a single chamber was made by placing stacks of 3-5 small electricity tape on both sides of a 25.4X76.2 mm microscope slide. A selected embryo was transferred to the chamber in a small drop of 50% glycerol/PBS and oriented with a needle. A 22X44 mm cover glass with a small drop of the same buffer was placed over the embryo to secure its position. The orientation of the embryo was adjusted by gently moving the cover glass. For flat mounting specimens, the yolk of the selected embryo was removed completely with 23G needles. The embryo without yolk was then placed onto a slide with a small drop of 50% glycerol/PBS and adjusted to a proper orientation by removing excess liquid and with the help of needles. A small fragment of cover glass (a bit larger than the specimen) was placed on top of the embryo. Care was taken to avoid bubbles and a drop of 50%

glycerol/PBS was added to fill the space under the cover glass. This specimen was sealed with nail polish along the edge of the cover glass to prevent it from drying. Photographs were taken with a Zeiss Axioplan fitted with a Zeiss AxioCam with either 20X or 40X objectives. Images were taken by DIC in bright field illumination or with FITC to observe GFP expression.

Statistical Analysis

The significance of the mean differences between various experimental groups was determined by one-way ANOVA followed by Tukey test analysis. A *P* value <0.05 was considered statistically significant.

Results

Expression of PRLRα in Pancreatic β-cells

Based on WISH, no significant expression of the PRLRα was observed in organs/tissues other than the pancreas and kidney in embryos ranging from 15 hpf to 5-day-old embryos (Fig. 2.1-9; Fig. 2.1-10). The expression of PRLRα was readily detectable in both the pancreas and kidney in zebrafish embryos examined between 17 hours post-fertilization (hpf) to 3 days post-fertilization (dpf) (Fig. 2.1-9D-K, M; Fig. 2.1-10A-E, K), whereas PRLRβ is restricted to only the kidney (Fig. 2.1-11A-J). We hypothesized that expression of both PRLRα and PRLRβ in the kidney are related to the well-known function of PRL in regulating water/ion balance in the embryos, while the expression of PRLRα in the pancreas is related to a novel function of PRL during embryonic development of the pancreas. Expression of PRLR in the pancreas is well established in adult vertebrates, with localization restricted to the β-cells. Therefore, we focused on examining the roles of PRLRα in the pancreas. In order to avoid potential cross-reactivity between insulin and PRLRα as might be the case when using double *in situ* hybridization, we combined WISH and immunohistochemistry. We first verified that expression of GFP in the

tg(ins:gfp) line truly represents endogenous insulin expression by co-localizing GFP (using an anti-GFP antibody) with insulin transcripts by means of WISH. The GFP expression in *tg(ins:gfp)* co-localized well with native insulin transcripts based on overlap between signals detected from GFP and insulin (Fig. 4-2A-C), indicating that the *tg(ins:gfp)* line is a useful model to visualize the effects of PRL/PRLRα on β-cells. PRLRα-expressing cells were again ascertained by WISH and co-localization with GFP in the *tg(ins:gfp)*, and demonstrating that PRLRα is expressed in β-cells (Fig. 4-2D-F).

Effects of PRL/PRLRα on the Number of Pancreatic β-cells during Embryonic Development

The effects of PRL and PRLRα on the number of pancreatic β-cells were examined by knockdown of PRL (referred to as PRL morphants), over-expression of PRL, or expression of non-functional PRLRα (*nPrlrα*). The number of β-cells increased in control embryos (non-injected and mismatch-MO) between 17 to 72 hpf (Fig. 4-3C). Embryos injected with the mismatch-MO were generally developmentally delayed compared to the non-injected control embryo, showing a slight decrease but no significant difference in β-cell number compared to the non-injected control embryos (Fig. 4-3C). Surprisingly, knockdown of PRL increased the number of β-cells between 17 and 72 hpf, while over-expression of PRL decreased the number of β-cells between 17 and 24 hpf compared to the mismatch-MO and non-injected control (Fig. 4-3B, C). In contrast, expression of *nPrlrα* exhibited a significantly reduced β-cell number compared to the non-injected control between 24 and 72 hpf (Fig. 4-3C); and with mismatch-MO at 72 hpf but not at 24 and 48 hpf. GFP expression of *nPrlrα* was not detectable at 17 hpf, thus we were unable to examine the effects of *nPrlrα* on β-cell number.

Effects of PRL Expression on Insulin Transcripts and Protein during Development

Expression of insulin transcripts in the developing pancreas was examined using qRT-PCR to determine whether increase in numbers of pancreatic β-cells also resulted in increased production of insulin mRNA. Zebrafish possesses two isoforms of insulin, *insa* and *insb*. Both forms of insulin are expressed in the pancreas, but expression of *insb* has been shown to decrease between 6-72 hpf (Papasani et al., 2006). So, we focused on the expression of *insa*. The *insa* transcript was low at 12 hpf and was at least ~2-fold less compared to later stages where PRLRα is expressed and β-cells have differentiated. PRL knockdown significantly increased the *insa* transcript ~1.8- to ~2-fold between 36 and 72 hpf compared to the non-injected (NI) control, whereas the overexpression of PRL reduced *insa* transcripts ~1.9-fold at 24 hpf compared to the NI control, but recovered after 48 hpf to comparable levels with non-injected embryos (Fig. 4-4B). This result is consistent with the observed increase in the number of β-cells in the same time window (Fig. 4-3C), i.e. from 24 to about 48 hpf. Both *insa* transcript and β-cell number recovered to comparable levels with NI controls or mismatch-MO controls in *prl* mRNA injected embryos after 48 hpf. It is likely that the injected *prl* mRNA does not persist in the embryos beyond 48 hpf, leading to the recovery in the number of β-cells in the injected embryos. Accounting for this, the result is consistent between gain-of-function and loss-of-function of PRL signaling, and suggests that PRL may play a role in determining the number of β-cells in the developing pancreas.

Due to lack of any specific antibody for zebrafish *insa*, we examined the *insa* protein level indirectly by measuring the level of GFP produced in *tg(ins:gfp)* from non-injected controls and PRL morphants. Expression of GFP in *tg(ins:gfp)* is under the control of the insulin promoter, and provides a relative measurement of endogenous insulin production in pancreatic β-

cells. Increases in GFP protein were observed in PRL knockdown embryos compared to the control at all time points examined, from 24 to 72 hpf (Fig. 4-4A).

Generation of Non-functional PRLRα (*nPrlrα*) and Analysis of its Impact on β-Cell Development and Function

The LexPR:LexOP construct was utilized because it enabled spatio-temporal control of transgenes, and in our case, the expression of a chimeric PRLRα-GFP gene directed to the β-cells. We replaced the intracellular domain of PRLRα with *gfp* and generated several stable transgenic lines (*ins:nPrlrα:gfp*) for the expression of non-functional PRLRα (*nPrlrα*) specifically in the insulin-producing β-cells (see Materials and Methods for detail). One major advantage of this system is that our transgene is strictly controlled in a ligand-dependent (Mifepristone, RU486) and cell-specific manner (insulin promoter). Induction of *nPrlrα* expression is easily achieved by administration of RU486 in the fish water, allowing temporal control to study different developmental or physiological state at any time point within the life cycle of the zebrafish.

The founder fish with the chimeric *nPrlrα* transgene potentially integrated into their genome was crossed with AB wild-type fish and identified to be a founder by administration of RU486 into the water of their progeny (F_1 offspring) at 24 hpf. After 6 hrs of RU486 treatment, GFP expression within the pancreas was used as an indicator for proper *nPrlrα* integration and were classified as F_1 offspring that were raised to sexual maturity. The process was repeated again to identify F_2 offspring. From our screen of 200 fish, four GFP transgenic lines were analyzed (Table 4-1). The ratios of GFP expressing progeny in F_1 offspring ranged from 16.7%-38% and were generally higher in F_2 offspring (Table 4-1). The ratio of F_1 offspring was less than the expected 50% for transgenes that integrate in all germ cells, indicating that our founder

fish experience the commonly observed mosaic nature of transgenes integration only into a subset of germ cells (Udvadia and Linney, 2003). Crossing GFP expressing F_1 with AB wild-type did produce the approximately 50% GFP expressing progeny (F_2) as would be expected for Mendelian inheritance of transgenes (Udvadia and Linney, 2003).

The expression of *nPrlrα* was similar to the endogenous insulin expression reported previously (Argenton et al., 1999; Biemar et al., 2001), and localized at the cell surface (Fig. 4-5). Based on the GFP expression, we isolated F_2 embryos that expressed *nPrlrα* and analyzed them for changes in β-cell population. Significant reductions in the numbers of insulin positive β-cells were observed in embryos induced to express *nPrlrα* by treatment with RU486 (Fig. 4-3C; Fig. 4-6C, G, K). F_2 embryos that were not administered RU486 did not exhibit a reduction in β-cell number (Fig. 4-6A, E, I). Moreover, treatment of AB wild-type embryos with RU486 had no effect on β-cell population (Fig. 4-6B, F, J) compared with non-induced F_2 embryos. These results indicate that RU486 alone has no effect on pancreatic β-cell population and that the expression of *nPrlrα* is responsible for the observed decreased in β-cell number and insulin expression. The timing of RU486 treatment did not seem to have major effects on β-cell number because short exposure of F_2 embryos to RU486 at 24 hrs and 48hrs (for 24 hrs intervals) or continued treatment beginning at 12 hpf resulted in similar reduction in the numbers of pancreatic β-cells when examined at 48 and 72 hpf (Fig. 4-7). Interestingly, the number of β-cells appeared recover in F_2 embryos injected with PRL-MO when simultaneously induced with RU486 (Fig. 4-6D, H, L) compared to *nPrlrα* expressing embryos alone (Fig. 4-6C, G, K). The loss of β-cells in *nPrlrα* is inconsistent with results from PRL-MO and PRL mRNA injections. We expected the *nPrlrα* embryos to have similar effects as PRL knockdown.

Effects of PRL Knockdown and Over-expression on the Expression of PRLRα

The increase in β-cell number and insulin in PRL knockdown embryos and decrease of β-cell number and insulin in the embryos over-expressing PRL were surprising results to us because previous studies consistently demonstrated that PRL treatment increases β-cell number. We examined the expression levels of PRLRα transcript to determine whether PRL knockdown had any effect on PRLRα levels. The expression of PRLRα was much higher in the pancreas of PRL knockdown embryos compared to the NI control, while the expression of PRLRα in the kidney appeared to be similar (Fig. 4-8A). qRT-PCR in whole embryos indicated that the levels of PRLRα transcripts appeared to be higher in PRL knockdown embryos compared to the NI control embryos between 24 and 72 hpf; however, the differences between the two groups was not statistically different (Fig. 4-8B). Nevertheless, our data suggests that PRLR may be up-regulated when the level of PRL is diminished (as in the case of MO knockdown) in a compensatory manner.

Effects of PRL Knockdown and *nPrlrα* on *Hb9* and *NeuroD* Transcriptional Factors

No apparent difference was found in the β-cell population undergoing cell proliferation as a result of an absence in overlap between insulin-producing cells and the cell proliferation marker PH3 of PRL morphants compared to those in the NI control embryos (Fig. 4-9). To determine whether neogenesis may be involved in the increased β-cell population of PRL morphants, we examined two important transcription factors involved in the differentiation of endocrine pancreas precursors into β-cells, *hb9* and *neuroD*. The expression of *hb9* was up-regulated in PRL morphants (Fig. 4-10B, F, J), whereas *hb9* was down-regulated in *nPrlrα* embryos between 24 and 72 hpf (Fig. 4-10C, G, K) and was almost absent at 72 hpf in *nPrlrα*

compared to the NI control (Fig. 4-10K). Injection of PRL-MO in *nPrlrα* embryos increased *hb9* positive cells (Fig. 4-10).

An up-regulation in *neuroD* positive cells was observed in the pancreas between 18 and 21 hpf in PRL morphants (Fig. 4-11). In contrast, a reduced number of *neuroD* expressing cells was observed in *nPrlrα* embryos (Fig. 4-11).

Effects of PRL Knockdown and *nPrlrα* on Islet Cell Migration

Two transgenic lines (*ins:gfp* and *ins:rfp*) expressing fluorescent proteins in pancreatic β-cells were used for determining the migration of the primary islet cells. Newly differentiated insulin positive cells appeared as a bilateral column of cells in PRL morphants at 15 hpf, which was similar as those in the control embryos (data not shown). These β-cells migrated posteriorly and clustered into a single primitive islet at the midline at 24 hpf, and then migrated to the right side of the embryos at 48 hpf in both PRL morphants and *nPrlrα* similar to those in control embryos (Table 4-2).

Discussion

In an attempt to understand the roles for pituitary hormones during early development, we investigated the function(s) of PRL in the developing pancreas. To date, the PRL receptor (PRLR)-deficient mouse has served as the principal model for investigating functions of PRL on pancreas development *in vivo* (Freemark et al., 2002). However, the *in utero* development of the rodent model restricts analysis of PRL function to postpartum and later stages. Using zebrafish as an alternative model, we examined the effects of PRL and PRLRα signaling deficiency in early zebrafish pancreas development. Using the *tg(ins:nPrlrα:gfp)* line which expresses non-functional PRLRα (*nPrlrα*) within β-cells induced by RU486, we demonstrated the requirement for PRLRα to regulate normal β-cell number prior to the formation of the primary islet and

continuing throughout embryogenesis. Based on data from PRL knockdown and embryos expressing *nPrlrα*, maintenance of β-cell number by PRL/PRLRα appears to involve the β-cell differentiating factors *hb9* and *neuroD*. These data indicate a conserved function of PRLRα among vertebrates in regulating β-cell populations and suggests an alternative mechanism for maintenance of β-cell number during early pancreas development in zebrafish that is distinct from self-replication of pre-existing β-cells, which is the mechanism proposed for pregnant mammals.

In rodent and human fetuses, PRLR is initially expressed within the exocrine ductular epithelial cells and acinar cells at early gestation followed by a shift in expression to insulin-producing β-cells by late gestation and the perinatal period (Royster et al., 1995; Freemark et al., 1997). Zebrafish possess a second distinct form of PRLR, PRLRβ, but only PRLRα is expressed within the pancreas and appears to be the sole receptor responsible for PRL functions in the developing pancreas (Fig. 2.1-9; Fig. 2.1-10). In contrast to the mouse, PRLRα is initially expressed relatively early during embryogenesis at 17 hpf within the pre-pancreatic region located between the bilateral pronephric ducts, and is expressed in β-cells. It is initially detected in the exocrine tissue in the mouse. The differences in PRLRα expression patterns in the mouse relative to zebrafish are likely due to evolutionary differences in the systems that specify the different morphogenetic events of pancreas development in these species. In the zebrafish, differentiation of β-cells are initially observed by 15 hpf resulting in bilateral rows of cells adjacent to the midline that eventually coalesce to the midline by 24 hpf (Biemar et al., 2001), while markers for exocrine tissues are only evident much later at around 48 hpf at a time when the primary islet is surrounded by exocrine tissue (Field et al., 2003). In rodents, β-cells develop from pancreatic duct epithelial cells, which remain near the pancreatic ducts, and eventually

migrate into the dorsal bud to develop into mature islets during late gestation (reviewed by Edlund, 2002; Hills, 2005). It is possible that the PRLR detected in the exocrine tissue of both rats and humans represent newly differentiated β-cells that have not migrated into the dorsal bud, rather than exocrine cell types. This hypothesis is supported by the identification of insulin-positive cells in the exocrine tissue or around duct cells in mammals (Wang et al., 1995; Bonner-Weir et al., 2000; Bogdani et al., 2003).

In both zebrafish and mammals, expression of PRLR was observed after the initial differentiation of β-cells, suggesting no involvement in morphogenesis, specification of the pancreas or initial β-cell differentiation. After the first appearance of β-cells, the β-cell population continues to expand by differentiation from precursors and ultimately migrates to the head region of the developing exocrine tissue (Biemar et al., 2001). We examined the effect of PRL on β-cell migration and found that the movement of β-cells into the exocrine compartment is independent of PRL. β-cells coalesce to the anterior exocrine compartment situated on the right side of the body-axis in both PRL morphants and embryos expressing *nPrlrα*. Although β-cell migration was not monitored in the mouse, islets were observed appropriately embedded in the head of the exocrine tissue postpartum in PRLR-/- null mice, suggesting no abnormality in β-cell migration in the mouse (Freemark et al., 2002). Together, these results suggest PRL/PRLRα function does not involve the regulation of β-cell migration during early pancreas development of either mice or zebrafish.

In the vertebrate pancreas, PRL and growth hormone (GH) have been repeatedly demonstrated to act as potent stimulators of β-cell proliferation and insulin gene transcription, which enhances insulin secretion by lowering the threshold for glucose-stimulated insulin secretion (Parsons et al., 1992; Brelje et al., 1993; Sorenson and Brelje, 1997; Friedrichen et al.,

2003). In addition, hypopituitary dwarf mice (deficient in both PRL and GH) and mice made deficient in PRLR by targeted deletion are consistently associated with impaired insulin production and reduction in β-cell mass (Parson et al., 1995; Dominici et al., 2002; Freemark et al., 2002; Huang et al., 2009), which is indicative that PRL operates in association with its cognate receptor to play an important role in the establishment or maintenance of normal β-cell function in the developing pancreas. Contrary to these findings, we were surprised to observe an increase in β-cell number, insulin transcripts, and insulin promoter regulated *gfp* expression in PRL morphants, while over-expression of PRL mRNA reversed these effects. It is important to note that antisense morpholino-mediated knockdown of PRL does not completely abolish endogenous levels of PRL. Thus, the seemingly contradictory results may be explained by a compensatory mechanism mediated by residual PRL or other growth factors such as GH. Examination of PRLRα levels between control and PRL morphants revealed an increase in PRLRα transcripts within the pancreas of the PRL knockdown embryos, but not in the kidneys (Fig. 4-8A). The up-regulation of PRLRα gene expression in this case may reflect the ability of residual PRL and/or GH, to stimulate PRLR transcription as previously described (Moldrup et al., 1993; Brelje et al., 2002). The mechanism leading to PRLR gene transcription involves hormone activation of PRLR and/or GHR, leading to the stimulation of the JAK2/STAT5 signaling pathway directly targeting the PRLR promoter (Galsgaard et al., 1999). Similarly, the increase in insulin transcripts observed in PRL morphants may also up-regulate insulin transcription by virtue of the same mechanism; STAT5 binding to the insulin promoter via the γ-interferon activating sequences (GAS), a known STAT5 DNA binding domain (Galsgaard et al., 1996). Alternatively, the increase in β-cell number and insulin transcripts in PRL morphants suggests that PRL may have a repressive effect on PRLRα such that high PRL levels repressed

PRLRα, which is relieved when PRL levels drop. Although the mechanism regulating the potential feedback loop between PRL and PRLRα in β-cells is not known, the consequence of the resulting up-regulation of PRLRα under this scenario remains the same: increase in insulin transcripts and β-cell number. Future research will be required to determine the mechanism, residual PRL/GH or the feedback relationship between PRL and PRLRα or both, that could explain for the observed β-cell phenotype in PRL morphants.

To further address the paradoxical differences between the effects on pancreas development of zebrafish PRL knockdowns and PRLR-/- mice, we examined the effect of PRL on β-cell development in the context of PRLRα signaling deficiency in zebrafish. Mifepristone (RU486) induction of the *tg(ins:nPrlrα:gfp)* line specifically expressed a chimeric PRLR in which the intracellular domain of PRLRα was replaced with a *gfp* sequence, rendering the chimeric PRLR non-functional in β-cells due to the lack of the Box 1 domain which is essential for the constitutive association of JAK2 and PRLR signaling (Bole-Feysot et al., 1998). Embryos expressing *nPrlrα* exhibited a dramatic reduction in total β-cell number at all time points examined, consistent with the mouse knockout model (Freemark et al., 2002; Huang et al., 2009). A previous study in zebrafish demonstrated that knockdown of PRLRα, and consequently down-regulating PRLRα signaling, stimulated the production of PRL in lactotrophs of the pituitary (Liu et al., 2006). Based on the previous results, reduced levels of PRLR signaling due to the expression of *nPrlrα*, would also be expected to stimulate the production of PRL in our model. Unlike the up-regulation of PRLRα observed in PRL morphants, the expected increase in PRL hormones in the pituitary of *nPrlrα* embryos appears to be insufficient to compensate for the reduced PRLRα signaling. PRLRα signaling is initiated by PRL binding to two PRLRs, leading to receptor dimerization that activates JAK2 autophosphorylation and other post-receptor

signaling (Rui et al., 1994). In view of the well characterized mechanism of PRL-activated PRLR signaling, three potential avenues of PRL binding in β-cells of RU486-induced *nPrlrα* expression exists: 1.) homodimerization of endogenous PRLRα, 2.) homodimerization of *nPrlrα*, and 3.) heterodimerization of *nPrlrα* with endogenous PRLRα. The fact that *nPrlrα* embryos exhibited a reduced number of β-cells similar to PRLR null mutant mice (Freemark et al., 2002) suggest the latter two mechanisms are likely activated, preventing any compensatory activity related to increased PRL hormones in these embryos. Interestingly, morpholino-mediated PRL knockdown in *nPrlrα* expressing embryos appears to restore normal insulin expression and β-cell number. It should be noted that PRL morphants increased PRLRα transcripts (Fig. 4-8A), and conversely, *nPrlrα* is expected to increase PRL. The increase in endogenous PRLRα transcripts in response to reduced PRL hormones could potentially increase PRL-mediated homodimerization of endogenous PRLRα, leading to normal PRLRα signaling; with simultaneous increase in PRL, as a consequence of *nPrlrα*, enhancing these effects. Moreover, despite an up-regulation of PRL production in PRLRα morphants, Liu et al. (2006) indicated that lactotrophs failed to respond to environmental salinity, implying that PRLRα is critical in mediating PRL functions. This is consistent with our observation that increases in PRLRα in the pancreas of PRL morphants enhanced β-cell number although PRL levels are low, while expression of *nPrlrα* reduced β-cell number even with the expected increase in PRL. However, it remains unclear how the two opposing compensatory mechanisms of PRL knockdown and *nPrlrα* contribute to the recovery of β-cell number when combined, compared to their actions alone. Future studies will be required to better understand the compensatory mechanism of the PRL/PRLRα system in β-cells.

Interestingly, in all *tg(ins:nPrlrα:gfp)* examined, only a subset of β-cells were found to express *nPrlrα*. Expression of *nPrlrα* was generally associated with weaker staining of insulin positive cells. It is likely that the expression of *nPrlrα* in the β-cells directly interfered with JAK2/STAT5 mediated insulin transcription (Galsgaard et al., 1996) resulting in weaker staining of insulin. Alternatively, stronger stained β-cells for insulin could represent recently differentiated β-cells because they lack expression of *nPrlrα* which is also under the regulation of the insulin promoter and may require more time for the translation and translocation of *nPrlrα* to the membrane. Starting at 48 hpf, the primary islet is exclusively clustered in the center of the exocrine tissue head (Field et al., 2003). In comparison to the control embryos, the remaining insulin-positive cells in *nPrlrα* embryos were observed to be loosely clustered with some scattered β-cells located at the periphery of the islet. It is plausible that the β-cells located adjacent to the exocrine compartment are recently differentiated β-cells derived from a pool of pancreatic precursors at the margin of the primary islet. Recently, *E. coli* nitroreductase induced β-cell ablation in zebrafish demonstrated that the mechanism for β-cell recovery is partly due to non-insulin producing progenitor cells proliferating and differentiating into mature β-cells (Pisharath et al., 2007). These non-insulin producing β-cell progenitors were generally located at the periphery of the islet (Pisharath et al., 2007) as were the proliferating cells we observed (Fig. 4-9). Several other groups have also demonstrated the existence of intra-islet precursors that are capable of generating new β-cells (Tsanadis et al., 1995, Fernandes et al., 1997; Guz et al., 2001; Pang et al., 1994). Therefore, the restricted expression of *nPrlrα* to weakly stained insulin-positive cells and scattered residual β-cells in *nPrlrα* embryos located peripherally within the islet, suggests that the lack of *nPrlrα* expression in strongly stained β-cells is due to their recent differentiation from non-insulin producing cells.

In fetal pancreas development, it is well known that β-cells are generated from a population of pancreatic progenitor cells (Edlund, 2002; Wilson et al., 2003; Piper et al., 2004). These progenitor cells differentiate into mature β-cells and persist throughout embryogenesis as a source for the expansion of β-cell mass (Jensen et al., 2000; Gu et al., 2003). Despite an increase in β-cell number in our PRL morphants, we did not observe a significant increase in β-cells positive for the cell proliferation marker PH3 (Fig. 4-9), suggesting a mechanism independent of β-cell replication from pre-existing β-cells. This is in good agreement with Yee and colleagues (2001) who also demonstrated that β-cell proliferation was not a major mechanism leading to increased β-cell mass during normal zebrafish embryogenesis. More recently, it was suggested that β-cells of the primary islet initially arise by differentiation of pancreatic precursors into β-cells in early zebrafish pancreas development, with a separate smaller population of β-cells arising from mitotic expansion of pre-existing β-cells (Moro et al., 2009). Cyclin D2-/- mutant mice clearly showed that cell replication is dispensable for β-cell mass formation during embryonic development but is essential for postnatal β-cell expansion (Georgia and Bhushan, 2004). Together, these results suggest that early β-cell development during zebrafish embryogenesis could be regulated at least partially by neogenesis of endocrine pancreas precursor cells while cell proliferation contributes to the β-cell mass expansion of adult zebrafish islets.

If the population of β-cells in the embryonic pancreas increases in number as a result of differentiation from non-insulin producing precursor cells, it is important to examine the pancreatic progenitor cell differentiation factors, *hb9* and *neuroD*. We would expect levels of *hb9* and *neuroD* to be up-regulated in the presence of PRL/PRLRα and down-regulated in the absence of PRLRα signaling. *Hb9* (HLXB9 in mammals) is an important transcription factor for

the initial morphogenesis of the pancreas and is required for the differentiation of β-cells in rodents (Harrison et al., 1999; Li et al., 1999). In zebrafish, *hb9* also appears to be required for the differentiation of insulin-producing cells, with *hb9* knockdown reducing or eliminating β-cell populations (Wendik et al., 2004). Samples prepared from *nPrlrα* expressing embryos exhibiting a reduced number of β-cells also showed a reduction in *hb9* expression within the pancreatic islets throughout embryogenesis. The second transcription factor we monitored was *neuroD*. Targeted deletion of *neuroD* resulted in a severe reduction in insulin-producing β-cells (Naya et al., 1997). As expected, *nPrlrα* embryos had a reduced number of *neuroD* positive cells (Fig. 4-11), which is likely accompanied by decreased insulin levels because *neuroD* also regulates insulin gene transcriptional activity (Naya et al., 1995). This observation is consistent with the decrease β-cell number and less intense staining for insulin in *nPrlrα* embryos (Fig. 4-6A, G, K). Increases in β-cell populations of PRL morphants, on the other hand, was demonstrated to increase the level of *hb9* and *neuroD*. The up-regulation of both these transcription factors is consistent with the observed increase in β-cell number and PRLRα transcripts. These results suggest that the increase in β-cell number of PRL morphants or decrease in β-cell number of *nPrlrα* embryos is accompanied by a corresponding increase or decrease in the expression of the β-cell differentiation factors, *hb9* and *neuroD*.

The regulation of β-cell function and development mediated by PRL/PRLRα is complex, but we attempt to consolidate these data by proposing a mechanism of PRL/PRLRα function in relation to β-cell population during early zebrafish pancreas development (Fig. 4-12). The cellular mechanisms mediating PRL functions on β-cells have been extensively studied. PRL has been demonstrated to mediate its biological functions on β-cells through the PRLR, leading to the activation of the JAK2/STAT5 pathway (Sorenson and Stout, 1995; Brelje et al., 2002, 2004).

Subsequently, phosphorylation of STAT5 complex binds to the promoters of PRLR, insulin, glucokinase and cyclin D2 that contain functional STAT5 binding sites in β-cells leading to their transcriptional activation (Galsgaard et al., 1996, 1999; Friedrichsen et al., 2003, Weinhaus et al., 2007). Furthermore, the use of constitutively active STAT5 and dominant negative STAT5 (Friedrichsen et al., 2001) produced effects similar to those exerted by lactogenic hormones, supporting the importance of STAT5 in mediating PRLR signaling in β-cells. During zebrafish β-cell development, we observed that expression of *nPrlrα*, which lacks JAK2 in the intracellular domain, produced embryos with fewer β-cells and less insulin expression (Fig. 4-6C, G, K). This suggests that the presence of *nPrlrα* interfered with normal JAK2 activation and consequently STAT5 which renders the β-cells less active in stimulating insulin transcription. The defect in JAK2/STAT5 pathway in *nPrlrα* embryos could similarly reduce the transcriptional activity of PRLRα, glucokinase, and cyclin D2 during zebrafish embryogenesis but additional investigation will be required to determine the direct effect of PRLRα on these genes in zebrafish β-cell development.

Although the JAK2/STAT5 mechanism is the principal pathway mediating PRL function, there are examples that indicate insulin transcription is independent of STAT5 (Fleenor and Freemark, 2001). The human insulin promoter and rat insulin-2 promoter do not possess the classical STAT5 recognition sites, but treatment with PRL increases the transcriptional activity of insulin in rat insulinoma cells (Fleenor and Freemark, 2001). Similarly, PRL induces the pancreas specific *pdx-1* promoter that also lacks STAT5 binding sites (Nasir et al., 2005). Interestingly, PRL (Galsgaard et al., 1996; Weinhaus et al., 2007), *pdx-1* (Babu et al., 2007), and *neuroD* (Naya et al., 1995; Moates et al., 2003) share two downstream targets involved in β-cell function, insulin and glucokinase. Recently, it was demonstrated that *pdx-1* and *neuroD*

physically interacted at a response element on the insulin promoter in β-cells which might be important for regulating insulin transcription (Babu et al., 2008). It is possible that PRLRα may activate both *pdx-1* and *neuroD* in β-cells during early zebrafish pancreas development either working synergistically or complementarily to regulate insulin gene transcription. Targeted deletion of *hb9* in mice resulted in a decrease in both *pdx-1* and insulin expression, which would suggest that *hb9* acts upstream of *pdx-1* (Harrison et al., 1999). However, ectopic expression of *pdx-1* in the chick indicated that *pdx-1* is capable activating *hb9* (Grapin-Botton et al., 2001). Similarly, *neuroD* was shown to activate *hb9* expression which was enhanced with *isl-1* and *lhx-3* (Lee et al., 2004) in motorneuron cells. *Isl-1* is also an important transcription factor involved in various aspects of pancreas development (Du et al, 2009), suggesting that *neuroD* and *isl-1* could also regulate *hb9* expression in pancreatic β-cells. Furthermore, knockdown of PRL2 in zebrafish resulted in a reduction in *isl-1* expression in the eyes (Huang et al., 2009), indicating that PRL may be an upstream regulator of *isl-1* gene activity. Together, these results suggests an alternative PRL mediated signaling pathway independent of JAK2/STAT5 that involves the activation of key transcription factors involved in β-cell development in zebrafish.

Nevertheless, it is currently unknown whether PRL/PRLRα directly or indirectly regulates the expression of *pdx-1*, *neuroD*, *hb9*, and *isl-1*. Although the transcriptional activities of these genes appear to be independent of STAT5, we cannot exclude the possibility that STAT5 may act as a co-factor for these transcription factors or activate other *trans*-acting activators important for insulin transcription. The mechanism of these PRL responsive genes may act synergistically, complementary, or even independent but awaits additional studies to determine their interactions in zebrafish β-cell development. It is also important to note that the JAK2/STAT5 pathway is the main mechanism responsible for the increase in β-cell population

during pregnancy (Sorenson and Brelje, 2009). In contrast, the β-cell differentiation factor, *pdx-1*, is not up-regulated in β-cells of PRL infused pregnant or virgin mice (Nasir et al., 2005). This suggests the possibility that PRL stimulates different mechanisms, neogenesis by up-regulating β-cell specific transcription factors vs. proliferation involving JAK2/STAT5 signaling, in response to the requirement for β-cell mass expansion at different developmental time points (embryonic vs. adults) in zebrafish. This suggestion would be consistent with recent findings that neogenesis of β-cells is the major mechanism of β-cell expansion in fetal rodents, while proliferation was the major contributing factor to β-cell number in adults (Georgia and Bhushan, 2004).

In conclusion, our study provides additional insights into the role of PRL during early endocrine pancreatic development. Our data corroborates previous findings with *in vitro* and *in vivo* mammalian experiments, indicating a conserved function of PRL in zebrafish. We show that PRL is not involved in early endocrine pancreas specification, cell migration, or the initial β-cell differentiation. PRL is, however, important for the maintenance of the primary β-cell population. It appears that both PRL and PRLRα are critical for mediating signal transduction that leads to increases in β-cell number and insulin transcription. The mechanism mediating changes in β-cell mass likely involves both neogenesis from pancreatic precursors and β-cell proliferation during embryonic pancreas development in zebrafish. It is currently unclear which mechanism predominates or whether PRL/PRLRα controls β-cell populations similar to the mechanism observed in pregnant mice models and presents an interesting area for future research.

In addition, we developed the first functional and β-cell specific *tg(ins:nPrlrα:gfp)* line that will facilitate future studies on PRL function within the endocrine pancreas at various developmental windows and physiological states.

Fig. 4-1. Schematic diagram illustrating the structure of non-functional PRLRα plasmid DNA construct. A 900 bp insulin promoter was inserted upstream of the LexPR transactivator sequence to specify expression of the transgene (*nPrlrα*). The LexPR transactivator is a hybrid element containing a bacterial LexA DNA binding domain (LexA-DBD), a truncated human progesterone receptor ligand binding domain (hPR-LBD), and the activation domain of the human NF-kB/p65 (NF-kB-AD). Addition of mifepristone (RU486) binds to the hPR-LBD of the LexPR transactivator which subsequently binds to the LexA operator-promoter (LexA-OP) via the LexA-DBD. The result is transcription of the *nPrlrα* placed downstream of the LexA-OP.

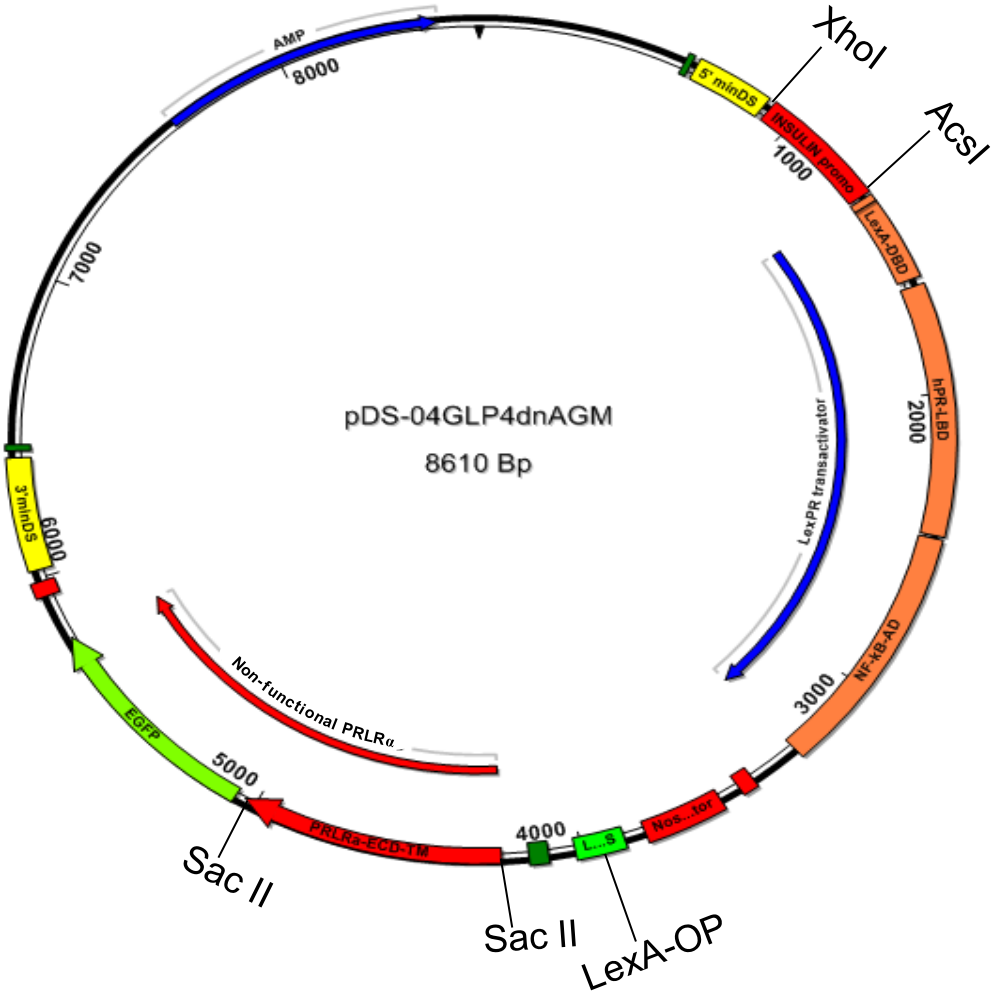


Table 4-1.Transgene transmission rates of *tg(ins:nPrlrα:gfp)* lines in F1 and F2.

Founder	Sex	F1 (%)	F2 (%)
A	Male	38/100 (38.0%)	30/55 (54.5%)
B	Male	24/79 (30.4%)	65/110 (59.1%)
C	Male	45/130 (34.6%)	54/110 (49.1%)
D	Male	20/120 (16.7%)	33/90 (36.7%)

Fig. 4-2. Expression of prolactin receptor α (PRLRα), insulin (*ins*), and *gfp* in pancreatic β-cells of transgenic zebrafish, *tg(ins:gfp)*. A: a representative flat mount image from whole mount *in situ* hybridized (WISH) of embryos with insulin antisense probe for detecting insulin transcript; B: A representative flat mount image of the same embryo stained with anti-GFP antibody for detection of the expression of GFP protein under the control of insulin promoter (see material and methods for detail). C: A superimposed image of panel A and panel B. D-L: Co-localization of *prlrα* and GFP expressions in representative images from sections of zebrafish embryos following WISH and immunohistochemistry. The expression of *prlrα* transcript was detected by WISH (D; purple) and GFP was detected with anti-GFP antibody staining (E; green). F: Superimposed images of (D) and (E). Arrowheads indicates the pancreatic β-cells (B and E) and arrows indicate *prlrα* expression in the kidney. Auto-fluorescent of the yolk is noted by *. Scale bar: 50 μm.

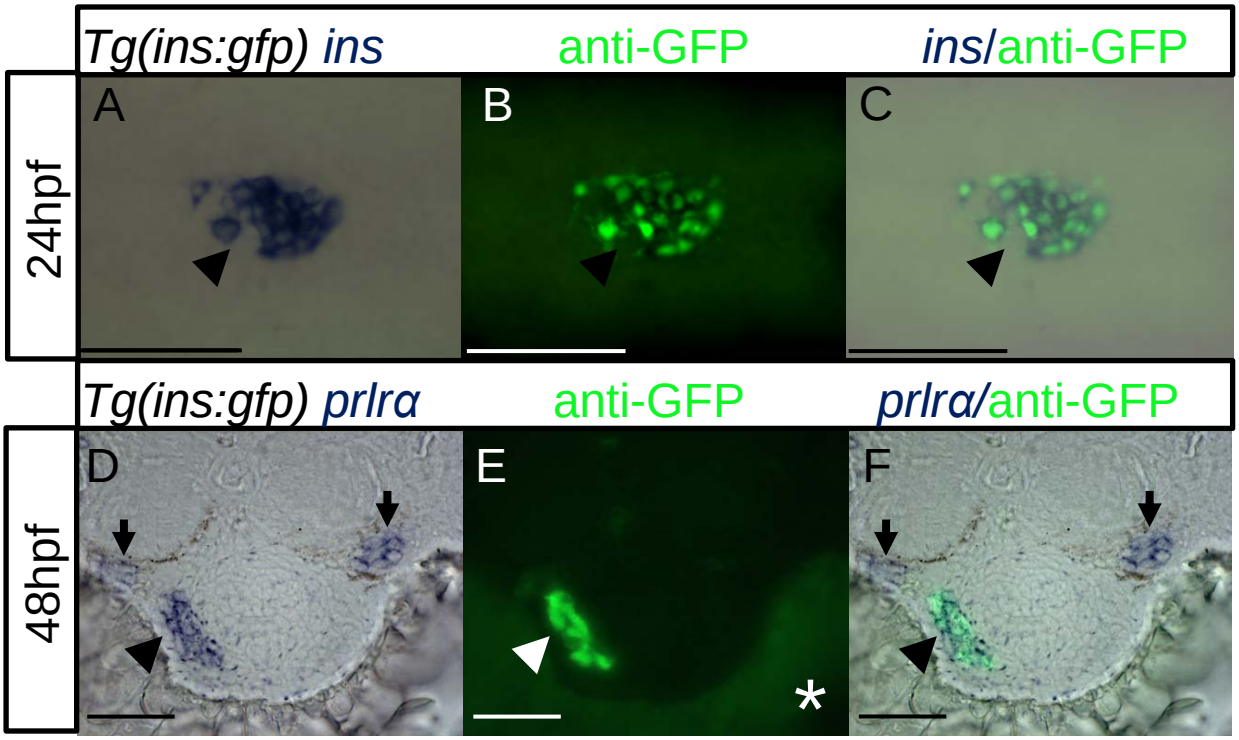


Fig. 4-3. Effects of prolactin antisense morpholino (PRL-MO), over-expression of prolactin (*prl* mRNA), and expression of non-functional prolactin receptor α (*nPrlrα*) on the number of insulin-producing β-cells in the zebrafish pancreas. A: Representative section images of embryo subjected to whole mount *in situ* hybridization using insulin antisense probe followed by nuclei staining with DAPI (4',6-diamidino-2-phenylindole; fluorescent dye that binds to DNA). B: Representative images of insulin-positive cells at 17 hpf from NI control, mismatch-MO, PRL-MO, and over-expression of PRL mRNA. C: Results shown as mean±SEM of total number of β-cells in serial sections of individual embryos (n=3-5). Significance ($P<0.05$) is denoted by * in comparison with NI controls. *s*, somite; *n*, notochord, *sb*, swimbladder, *G*, gut.

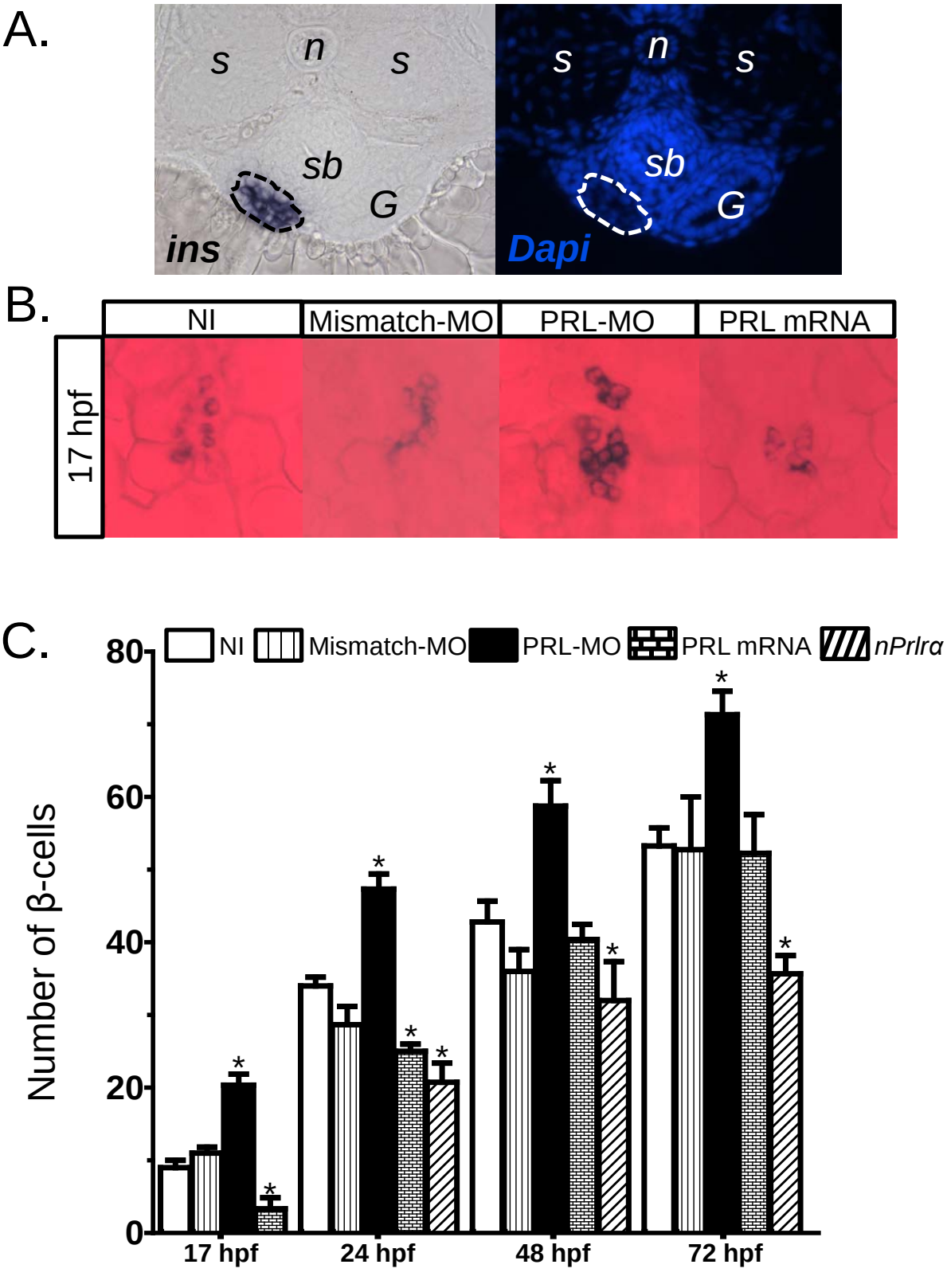

A.
s
n
s
sb
G
ins
Dapi
B.
NI
Mismatch-MO
PRL-MO
PRL mRNA
17 hpf
C.
NI
Mismatch-MO
PRL-MO
PRL mRNA
nPrlrα
Number of β-cells
0
20
40
60
80
17 hpf
24 hpf
48 hpf
72 hpf
Developmental Time Points

Fig. 4-4. Effects of prolactin antisense morpholino (PRL-MO) and over-expression of PRL on the expression of insulin. A: Representative Western analysis of PRL-MO knockdown on the expression of GFP protein controlled by an insulin promoter in zebrafish embryos; Lane1: non-injected control embryos, Lane2: PRL-MO. Anti-β-tubulin was used as the loading control. B: Effects of PRL-MO knockdown and over-expression of PRL on insulin a (*insa*) transcript analyzed by quantitative real-time PCR. The expression levels of *insa* were normalized by the EF1α, and shown as (mean±SE) from three independent experiments. Significance ($P<0.05$) is denoted by *.

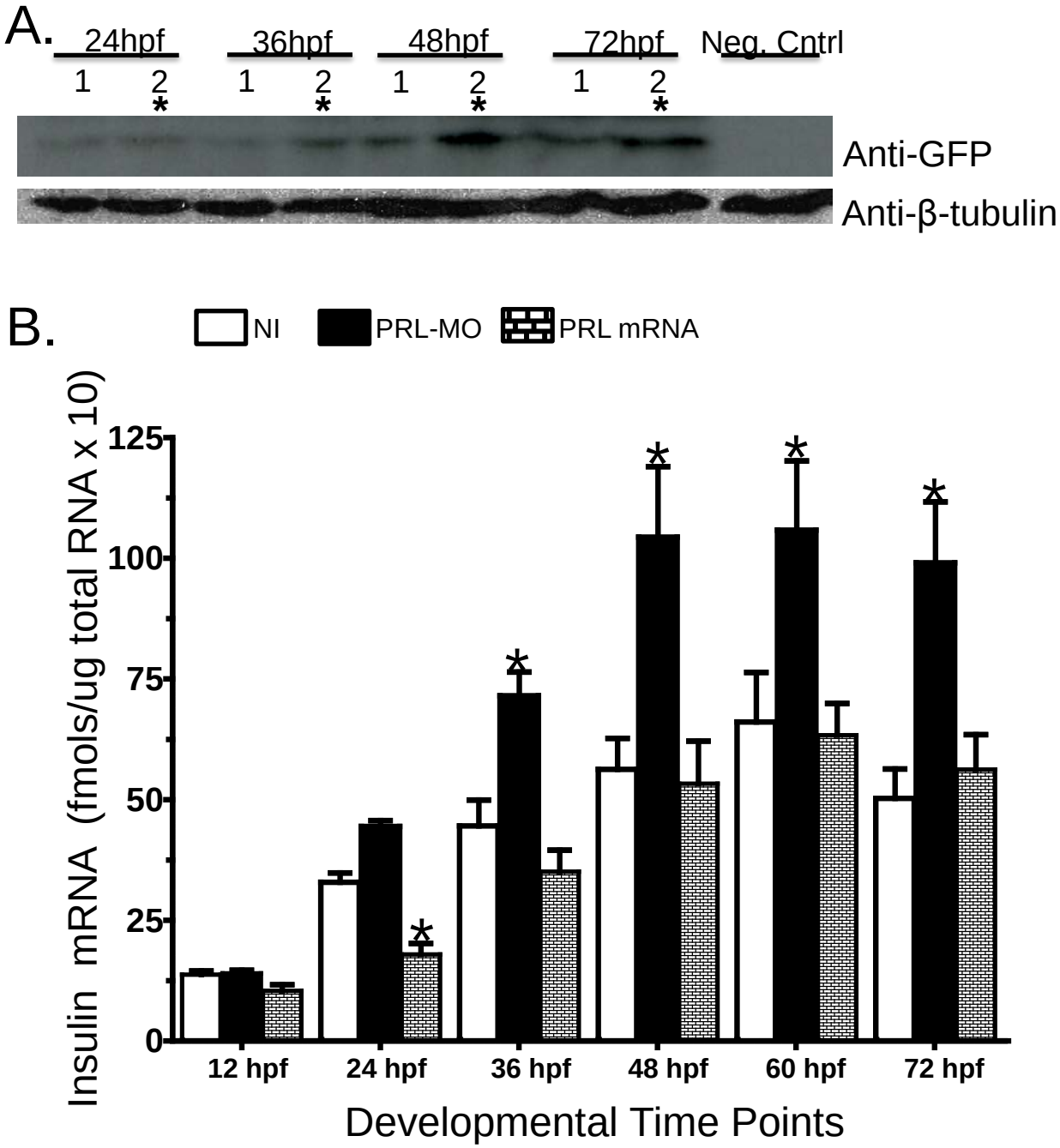

A.
24hpf
36hpf
48hpf
72hpf
Neg. Cntrl
1
2
*
Anti-GFP
Anti-β-tubulin
B.
NI
PRL-MO
PRL mRNA
Insulin mRNA (fmols/ug total RNA x 10)
125
100
75
50
25
0
12 hpf
24 hpf
36 hpf
48 hpf
60 hpf
72 hpf
Developmental Time Points

Fig. 4-5. Expression of non-functional prolactin receptor α (*nPrlrα*) in pancreatic β-cells at 24 hours post-fertilization (hpf). A: A representative image of a section prepared from insulin-positive cells after whole mount *in situ* hybridization (WISH) using an insulin antisense ribopobe. B: The same section as shown in panel A immunostained with an anti-GFP antibody to detect in intracellular domain of the *nPrlrα* and co-stained with DAPI. C: This panel shows an image in which panels A and B were superimposed.

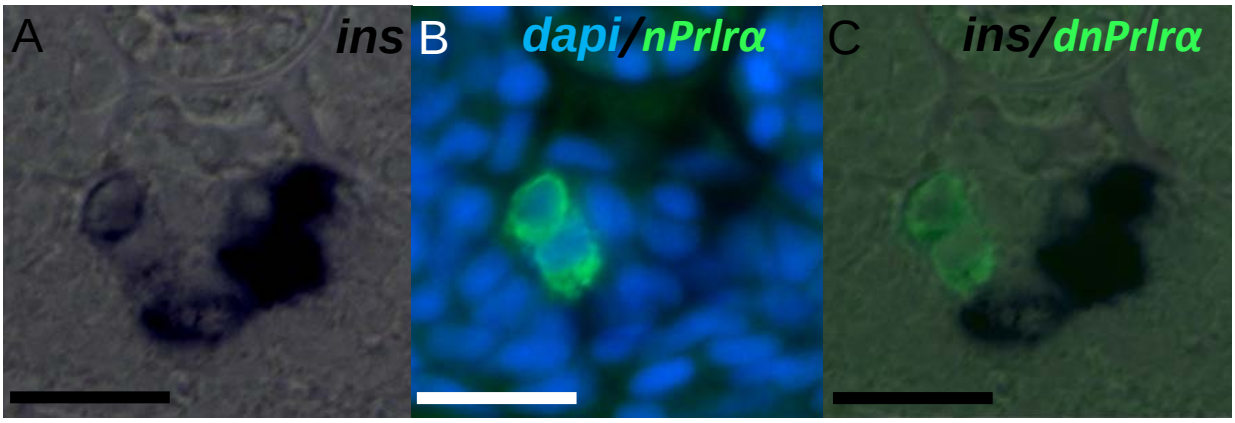


Fig. 4-6. Effects of expression of non-functional prolactin receptor α (*nPrlrα*) and prolactin antisense morpholino (PRL-MO) on the expression of insulin during zebrafish pancreas development. Dotted-line indicates the border of the developing exocrine pancreas. *Tg(ins:nPrlrα:gfp)* were treated with RU486 (*nPrlrα* Pos.; C, G, K) and without RU486 (*nPrlrα* Neg.; A, E, I). Only *tg(ins:Prlrα:gfp)* exposed to RU486 expressed *nPrlrα*. Wild-type embryos were exposed to RU486 served as the control for non-specific effect due to chemical treatment (AB-WT+RU486, B, F, J). *nPRLRα* Pos.+PRLMO represents *tg(ins:nPrlrα:gfp)* embryos injected with PRL-MO and treated with RU486 (D, H, L).

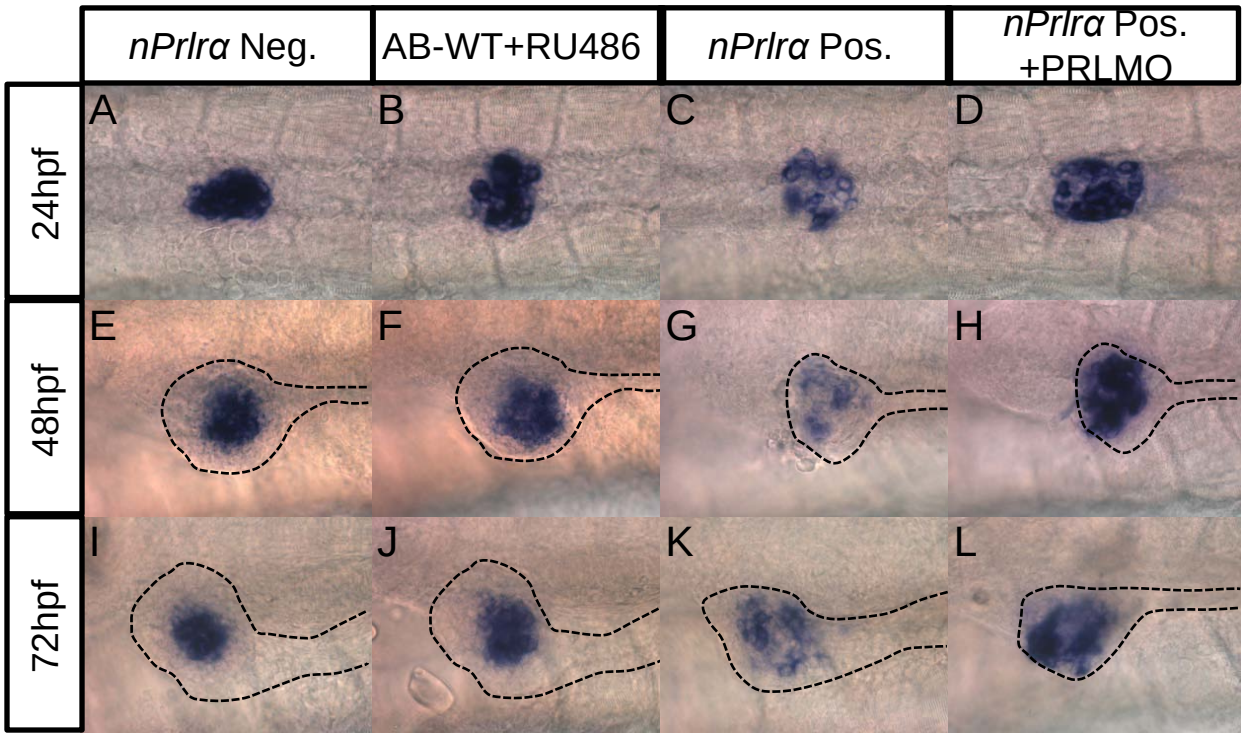


Fig. 4-7. Effect of different RU486 exposure times on insulin-positive cells in *tg(ins:nPrlrα:gfp)* line at 48 and 72 hpf. *Tg(ins:nPrlrα:gfp)* embryos were raised without RU486 (A, D), exposed to RU486 at 12 h (B, E), 24 h (C), and 48 h (F). Embryos were processed at 48 and 72 hpf for whole mount RNA *in situ* hybridization with an antisense insulin riboprobe followed by sectioning at 12 um thickness. Arrowheads indicate insulin-positive cells. Scale bar, 50μm.

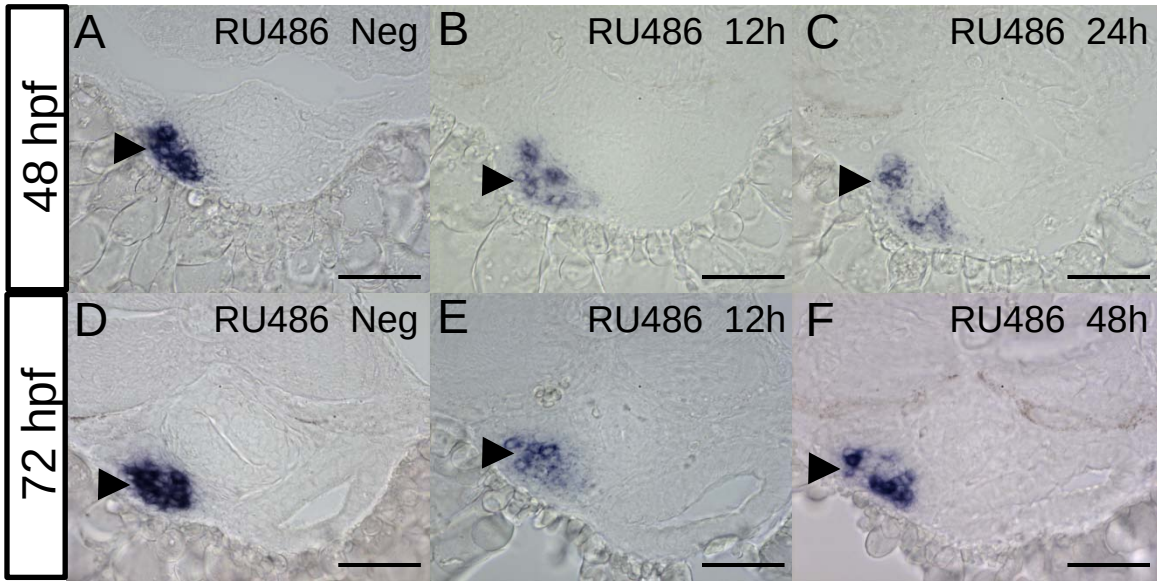


Fig. 4-8. Effects of prolactin antisense morpholino (PRL-MO) on the expression of PRLRα transcripts in zebrafish embryos. A: Representative images of whole mount *in situ* hybridized (WISH) embryos using an antisense PRLRα riboprobe. Arrowheads indicate pancreas. Arrows indicate kidney. B:Real-time quantitative analysis of *prlrα* in PRL-MO knockdown and over-expression of PRL compare to those in the control embryos. Scale bar: 50 μm.

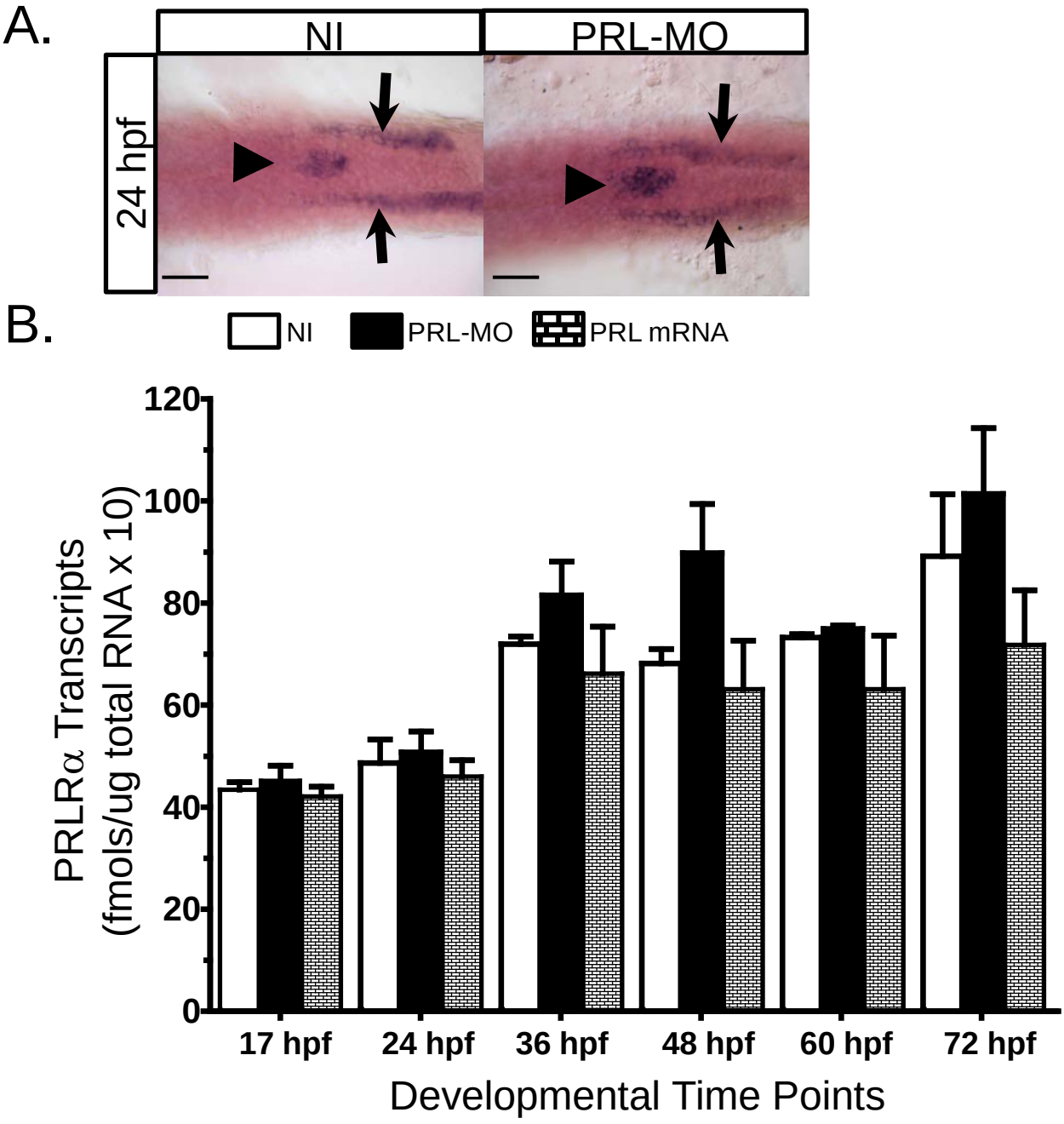


Fig. 4-9. Comparison of cell number between non-injected and PRL-MO-injected embryos at 48 hours post-fertilization (hpf). Representative serial sections of NI and PRL-MO-treated 48 hpf embryos. β-cells were identified with anti-GFP antibody in *tg(ins:GFP)* lines (green) and proliferating cells with anti-PH3 antibody (red). Arrowheads indicate proliferating cells within the islet. Scale bar: 100 μm.

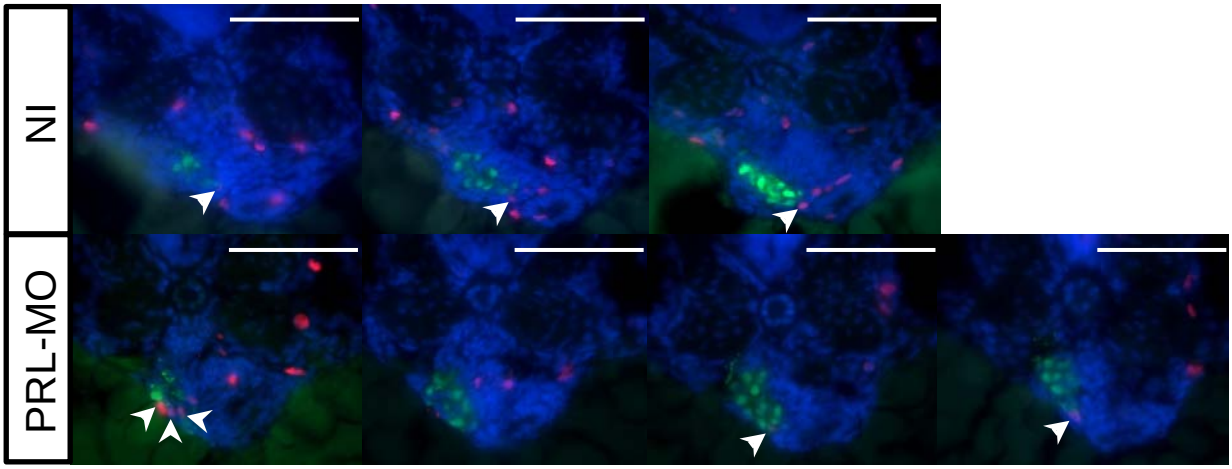


Fig. 4-10. Effects of prolactin antisense morpholino (PRL-MO) and expression of non-functional prolactin receptor α (*nPrlrα*) on the expression of *hb9* transcript analyzed by whole mount *in situ* hybridization. Arrows indicate gas bladder, and arrowheads indicate the endocrine pancreas.

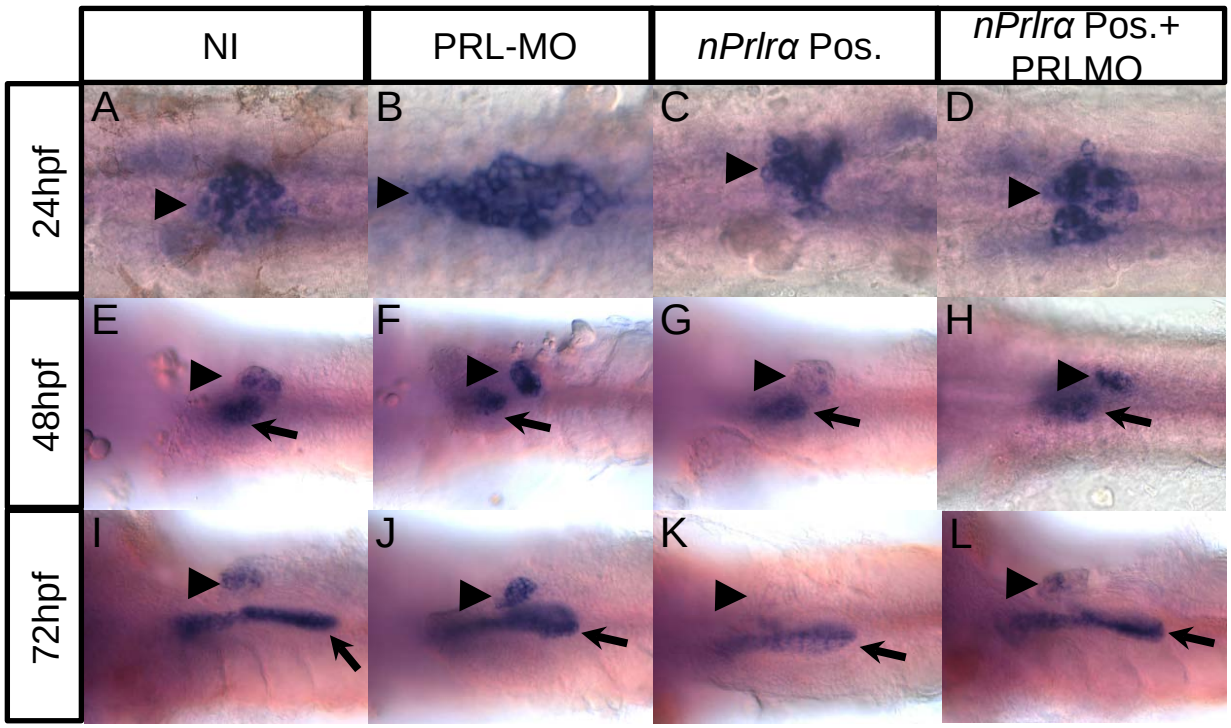


Fig. 4-11. Effects of prolactin antisense morpholino (PRL-MO) and expression of non-functional prolactin receptor α (*nPrlrα*) on the number of *neuroD* positive cells at 18 and 21 hours post-fertilization (hpf) in zebrafish embryos. Significance (P<0.05) was denoted by *.

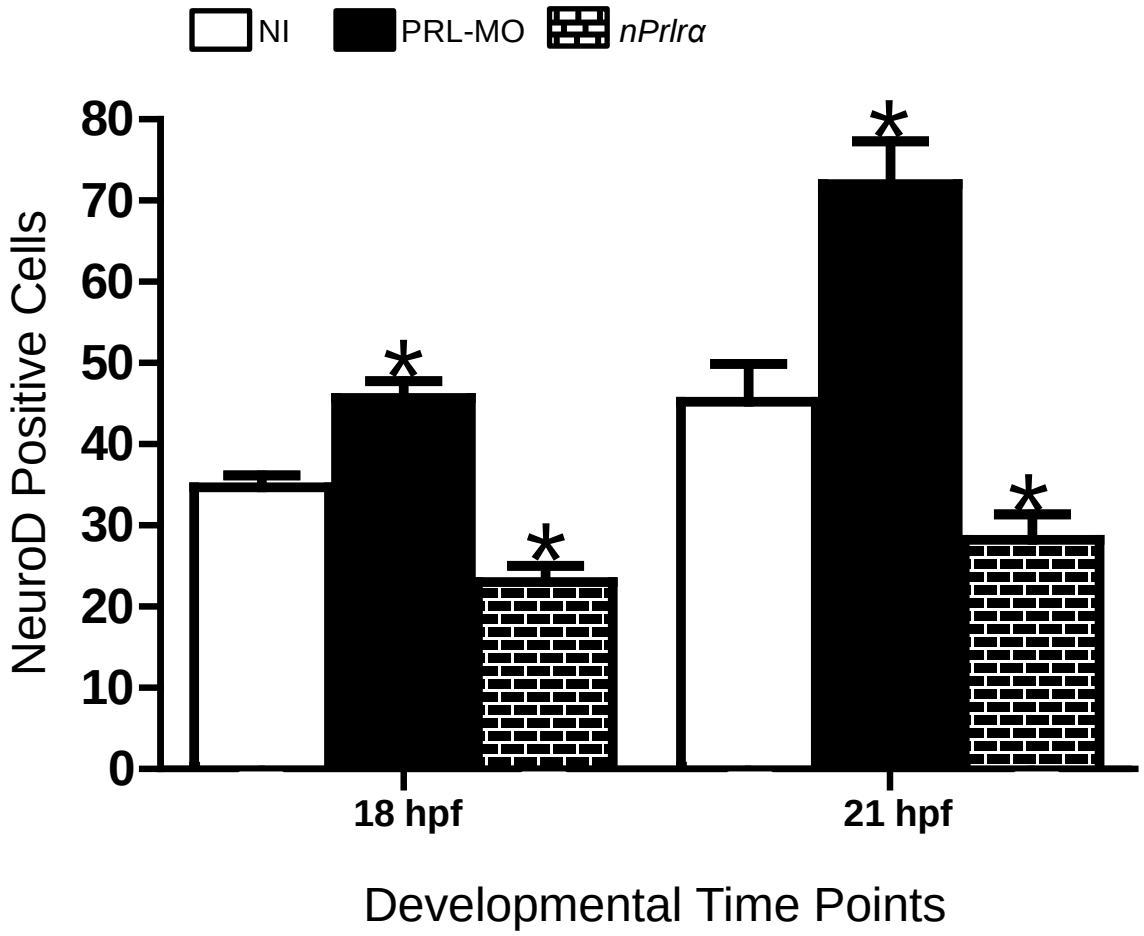
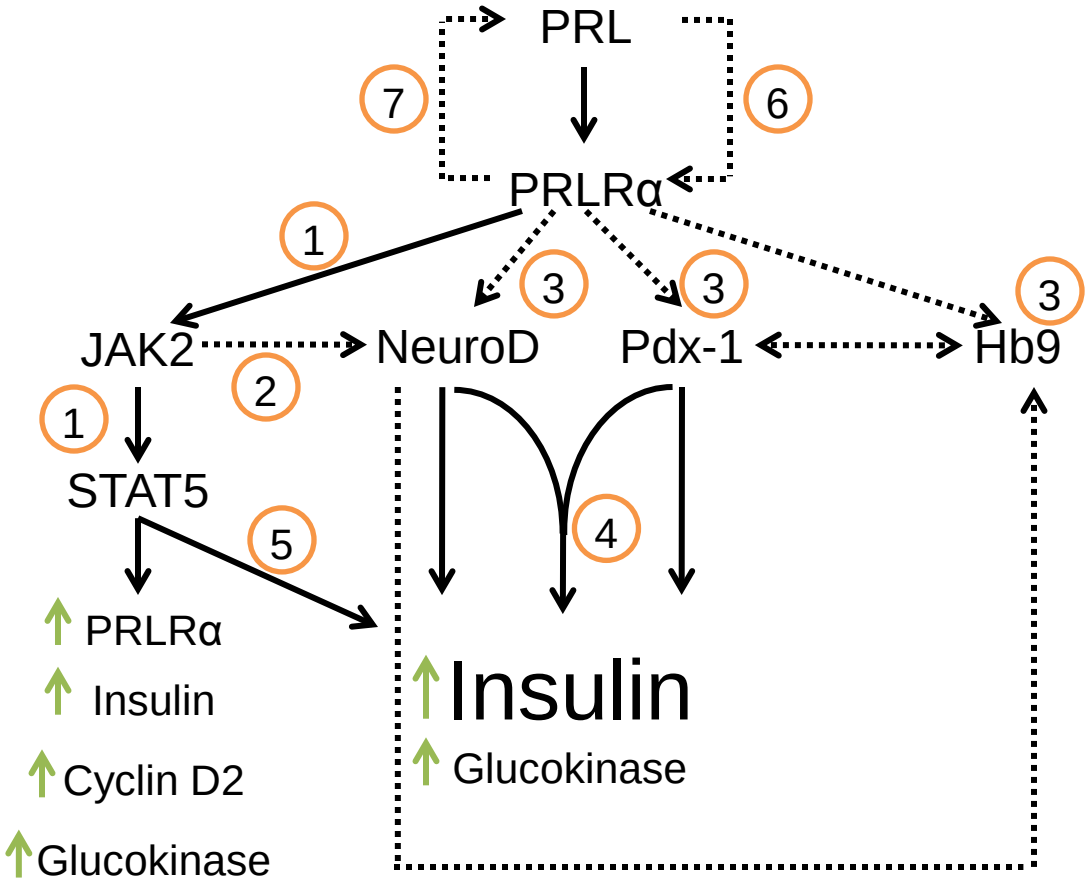


Table 4-2. Percentage of numbers of the primary islet (insulin-producing β-cells) migrated in embryos injected with prolactin antisense morpholino (PRL-MO) or expressing non-functional PRLRα (*nPrlrα*) compared to the non-injected control embryos.

Phenotype / Treatment	24hpf		48hpf			72hpf		
	No GFP	Midline	Left	Midline	Right	Left	Midline	Right
Non-injected	0.66%	99.3%	2.22%	0.00%	97.8%	0.00%	0.00%	100%
PRL-MO	0.00%	100%	1.00%	0.00%	99.0%	0.90%	0.00%	99.1%
nPrlra	0.00%	100%	1.11%	0.00%	98.9%	1.00%	0.00%	99%

Fig. 4-12. A proposed model of the PRL/PRLRα-mediated mechanism by which β-cells develop and/or functions during zebrafish embryogenesis. PRL binds to PRLRα on β-cells and activates: (1) the JAK2/STAT5 signaling pathway to directly induce target genes via promoters containing GAS motifs and leading to gene transcription and maintaining β-cell function; (2) the activation of JAK2 could lead to the activation of *neuroD*, *hb9*, and *pdx-1* which regulates insulin synthesis and promote a β-cell fate in pancreatic precursors; (3) PRLRα could regulate *neuroD*, *pdx-1*, and *hb9* independent of JAK2 where they control insulin and glucokinase transcriptional activity alone, simultaneously, or as cofactors (4) and possibly involving STAT5 (5). The extent of PRLRα signaling may be dependent on the feedback loop between PRLRα (6) and PRL (7) levels. Solid arrows represent proven pathways and dotted arrows are potential mechanisms.



CHAPTER 5: CONCLUSION AND FUTURE DIRECTION

In summary, I have demonstrated that prolactin (PRL) and its cognate receptor PRLRα, is an important anti-apoptotic factor potentially regulating anterior structure development and a potent hormone responsible for the maintenance of β-cell populations throughout zebrafish embryogenesis. The biological relevance of PRL during embryonic development is supported by the following evidence: 1.) PRL and the cognate receptors for PRL, PRLRα and PRLRβ, are expressed as early as the one-cell stage and are maintained or fluctuate throughout zebrafish embryogenesis; 2.) morphological abnormalities of PRL morphants are rescued using *in vitro* transcribed *prl* mRNA and constitutively active signaling molecules known to be activated by PRLRs (JAK2a, STAT5.1, PI3K, and AKT); 3.) physical defects of PRL morphants are independent of off-target effects resulting from the activation of the p53 pathway commonly disturbed by morpholino treatment; 4.) the amount of PRL protein is reduced in PRL morphants; and 5.) *in vivo* inhibition of PRLRα and PRLRβ phenocopied many defects observed in PRL morpholino-injected embryos.

PRL morphants exhibited an increase in cell death within the central nervous system (i.e. eyes and brain), as evident by activation of caspase-8 and caspase-3. This likely leads to the DNA damage detected by the Neutral Comet and TUNEL assay. The appearance of cell death in embryos experiencing a reduction in PRL and not GH, suggests a specific function of PRL as an anti-apoptotic factor in zebrafish embryos that is not shared with other members of the same hormone family. In addition, GHR and SLR knockdown, together or independently, did not produce any defects in eye or head size (data not shown). In contrast, the cell death in PRL morphants was rescued with *prl* mRNA and constitutively active JAK2a, indicating that PRLR activation and the JAK2a pathway is involved in mediating suppression of apoptosis. We cannot

exclude the possibility that the PI3K/AKT pathway also plays a role in cell survival. Activation of the PI3K/AKT pathway has been shown to mediate critical events leading to cell growth and survival (Hennessy et al., 2005). PRLR could simultaneously activate both the JAK2/STAT5 and PI3K/AKT signaling transduction to promote cell survival or these different pathways could be up-regulated in a cell-specific manner. Closer examination of these signaling pathways within specific cells in the eyes and brains will help resolve this issue.

The pancreas and kidneys are two additional targets of PRLRα but no apoptotic cells were observed in these regions. The absence of apoptosis within these organs may reflect differences in the effects of PRL on these tissues compared to the eyes and brain. Although the development of the vertebrate eye is not exclusively derived from the ectoderm, the retina and central nervous system originate from neuroepithelial tissues (Baily et al., 2004). During neural plate formation, various neuronal cell types are overproduced and approximately 70% of these cells undergo programmed cell death through the course of development to define specific neuronal sub-populations (Roth and D'Sa, 2001). The survival of these neuron populations is dependent on proper regionalization and innervation to target cells for exposure to trophic factors. In this scenario, PRL may be acting as one of these anti-apoptotic trophic factors and suggests neuronal cells are more sensitive to apoptosis due to the complex network of neuronal interactions compared with other cell types. Furthermore, the function of PRL could be mediated indirectly through the IGF system. In contrast, the kidney and pancreas are derived from the intermediate mesoderm and endoderm, respectively. Formation of these organs develops from mesenchymal-epithelial inductive interactions that lead to specialization of epithelial cells to a nephrogenic fate or pancreatic fate (Burrow, 2000; Drummond, 2003). The outcome of mesenchymal-epithelial induction is to generate specialize cells that will eventual proliferate to

increase the population, thus apoptosis is not likely to occur. Nonetheless, programmed cell death was observed in two regions during vertebrate kidney development: the nephrogenic zone of the developing kidney cortex, and the medullary papilla (Coles et al., 1993). The equivalent structures in the zebrafish kidney are not developed until 40 hpf (Drummond, 2003). We did not examine apoptosis beyond 35 hpf, and it remains to be answered if PRL also acts as an anti-apoptotic factor in these equivalent structures, but it is unlikely because PRLRs are restricted to the pronephric tubules. Unlike the kidney, apoptosis appears to be a rare event in the adult pancreas (Tetra et al., 2005) and is only observed during the neonatal period with simultaneous activation of proliferation, suggesting a remodeling event of the endocrine pancreas for adult function (Kaung, 1994; Scaglia et al., 1997). The apoptosis assays were performed on PRL morphants and we know now that there was an increase in PRLRα transcripts. It is possible that the absence of apoptosis in the pancreas may be due to the PRLRα-mediated up-regulation of the JAK2/STAT5 transcripts and the pro-survival molecule Bcl-xL (Fujinaka et al., 2007). However, heterozygous and homozygous PRLR null mutant mice did not exhibit an increase in pancreatic cell apoptosis, during neonatal development or in pregnancy, indicating that the role of PRL in the pancreas does not involve regulation of cell death (Freemark et al., 2002; Huang et al., 2009).

Recently, a second PRL, PRL2 compared to our PRL1, was identified in non-mammalian vertebrates and shown to be expressed only in extra-pituitary tissues (eye, brain, and kidney) (Huang et al., 2009). It was demonstrated that PRL2 is potentially involved in retinal neuron differentiation, but no indication of the eye size or apoptosis was available. It is of great interest to re-examine the specific nuclear layers of the retina undergoing apoptosis in PRL morphants and compare the regions affected with results from PRL2 knockdown to better understand whether the duplicated PRLs in zebrafish possess overlapping and/or unique functions, as with

the PRLRs. In view of PRL2 acting as a retinal neuron differentiation factor and the observation of apoptotic cells in both the eye and optic tectum, there is also a need to analyze the topographic mapping of the retinotectal projections between the two structures. Proper interactions between the retina and optic tectum are essential for normal development of the visual system (D'Souza et al., 2005), and perhaps PRL is important in this process.

The pancreas is another important target of PRL and was demonstrated to exert a robust effect on the β-cell population during zebrafish development. PRLRα was expressed immediately after the initial β-cell differentiation and its expression co-localized with β-cells thereafter. The absence of PRLRβ within the pancreas indicates that PRLRα acquired an additional role in maintaining normal β-cell function after the divergence of fish from tetrapods and during the fish specific whole genome duplication event in the lineage leading to the modern teleost. *Tg(ins:nPrlrα:gfp)* lines treated with RU486 induced the expression of non-functional PRLRα (*nPrlrα*) in β-cells and the resulting embryos displayed a reduction in total β-cell number. The β-cell phenotype in *nPrlrα* embryos is reminiscent of the reduced β-cell population observed in targeted deletion of the PRLR gene in mice (Freemark et al., 2002; Huang et al., 2009), leading to the suppression of PRLR signaling. We suggest that the expression of *nPrlrα* also reduced PRLRα signaling through a mechanism of heterodimerization between native PRLRα and RU486 induced *nPrlrα* or homodimerization of *nPrlrα* in our system. In comparison, morpholino-mediated PRL knockdown unexpectedly increased β-cell population and insulin transcripts, but it was also accompanied with an increase in endogenous PRLRα mRNA within the endocrine pancreas. It is worth noting that while PRL morphants had an increase in β-cell number, insulin transcripts, and insulin promoter regulated *gfp* protein, it resulted in overall smaller body and reduced anterior structures. In contrast, a reduction of the β-

cell population was seen in *nPrlrα* embryos, yet the body size was relatively normal compared to controls (data not shown). Future studies should aim to identify the physiological significance of reduced β-cell number on the metabolic state of the embryo throughout embryogenesis, the effect of reduced β-cells in relation to adult pancreas development and function and the potential relevance to organ development in zebrafish.

In addition to reduced β-cell number, *nPrlrα* embryos also revealed a severe reduction in β-cell differentiation transcription factors, *hb9* and *neuroD*. These results suggest a novel mechanism for PRL in regulating β-cell number that contrasts with its defined role in β-cell proliferation in postnatal and pregnant mice. Within the endocrine pancreas of zebrafish, PRL targets β-cells through the PRLRα. It is not known whether PRL also activates the JAK2/STAT5 signaling mechanism that controls expression of PRLR, insulin, cyclin D2, and glucokinase during zebrafish embryogenesis as it does in isolated islets or *in vivo* in rodent models. Furthermore, no evidence is available to indicate the involvement of the JAK2/STAT5 in regulating the transcription of *hb9* or *neuroD*. However, it was recently demonstrated that epidermal growth factor, which belongs to the same cytokine family with PRL, could transiently express *ngn3* through the JAK2/STAT3 *in vitro* (Baeyens et al., 2006). PRL could potentially function through a similar mechanism to increase *ngn3*, which is an upstream regulator of *neuroD*, and subsequently activate *neuroD*. *Ngn3* is considered the marker for all endocrine pancreas precursor cells and is required for differentiation of all islet cell types (Gradwohl et al., 2000). We observed that in some instances, PRLRα was expressed in pancreatic cells that did not express insulin. These non-insulin expressing cells could potentially be endocrine pancreas precursor cells that require PRLR to regulate differentiation into mature β-cells by stimulating the transcriptional activity of *neuroD*, *pdx-1*, and *hb9*. Our study also indicated the continued

presence of β-cells in *nPrlrα* embryos, albeit fewer than wild-type animals. The locations of the remaining β-cells appeared to be dispersed, with many residing at the periphery of the islet, unlike the tight core of β-cells observed in normal embryos. This might indicate the continued presence of β-cell progenitors within the developing zebrafish pancreatic islet to compensate for the loss of β-cells observed in *nPrlrα* expressing embryos. Identification of these potential β-cell progenitors and analysis of the relationship between PRL, PRLRα, *ngn3*, *hb9*, and *neuroD* expression within these non-insulin producing cells would help determine the involvement of PRL/PRLRα in β-cell differentiation and provide a better understanding on the mechanism of β-cell expansion during early pancreas development in zebrafish.

Finally, there appeared to be an intrinsic feedback relationship between PRL and PRLRα related to β-cell development during zebrafish embryogenesis. PRL knockdown mediated by antisense morpholino produced embryos with an increase in β-cell number and PRLRα transcripts within the pancreas. The up-regulation of PRLRα is reminiscent of a previous study in which inhibition of endogenous PRL secretion by bromocriptine in mice stimulated the expression of PRLR in the fallopian tube (Shao et al., 2008). In contrast, treatment of PRL by subcutaneous injections inhibited PRLR expression (Shao et al., 2008). The observed decreased in β-cell populations of our embryos over-expressed with *prl* mRNA would be consistent with a decreased in PRLRα expression. These results suggest that high levels of PRL inhibits, while low levels of PRL stimulates expression of PRLRα within the pancreas. The level of PRLRα expression has also been demonstrated to regulate PRL production. In zebrafish, PRLRα knockdown mediated by antisense morpholino demonstrated an increase in PRL transcripts in the pituitary gland (Liu et al., 2006). Along the same line, disruption of PRLR signaling by targeted deletion of PRLR in mice caused an increase in circulating PRL levels (Binart et al.,

2000; Halperin et al., 2007). Expression of *nPrlrα* in our embryos maintained a reduced β-cell number phenotype, suggesting that the expected up-regulation of PRL is insufficient to compensate for the reduced PRLRα signaling. It has been demonstrated that the increase in PRL levels of PRLR null mutant mice is controlled at the hypothalamus and pituitary level (Schuff et al., 2002), but the expression of *nPrlrα* in our model is restricted to the pancreas and it is unclear how PRL is suspected to be up-regulated in these embryos. Interestingly, injection of PRL-MO in *nPrlrα* expressing embryos exhibited a recovered β-cell population comparable to the control embryos. The exact mechanism controlling the feedback loop between PRL and PRLRα in relation to the β-cell number is unclear during zebrafish embryogenesis, but investigation into this relationship will be important to further define the function of PRL on β-cell development.

In conclusion, this study has provided strong evidence to define functional roles for PRL in embryonic development, which has been argued for many years. PRL and PRLRα were demonstrated to serve at least two functions during embryogenesis in zebrafish, an anti-apoptotic factor and a regulator of β-cell populations. The observation that PRL and PRLRα signaling were capable of interacting with developmental genes (*hb9* and *neuroD*) *in vivo* in zebrafish has provided a suitable alternative model to study the emerging field of developmental endocrinology. The establishment of the *tg(ins:nPrlrα:gfp)* line also provides a valuable tool to further examine the specific functions of PRL/PRLRα in β-cell function, development and/or regeneration in a cell and developmental stage specific manner. This is the first study that examined specific functions of PRL during embryogenesis, and future studies will be required to better understand the complexity of PRL function in a variety of biological settings during early vertebrate development.

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Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

February 6, 2008

Yong Zhu, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. Zhu:

Your Animal Use Protocol entitled, "Studies of Hormones and Receptors in Zebrafish (Danio Rerio)," (AUP #D185b) was reviewed by this institution's Animal Care and Use Committee on 2/6/08. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

East Carolina University
Animal Use Protocol (AUP) Form
Revised, October 5, 2007

Project Title: Studies of Hormones and Receptors in Zebrafish (*Danio Rerio)*

1. Personnel

1.1. Principal investigator and email: Yong Zhu, zhuy@ecu.edu

1.2. Department, office phone: Biology, Howell Science N401, 328-6504

1.3. Emergency numbers:

	Principal Investigator:	Other (Co-I, technician, student)
Cell: Pager: Home:	Yong Zhu 252-215-6504 (office) 252-215-9071 (home)	PhD candidate, Nhu Nguyen (Cell 252-412-9439) PhD candidate: Richard Hanna (cell 443-514-6229) MS candidate: Melina Pereira (252-258-8202) MS candidate: Sean Daly (cell 814-3952) MS candidate: Raymond Stewart Lyon Jr (cell 919-219-3288) MS candidate: Brian D. Sufrinko (252-452-4866)

FOR IACUC USE ONLY

AUP # D1856
New/renewal: Renewal
Date received: 2/4/08
Full Review and date: Designated Reviewer and date:
Approval date:
Pain/Distress category: A
Surgery: ✓ Survival: Multiple:
Prolonged restraint:
Food/fluid restriction:
Hazard approval/dates: Rad: IBC: EH&S:
OHP enrollment/mandatory animal training completed :
Amendments approved:

1.4. Co-Investigators if any:

1.5. List all personnel (PI, Co-I, technicians, students) that will be performing procedures on live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:

Name	Relevant Animal Experience
PI: Yong Zhu	22 years
PhD student: Nhu Nguyen	5 years
PhD student: Richard Hanna	4 years
MS student: Melina Pereira	5 years
MS student: Sean Daly	1 year (will be trained by PI)
MS student: Stewart Lyon Jr	1 year (will be trained by PI)
MS student: Brian Sufrinko	1 year (will be trained by PI)

2. Regulatory Compliance

2.1 Non-Technical Summary

Using language a non-scientist would understand, please provide a 6 to 8 sentence summary explaining the overall study objectives and benefits of proposed research or teaching activity, and a brief overview of procedures

involving live animals (more detailed procedures are requested later in the AUP). Do **not** cut and paste the grant abstract.

Using zebrafish as a model, my laboratory focus on: 1) examining physiological functions of pituitary hormones, especially roles of the prolactin/growth hormone superfamily in early development in vertebrates; 2) investigating non-genomic actions of steroids with a focus on physiological functions and signaling mechanisms of the membrane and nuclear progestin receptor family. These studies conducted in my laboratory are novel and using cutting-edge technologies. In past 5 years, we have provided opportunities to a large number of graduates and undergraduates to engage in nationally and internationally recognized research to help them achieve their career objectives and to gain an appreciation for the value of basic science research in our society. We will continue our effort to prepare our students well for the future by providing them with up-to-date knowledge, advanced technical skills and a strong sense that they are participating in exciting science. The basic procedures include rearing and maintaining of health zebrafish colony, embryos, and larvae. Sometime, fish will be subjected to experimental treatments; tissues from various treated fish will require being isolated for further analyses.

2.2. Unnecessary Duplication

Does this study duplicate existing research? Yes ☐ No ■.

If yes, why is it necessary? (note: teaching by definition is duplicative)

2.3 Literature Search to ensure that there are no alternatives to the use of animals

List the following information for each search (please do not submit search results but retain them for your records):

Date Search was performed: 02/04/2008
Database searched: Medline and Premedline
Period of years covered in the search: 1966-present
Keywords used and strategy (must include the word alternatives): growth hormone, prolactin, embryonic development, organogenesis, steroid, progestin, membrane, nuclear, receptor, nonclassical, nongenomic, genomic, animal modes, zebrafish, and alternative methods.

Other sources consulted: publications and communications in related professional journals, and local, national, and international conferences.
No alternatives have been found from search of journal sof Science, Nature, Proceeding of the National Academy of Sciences of the United States of America, Endocrinology, Molecular Endocrinology, Journal of Endocrinology, Biology of Reproduction, General and Comparative Endocrinology. Currently, my lab is the only laboratory in the world conducting the proposed studies using zebrafish as a model.

Narrative indicating the results of the search (2-3 sentences) and why there are no alternatives to your proposed use of the animals in this protocol. If alternatives exist, describe why they are not adequate:
The above keywords were combined in various amalgamations. No publication or communication was found from other laboratories describing the characterizations of membrane progestin receptor in zebrafish.

2.4 Hazardous agents

2.4a. Protocol related hazards

Will any of the following be used in live animals and therefore pose a potential risk for animal care and research personnel:

	Oversight committee/ approval date	Safety procedures attached (Yes/No)
Radioisotopes	Radiation/	No
Ionizing radiation	Radiation/	No
Infectious agents	Biosafety/	No
Toxins (venoms, etc)	Prospective Health/	No
Oncogenic/toxic/mutagenic agents	EH&S/	No
Material of human origin	Biosafety/	No
Cell lines injected or implanted (MAP test)	DCM/	No
Recombinant DNA in animals	Biosafety/	Yes
Nanoparticles	EH&S/	No
Other agents		No

If any hazardous agents are used, please fill out the attached Hazardous Agents Form (Appendix 1).

2.4b. Incidental hazards

Will personnel be exposed to any incidental zoonotic diseases or hazards during the study (field studies, primate work, etc)? If so, please identify each and explain steps taken to mitigate risk:

No

3. Animals and Housing

3.1. Species and strains:	Wild type, mutant or transgenic zebrafish. Wild type zebrafish are normally obtained from local pet stores, and propagated in the laboratory. Disease free mutant or transgenic zebrafish are normally obtained from Zebrafish International Resource Center or other laboratories around the world
3.2. Weight, sex and/or age:	0.001-5 gram, mixed sex, 0-4 years

Total number of animals in treatment and control groups	Additional animals (Breeders, substitute animals)	Total number of animals used for this project
2000	+ 4000	=6000

3.3. Justify the species and number (statistical justification if applicable) of animals requested:

Zebrafish is easy to maintain and the adult fish spawn daily under laboratory conditions. Zebrafish is a well-accepted model for studying the processes of the development and the reproduction, and has become an alternative model for studying gene functions in vertebrates. My laboratory focuses on physiological functions and molecular mechanisms of the hormones and receptors in vzebrafish using various probes including hormones and receptors that we have developed recently in zebrafish.

Because of small size of the fish, relative large number of fish will be required to provide the minimal amount of tissues for in vitro incubations and manipulations. Number of sampled animals will be varied according to the various experiments. Approximately 5-100 individuals will be sampled each time.

3.4. Justify the number and use of any additional animals needed for this study (i.e. breeder animals, inappropriate genotype/phenotype, extra animals due to problems that may arise, etc.):

Normally, we keep about 100-200 adult breeders for raise zebrafish larvae. Each female capable spawn 110-200 eggs daily. Our experiments need to be repeated at least 3 times in order to accept and reject the results.

3.5. Will the phenotype of mutant, transgenic or knockout animals predispose them to any health problems? Yes ☐ No ■
(if yes, describe)

3.6. If wild animals will be captured or used, provide permissions (collection permit # or other required information:

N/A. Wilde type zebrafish were purchased from local pet store

3.7. Are there any unusual husbandry and environmental conditions required? Yes ☐ No ■ If yes, then describe conditions and justify the exceptions to standard housing (temperature, light cycles, sterile cages, special feed, wire-bottom cages, no enrichment, social isolation, etc.):

3.8. List all laboratories or locations outside the animal facility where animals will be used. Note that animals may not stay in areas outside the animal facilities for more than 12 hours without prior IACUC approval. For field studies, list location of work/study site.

4. Animal Procedures

4.1. Will procedures other than euthanasia and tissue collection be performed? Yes ☐ No ■
If animals will be used exclusively for tissue collection following euthanasia (answer "no" above), then skip to Question 5 (Euthanasia).

4.2. Outline the Experimental Design including all treatment and control groups and the number of animals in each. Tables or flow charts are particularly useful to communicate your design.

Two typical types of experiments are carried out in my laboratory. The first type of experiment is involvement of studies in zebrafish development. Normally, 8 females and 4 males will be placed in 20-gallon tanks. Two layers of marbles are laid at bottom of the tanks the night prior to collection of embryos. We pay special care on minimal disturbance to parent zebrafish in order to obtain health and a large number of embryos. No tissues collection and no scarifying of adult zebrafish will be conducted in this kind of experiment.
The second type of experiments involved collection of tissues from various stages of zebrafish. For single treatment or one time point analyses, tissues from 2-3 fish will be collected following appropriate anesthesia. Additional 4-15 fish will needed in order to repeat the experiment and conduct statistical analyses. For multiple treatments and multiple time points such as monitoring change of receptors and hormones during daily cycle, normally 5-8 fish are needed for each treatment or each time point (in order to be able to conduct statistical analyses). Typically, 6 treatments or 6 time points (24 hours a day, sampling every 4 hours) are required for each experiment. Total 30-48 fish will be needed for one experiment, and the experiment needs to be repeated at least 3 times in order to publish the results. So, approximately 90-144 individual fish are required for one experiment.

In sections 4.3-4.19 below, please respond to all items relating to your proposed animal procedures. If a section does not apply to your experimental plans, please leave it blank.

4.3. <u>Anesthesia/Analgesia/Tranquilization/Pain/Distress Management (for procedures other than surgery)</u>
Adequate records describing anesthetic monitoring and recovery must be maintained for all species.

If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary.

	Agent	Concentration	Dose (mg/kg)	Volume	Route	Frequency	Duration
Pre-emptive analgesic							

Pre-anesthetic							
Anesthetic							
Analgesic Post procedure							
Other							

a. Reason for administering agent(s):

b. For which procedure(s):

c. Method of monitoring anesthetic depth:

d. Methods of physiologic support during anesthesia and recovery:

e. Duration of recovery:

f. Frequency of recovery monitoring:

g. Specifically what will be monitored:

h. When will animals be returned to their home environment:

i. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:

4.4 Use of Paralytics

Will paralyzing drugs be used?

For what purpose:

Please provide scientific justification for paralytic use:

Paralytic drug:

Dose:

Method of ensuring appropriate analgesia during paralysis:

4.5. Blood or Body Fluid Withdrawal/Tissue Collection/Injections/Tail Snip/Gavaging

Please fill out appropriate sections of the chart below:

	Location on animal	Needle/ catheter/ gavage tube size	Route of administration	Biopsy size	Volume collected	Compound and volume administered (include concentration and/or dose)	Frequency of procedure
Body Fluid Withdrawal							
Tissue Collection							
Injection/Infusion							
Tail clip							
Gavaging							
Other							

4.6. Prolonged restraint with mechanical devices

Restraint in this context means beyond routine care and use procedures, and includes rodent and rabbit restrainers, primate chairs, stocks, slings, tethers, metabolic crates, inhalation chambers, and radiation exposure restraint devices).

a. For what procedure(s):

b. Restraint device(s):

c. Duration of restraint:

d. Frequency of observations during restraint/person responsible

e. Frequency and total number of restraints:

f. Conditioning procedures:

g. Steps to assure comfort and well-being:

h. Adverse effects/humane endpoints:

4.7 Tumor and Disease Models/Toxicity Testing

a. Describe methodology:

b. Expected model and/or clinical/pathological manifestations:

c. Signs of pain/discomfort:

d. Frequency of observations:

e. Adverse effects/humane endpoints:

4.8 Treadmills/Swimming/Forced Exercise

a. Describe aversive stimulus (if used):

b. Conditioning:

c. Safeguards to protect animal:

d. Duration:

e. Frequency:

f. Total number of sessions:

g. Adverse signs/humane endpoints:

4.9 Projects Involving Food and Water Deprivation or Dietary Manipulation

(Routine pre-surgical fasting not relevant for this section)

a. Food Restriction

i. Amount restricted and rationale:

ii. Duration (hours for short term/weeks or months for long term):

iii. Frequency of observation/parameters documented (weight, etc):

iv. Adverse effects/humane endpoints:

b. Fluid Restriction

i. Amount restricted and rationale:

ii. Duration (hours for short term/weeks or months for long term):

iii. Frequency of observation/parameters documented:

iv. Adverse effects/humane endpoints:

c. Dietary Manipulations

i. Compound supplemented/deleted and amount:

ii. Duration (hours for short term/weeks or months for long term):

iii. Frequency of observation/parameters documented:

iv. Adverse effects/humane endpoints:

4.10 Endoscopy/Fluroscopy/X-Ray/Ultrasound/MRI/CT/PET/Other Imaging

a. Describe animal methodology:

b. Duration of procedure:

c. Frequency of observations during procedure:

d. Frequency/total number of procedures:

e. Method of transport to/from procedure area:

f. Please provide or attach appropriate permissions/procedures for animal use on human equipment:

4.11 Polyclonal Antibody Production

a. Antigen/adjuvant used:

b. Needle size:

c. Route of injection:

d. Site of injection:

e. Volume of injection:

f. Total number of injection sites:

g. Frequency and total number of boosts:

h. What will be done to minimize pain/distress:

i. Adverse effects/humane endpoints:

4.12 Monoclonal Antibody Production

a. Describe methodology:

b. Is pristane used: [ ] Yes [ ] No

- Volume of pristane:

c. Will ascites be generated: [ ] Yes [ ] No

d. Criteria/signs that will dictate ascites harvest:

e. Size of needle for taps:

f. Total number of taps:

g. How will animals be monitored/cared for following taps:

h. What will be done to minimize pain/distress:

j. Adverse effects/humane endpoints:

4.13 Temperature/Light/Environmental Manipulations

a. Describe manipulation(s):

b. Duration:

c. Intensity:

d. Frequency:

e. Frequency of observations/parameters documented:

f. Adverse signs/humane endpoints:

4.14 Behavioral Studies

a. Describe methodology/test(s) used:

b. If aversive stimulus used, frequency, intensity and duration:

c. Frequency of tests:

d. Length of time in test apparatus/test situation:

e. Frequency of observation/monitoring during test:

g. Adverse effects/endpoints:

4.15 Capture with Mechanical Devices/Traps/Nets

a. Description of capture device/method:

b. Maximum time animal will be in capture device:

c. Frequency of checking capture device:

d. Methods to ensure well-being of animals in capture device:

e. Methods to avoid non-target species capture:

h. Method of transport to laboratory/field station/processing site and duration of transport:

g. Methods to ensure animal well-being during transport:

h. Expected mortality rates:

i. Endpoints for injured/ill animals:

4.16 Field Site/Laboratory Processing

a. Parameters to be measured/collected:

b. Approximate time required for data collection per animal:

c. Method of restraint for data collection:

d. Methods to ensure animal well-being during processing:

e. Disposition of animals post-processing:

f. Endpoints for injured/ill animals:

4.17 Wildlife Telemetry/Other Marking Methods

a. Describe methodology (including description of device):

b. Will telemetry device /tags/etc be removed? If so, describe:

c. Adverse signs/humane endpoints:

4.18 Other Animal Manipulations

a. Describe methodology:

b. Steps to ensure animal comfort and well-being:

c. Adverse effects/humane endpoints for ill/injured animals:

4.19 Surgical Procedures

All survival surgical procedures must be done aseptically, regardless of species or location of surgery. Adequate records describing surgical procedures, anesthetic monitoring and postoperative care must be maintained for all species.

A. Location of Surgery:

B. Type of Surgery:

[ ] Nonsurvival surgery (animals euthanized without regaining consciousness)
[ ] Major survival surgery (major surgery penetrates and exposes a body cavity or produces substantial impairment of physical or physiologic function
[ ] Minor survival surgery

[ ] Multiple survival surgery
If yes, provide scientific justification for multiple survival surgical procedures:

C. Describe the pre-op preparation of the animals:

1a. Food restricted for _____ hours

1b. Food restriction is not recommended for rodents and rabbits and must be justified:

2a. Water restricted for _____ hours

2b. Water restriction is not recommended in any species for routine pre-op prep and must be justified:

D. Minimal sterile techniques will include (check all that apply):

Please refer to DCM Guidelines for Aseptic Surgery for specific information on what is required for each species.

[ ] Sterile instruments

- How will instruments be sterilized:

- If serial surgeries are done, how will instruments be sterilized between surgeries:

[ ] Sterile gloves
[ ] Cap and mask
[ ] Sterile gown
[ ] Sterile operating area
[ ] Clipping or plucking of hair or feathers
[ ] Skin preparation with a sterilant such as betadine
[ ] Practices to maintain sterility of instruments during surgery

E. Describe the following surgical procedures:

1. Skin incision size and site on the animal:

2. Describe surgery in detail (include size of implant if applicable):

3. Method of wound closure:
   a. Number of layers

b. Type of wound closure and suture pattern:

c. Suture type/size / wound clips/tissue glue:

d. Plan for removal of skin sutures/wound clips/etc:

F. Anesthetic Protocol:

	Agent	Concentration	Dose (mg/kg)	Route	Frequency	Duration
Pre-emptive analgesic						
Pre-anesthetic						
Anesthetic						
Analgesic Post Op						
Other						

1. Criteria to monitor anesthetic depth, including paralyzing drugs:

2. Methods of physiologic support during anesthesia and recovery:

3. Duration of recovery from anesthesia:

4. Frequency/parameters monitored during recovery:

5. When will animals be returned to their home environment:

6. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:

G. Recovery from Surgical Manipulations (after animal regains consciousness)

1. Following recovery, what parameters will be monitored:

2. How frequently will animals be monitored:

3. How long post-operatively will animals be monitored:

5. Euthanasia

Please refer to the 2007 AVMA Guidelines on Euthanasia and DCM Guidelines to determine appropriate euthanasia methods.

5.1 Euthanasia Procedure. If a physical method is used, the animal should be first sedated/anesthetized with CO_2 or other anesthetic agent. If prior sedation is not possible, a **scientific justification** must be provided. All investigators, even those doing survival or field studies, must complete this section in case euthanasia is required for humane reasons.

MS-222 (150-200 mg/Liter) will be administrated into buffered water (10% Hank's solution, The Zebrafish Book, Westerfield, 2007) for cardiovascular uptake. The fish will be remained in the solution for approximate 1-2 minutes until stopping of movement and heart beat, which can be easy visualized.

5.2. Method of ensuring death:

Anesthetic overdose (MS-222, see 5.1 for detail) followed by decapitation

5.3. For field studies, describe disposition of carcass following euthanasia:

I acknowledge that humane care and use of animals in research, teaching and testing is of paramount importance, and agree to conduct animal studies with professionalism, using ethical principles of sound animal stewardship. I further acknowledge that I will perform only those procedures that are described in this AUP and that my use of animals must conform to the standards described in the Animal Welfare Act, the Public Health Service Policy, The Guide For the Care and Use of Laboratory Animals, the Association for the Assessment and Accreditation of Laboratory Animal Care, and East Carolina University.

Please submit the completed animal use protocol form via e-mail attachment to iacuc@ecu.edu. You must also carbon copy your Department Chair.

PI Signature: by e-mail Date: 2/4/08

Veterinarian: Karen A. Dypolt Date: 2/6/08

IACUC Chair: Robert B Carroll, PhD jd Date: 2/6/08

APPENDIX 1 - HAZARDOUS AGENTS			
Principal Investigator:	Campus Phone:		Home Phone:
IACUC Protocol Number:	Department:		E-Mail:
Secondary Contact: Department:	Campus Phone:	Home Phone:	E-Mail:
Chemical Agents Used:		Radioisotopes Used:	
Biohazardous Agents Used:		Animal Biosafety Level:	Infectious to humans?
PERSONAL PROTECTIVE EQUIPMENT REQUIRED:			
Route of Excretion:			
Precautions for Handling Live or Dead Animals:			
Animal Disposal:			
Bedding / Waste Disposal:			
Cage Decontamination:			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment:			
Initial Approval Safety/Subject Matter Expert Signature & Date _____			