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Profiling estrogen-regulated gene expression change in neuroendocrine and reproductive systems

by

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ABSTRACT

Neuroendocrine and reproductive systems are strictly regulated by a series of sex hormones, especially 17-beta estradiol (E2). Through specific membrane or nuclear receptors, E2 initiates a series of diverse signaling pathways that regulates the expression of target genes. This transcriptomic output shapes the specific spatial (cell or tissue level) and temporal (seasonal level) E2 actions. In this study, I attempted to define estrogen-related gene expression changes in the neuroendocrine and reproductive systems.

Firstly, I targeted the physiological period of E2 action when gonad size is large just prior to spawning (March and April) in the goldfish *(Carassius auratus)* model. The effect of the aromatase inhibitor fadrozole and the resultant decline in E2 on neuroendocrine gene expression and reproductive development was determined using microarray analysis. Several regulatory themes for physiological E2 action in fish brain have been revealed from these novel E2 regulated genes, including regulation of the calcium signaling pathway and auto-regulation of nuclear estrogen receptor action.

Secondly, I aimed to define the seasonal gene expression characteristics that are associated with hormone profiles, typically E2 blood level change, during a breeding cycle in the goldfish. By using both theoretical and experimental strategies, I have identified a core set of genes in fish neuroendocrine brain that were differentially expressed between physiologically distinct stages including sexually mature prespawning, sexual regression, and early gonadal re-development. Moreover I demonstrated that gene expression changes between stages can be regulated by photoperiod.

Thirdly, to further understand the mechanism underlying fadrozole effects on gonadal development, I used a frog model *(Xenopus tropicalis)* to show that a germline specific piRNA (piwi-interacting RNA) pathway may be involved in the E2/testosterone regulation of gonadal development in the tadpole. Here, I investigated the effect of fadrozole or finasteride (5 alpha-reductase inhibitor), which are known to influence gonadal development, on the gene expression of a piRNA-specific protein Maelstrom (MAEL) and showed both treatments increased MAEL mRNA expression. Moreover, since the specific function of MAEL is unknown, I conducted a bioinformatics analysis to infer its putative function and

evolutionary history. This is one case study for our efforts to annotate some functionally unknown genes which are related to or regulated by E2 actions.

In conclusion, the physiological stage or seasonal specific gene expression information defined in this study provides a series of new functional insights into regulatory mechanisms of E2 and related hormones in the vertebrate neuroendocrine and reproductive systems.

RÉSUMÉ

Les systèmes neuroendocriniens et reproducteurs sont uniquement contrôlés par les hormones sexuelles, en particulier la 17-beta œstradiol (E2). Grâce à des récepteurs membranaires et nucléaires, l'E2 amorce plusieurs cascades de signaux qui contrôleront l'expression de gènes cibles. Le résultat de cette transcription permettra de définir les actions spatiales (dans les cellules ou organes) et temporelles (lors des saisons) de l'E2. Lors de mon doctorat, j'ai tenté d'identifier les changements de l'expression des gènes contrôlés par l'estrogène dans les systèmes neuroendocriniens et reproducteurs.

Premièrement, j'ai étudié l'action de l'E2 chez le poisson rouge (*Carassius auratus)* lors de la période physiologique où les gonades sont de grande taille, juste avant la ponte (mars et avril). L'analyse par biopuces a permis de déterminer les effets d'un inhibiteur de l'aromatase, le fadrozole, ainsi que les effets de la diminution de l'E2 sur l'expression génique neuroendocrinienne et sur le développement du système reproducteur. Plusieurs cascades commandant l'action physiologique de l'E2 ont été identifiées dans le cerveau du poisson incluant la cascade de contrôle du calcium et l'autorégulation du récepteur nucléaire de l'E2.

Deuxièmement, j'ai défini les caractéristiques de l'expression génique saisonnière associée aux profils hormonaux causées par le changement des concentrations de l'E2 sanguines durant le cycle de reproduction du poisson rouge. Des stratégies théoriques et expérimentales ont permis d'identifier un groupe de gènes dans le système endocrinien cérébral du poisson qui démontrait une expression différentielle selon différents stades physiologiques incluant entre autre la période de maturation sexuelle précédent la reproduction, la régression sexuelle et le période du développement des gonades immatures. De plus, j'ai démontré que cette expression génique différentielle pouvait être contrôlée par la photopériode.

Troisièmement, afin de déterminer le mécanisme d'action du fadrozole sur le développement des gonades, j'ai également étudié les effets de cette molécule chez les amphibiens *(Xenopus tropicalis).* Les résultats ont démontré que la cascade de commande des piARN (ARN interagissant avec Piwi) spécifique aux cellules germinales pourrait être impliquée dans le contrôle de l'E2/testostérone lors du développement des gonades chez le

têtard. J'ai examiné les effets du fadrozole et du finastéride (inhibiteur de la 5-alpha-réductase), deux molécules reconnues pour influencer le développement des gonades, sur l'expression du gène de la protéine spécifique piARN Maelstrom (MAEL). Les deux traitements ont causé une augmentation de l'expression de MAEL. Puisque les fonctions de MAEL sont inconnues, j'ai ensuite effectué des analyses en bioinformatique afin de déduire ses fonctions potentielles et son historique évolutif. Ceci constitue seulement un exemple de tous les efforts que j'ai appliqués pour déterminer quelques gènes fonctionnels inconnus sont reliés ou contrôlés par les actions de l'E2.

En conclusion, les profils d'expression génique spécifiques aux stades physiologiques ou aux saisons définis dans cette étude fournissent une panoplie de nouvelles possibilités quant aux cascades de commande de l'E2 et des autres hormones impliquées dans les systèmes neuroendocriniens et reproducteurs des vertébrés.

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ABBREVIATIONS

AC	adenylyl cyclase
AIB1	amplified in breast cancer 1
cAMP	cyclic adenosine monophosphate
CBP	cAMP response element binding protein-binding protein
CCK	cholecystokinin
C/EBPβ	CCAAT enhancer binding protein beta
CREB	cAMP response element binding protein
DHT	dihydrotestosterone, androgen
E2	17beta-estradiol
EDC	endocrine disrupting chemical
EE2	17alpha-ethinylestradiol
EEIG-1	early estrogen-induced gene 1
eNOS	endothelial nitric oxide synthase
ERE	estrogen response element
ERKO	estrogen receptor knockout
EST	expressed sequence tag
FDR	false discovery rate
FSH	follicle-stimulating hormone
GABA	gamma-aminobutyric acid
GH	growth hormone
GIRK	G protein-gated inwardly rectifying K^+ channel
GnRH	gonadotropin-releasing hormone
GO	gene ontology
GPCR	G protein-coupled receptor
GRIP-1	glucocorticoid receptor-interacting protein-1
GH	growth hormone
GSI	gonadotropin-releasing hormone
HCA	hierarchical clustering analysis
HYP	hypothalamus

LH	luteinizing hormone
MAEL	maelstrom
MAPK	mitogen activated protein kinase
mER	membrance estrogen receptor
miRNA	microRNA
nER	nuclear estrogen receptor
NPY	neuropeptide Y
ODC1	ornithine decarboxyase 1
PCA	principal component analysis
PGC-1	peroxisome proliferators-activated receptor gamma coactivator 1 alpha
PI3K	phosphatidylinositol 3-OH kinase
piRNA	PIWI-interacting RNA
PITX1	pituitary homeobox 1
PKA	protein kinase A
RIA	radioimmunoassay
RISC	RNA-induced silencing complex
rasiRNA	repeat-associated small interfering RNA
RTK	receptor tyrosine kinase
SAM	significance analysis of microarray
Sf-1	steroidogenic factor-1
SHC	src-homology and collagen homology
siRNA	short interfering RNA
SOS	son of sevenless
SRC-1	steroid receptor coactivator-1
SRF	serum response factor
T	testosterone
T3	thyroid hormone triiodothyronine
TEL	telencephalon
TF	transcription factor
TGFβ	transforming growth factor beta

TH	thyroid hormone
UBC	ubiquitin-conjugating enzyme

CHAPTER 1: INTRODUCTION

1.1 Objective and outline of the dissertation

The sex hormone estrogen, mainly 17-beta estradiol (E2), has diverse and important functions in both female and male vertebrates (Greco et al., 1993; Jones et al., 2006). While many genes and pathways potentially regulated by E2 have been identified in human cancer cells (Doisneau-Sixou et al., 2003; Basu and Rowan, 2005) or peripheral tissues of rodent models (Greco et al., 1993; Couse and Korach, 1999), much less is known about E2 action in the neuroendocrine brain, and especially in teleost models. I hypothesized that the specific functions of E2 in the neuroendocrine and reproductive systems are achieved by shaping their target gene expression in spatial (cell or tissue level) and temporal (seasonal or stage level) manners. Therefore, the overall objective of my Ph.D. research was to understand E2 action in these processes by identifying and profiling novel E2-regulated genes in fish and frog models. In this dissertation, three main data chapters will be presented as sample cases for four subjects.

Firstly, I used the goldfish model to identify the putative E2-regulated genes. Based on the seasonal profiles of plasma E2 levels, I have utilized two strategies. I used fadrozole, a specific aromatase inhibitor, to decrease E2 levels in the female goldfish during March and April when fish are sexually mature and have highest aromatase activity in brain and E2 level in blood. The other approach was to increase circulating E2 level in the male goldfish during its sexual regressed period (October) via silastic implants containing E2. At that time, goldfish are sexually regressed, have small testes, and have low plasma E2 levels. In both experiments, microarrays were used to profile the differentially expressed genes after treatments. The results from the fadrozole experiments are presented in Chapter 2, while the microarray and realtime PCR results from E2 treatment in male fish have already been published as a collaborative study (Marlatt et al., 2008). Moreover, based on our results, I identified some common genes whose expression was changed in fish after exposure to baclofen, a GABA (gamma-aminobutyric acid)-B receptor agonist and conducted another experiment to show a crosstalk in regulating expression of activin βA by both E2 and GABA. These data will be presented in Appendix A.

Since goldfish reproduction follows a seasonal rhythm in which E2 and other sex hormones exhibit seasonal profiles, I was interested in identifying associated gene expression patterns in the neuroendocrine brain. I have utilized a set of the female goldfish microarray data to extract such seasonal gene expression characteristics. Moreover, independent sampling and real-time PCR were used to verify our results. This is presented in the Chapter 3.

Next, driven by the evidence in Chapter 2 that fadrozole treatment can delay seasonal ovarian development in adult goldfish, I investigated one possible mechanism of E2 regulation of gonadal development in a vertebrate model of sexual differentiation. I hypothesized that E2 can affect this process by regulating a germline-specific piwi-interacting RNA (piRNA) pathway. Maelstrom (MAEL) is functionally involved in the piRNA pathway, and there is only one homolog in most eukaryotic species, indicating that it is a unique representative for the piRNA pathway. However, we could not identify MAEL in fish, so we utilized a frog model, *Xenopus tropicalis,* to further test the E2 regulation on piRNA pathway. The results are presented in the first part of Chapter 4.

My final effort to understand E2 action was to annotate the putative functions for some E2 regulated genes or genes related to the mechanism of estrogen action. Several genes have been retrieved by our studies; however their specific functions are still unknown. These include MAEL, PIH1, and early estrogen-induced gene 1(EEIG-1). I present the result for MAEL as a case study, since its expression has been changed by fadrozole treatments. This is the second part of Chapter 4. In the search for other candidate genes, using protein prediction models, I was able to identify a SANT-associated protein domain I named SANTA, possibly involved in chromatin remodeling. This latter study is published (Zhang et al., 2006) and is not presented in the thesis.

The background for each study is extensively described in each individual chapter. However, in this introduction to the thesis, I review the molecular mechanisms of estrogen regulation of gene expression which covers both nuclear receptors and membrane receptors. The discussed molecular E2 regulatory pathways facilitated my understanding E2 actions and its crosstalk with other hormones, and therefore interpretation of my data that is presented in the thesis. Part of this introduction has been published in a review paper (Zhang and Trudeau, 2006).

1.2 Nuclear receptor-mediated action of E2: the classical mechanism

The main estrogenic hormone, 17β-estradiol (E2), exerts important regulatory roles in a wide variety of biological processes including reproduction, differentiation, cell proliferation, apoptosis, inflammation, metabolism, homeostasis and brain function (Tsai and O'Malley, 1994). The classical mechanism of E2 action is mediated by transcriptional actions of the nuclear estrogen receptors (nERs), ERα and ERβ, which are members of a superfamily of nuclear receptors that function as ligand- or hormone-dependent transcription factors (McKenna and O'Malley, 2002). Two important functional domains in ERα and ERβ, the ligand binding domain (LBD) and the DNA binding domain (DBD), are well conserved both at the amino acid sequence and the structural levels. The two major nER subtypes have distinct expressions patterns in tissues (Couse et al., 1997; Shughrue et al., 1997; Shughrue et al., 1998) and ERα and ERβ-knockout (αERKO, βERKO) mice models exhibit different phenotypes (Couse and Korach, 2001; Hess, 2003; Hewitt et al., 2005), indicating distinct biological functions of the receptors. In contrast to the functional deficiencies in the reproductive system in both sexes of αERKO mice, male βERKO mice are fertile while females are subfertile.

Upon binding with E2, nERs can be released from inactive complexes containing heat-shock proteins and immunophilins (Ylikomi et al., 1998), homodimerize or heterodimerize (Cowley et al., 1997) and bind to a DNA estrogen response element (ERE) which is located in or nearby the promoter regions of target genes. Maximal transcriptional activity of nER requires recruitment of other transcription factors and multiple coactivators including SRC-1 (steroid receptor coactivator-1), GRIP-1 (Glucocorticoid receptor-interacting protein-1), AIB1 (amplified in breast cancer 1), CBP/p300 (cAMP response element binding protein-binding protein), PGC-1 (peroxisome proliferators-activated receptor gamma coactivator 1α) and p68 RNA helicase (McKenna and O'Malley, 2002; Metivier et al., 2003). Coactivator interaction and its availability can greatly influence nER action. In both male and female rodent brain, reduction of SRC-1 and CBP protein with antisense oligonucleotides disrupted nER-mediated induction of the progestin receptor gene (Molenda et al., 2002). In male Japanese quail, inhibition of SRC-1 significantly affected the estrogen-dependent male-typical sexual behavior, and is correlated with reduction in the

volume of the preoptic medial nucleus and the expression of aromatase and vasotocin proteins (Charlier et al., 2005).

Nuclear ER actions can be modulated by numerous cellular signaling pathways. Different kinases, such as PKA (Chen et al., 1999), mitogen activated protein kinase (MAPK) (Tang et al., 2004), and cyclin A-CDK2 (Rogatsky et al., 1999) are induced by extracellular signals (e.g., growth factors) which leads to phosphorylation of several N-terminal serine residues of ERα, for example, serines 104, 106, 118 and 167 (Lannigan, 2003). These phosphorylation modifications modulate receptor functions, including nER downregulation by the ubiquitin-proteasome pathway (Marsaud et al., 2003), nuclear localization of nER (Lee and Bai, 2002), nER dimerization (Chen et al., 1999), and transcriptional activity (Ali et al., 1993; Le Goff et al., 1994). Coregulators of nER are also targeted by these signaling pathways. Mutation of the ERK2-responsive phosphorylation site in GRIP-1 inhibits the ability of this coactivator to enhance ERα transcriptional activity stimulated by E2 in human Hela cells (Lopez et al., 2001) and MCF-7 cells (Frigo et al., 2006). Kinase-mediated signaling pathways most likely serve to induce differential activation and recruitment of nuclear receptors and coregulators, which will modulate gene expression in a cell-specific manner (McKenna and O'Malley, 2002; Smith and O'Malley, 2004; Wu et al., 2005).

Importantly, nER can also regulate gene expression without binding directly to DNA. Many promoters of E2 target genes have no consensus ERE and nER is assumed to act through specific protein-protein interactions with other classes of transcriptional factors to affect gene transcription. Best studied is the ERα-Sp1 interaction which induces expression of several genes, including E2F1 (Wang et al., 1999), LDL-R (Li et al., 2001), c-fos (Duan et al., 1998) and cyclin D1 (Castro-Rivera et al., 2001). Interactions between nER and AP-1 proteins (Fos and Jun) increase transcription of ovalbumin (Gaub et al., 1990), IGF-I (Umayahara et al., 1994), collagenase (Webb et al., 1995) and cyclin D1 (Sabbah et al., 1999; Liu et al., 2002), whereas ER-NFκB-C/EBP interaction decreases the expression of interleukin-6 gene(Stein and Yang, 1995).

1.3 Membrane receptor-mediated actions of E2

Rapid membrane-initiated actions of E2 have been observed since the late 1960s (Lincoln, 1967; Dufy et al., 1976; Pietras and Szego, 1977; Nakhla et al., 1994; Watters et al.,

1997; Kelly and Levin, 2001). E2 exposure can rapidly induce activation of many protein kinases and modulate ion fluxes (i.e. Ca^{2+}, K^{+}) across the plasma membrane. Specific mERs are believed to be involved since such transient events are not prevented by protein synthesis inhibitors. The existence of mERs is also supported by the evidence that treatment with membrane-impermeable estrogen, typically E2 conjugated to bovine serum albumin (E2-BSA) in a short time course (seconds to minutes), can mimic the rapid effects of E2 on signal transduction pathways.

1.3.1 Membrane receptors for E2: classical estrogen receptors or GPCR30?

For a long time, people have tried to verify the nature of the mER, although it still remains controversial. Some experiments favor that the classical nER acting at the level of the plasma membrane may contribute to rapid action of E2. Antibodies directed against different epitopes of nERα have been used to localize it to the region of the plasma membrane (Pappas et al., 1995; Norfleet et al., 1999). Moreover, it has been shown that the translocation of ERα to the plasma membrane is facilitated by two important adaptor proteins, Shc (Src-homology and collagen homology) and IGF-1R (insulin-like growth factor 1 receptor) (Song et al., 2004b; Zhang et al., 2004b). Using a yeast two-hybrid system, Lu et al. (Lu et al., 2004) identified another important protein, Striatin, which acts as a molecular anchor for ERα in the plasma membrane. A critical role of classical ERs in the rapid action of estrogen was also supported by the data from ERα- and ERβ-knockout mice in which the rapid induction of MAPK or PI3K phosphorylations by E2 was lost in either the medial preoptic nucleus (Abraham et al., 2004) or the endothelial cells (Pedram et al., 2002). Moreover, mass spectrometry analysis has verified that ERα is the main protein that locates at plasma membrane and interacts with E2 (Pedram et al., 2002).

Other distinct membrane ER (mER) isoforms which are different from the classical ERα and ERβ are also likely to exist. Some cell types that do not express either ERα or ERβ can still exhibit rapid responses to E2. For example, mouse pancreatic β cells exhibit rapid membrane-mediated non-genomic responses to estrogens and xenoestrogens yet these cells are immunonegative for membrane-localized classical nERs (Nadal et al., 2000). Filardo et al. (2000) for the first time suggested that G-protein coupled receptor (GPCR) 30, an orphan receptor member of GPCR family, may be such a mER. It has been shown that rapid estrogen

activation of Erk1/2 phosphorylation is lost in MDA-MB-231 cells where GPCR30 is not expressed; however such action can be recovered by transfection of GPCR30 (Filardo et al., 2000). Thereafter, Thomas et al (2005) demonstrated that GPCR30 has all the binding and signaling characteristics of a distinct mER in the SKBR3 breast cancer cell line that does not express either ERα or ERβ. This is further supported by several studies in which silencing of GPCR30 with either antisense oligodeoxynucleotide (Maggiolini et al., 2004; Vivacqua et al., 2006; Sirianni et al., 2008), siRNAs (Hsieh et al., 2007), or mouse knockouts (Wang et al., 2008a) suppresses the E2-induced rapid responses. Moreover, a selective GPCR30 agonist, G-1, has been developed that shows similar potential as E2 in inducing signaling pathways including Ca^{2+} and PI3K activation, where it has no binding activity to nER (Bologa et al., 2006). It should be noted that there is a disagreement on the cellular location of GPCR30 in the published literature. In contrast with the plasma membrane location reported by Thomas et al. (2005) and Funakoshi et al. (2006), GPCR30 was subsequently found to localize to endoplasmic reticulum membranes of different types of cells (Revankar et al., 2005; Otto et al., 2009). There is also some disagreement regarding the nature of mER. Although a role of GPCR30 in E2 rapid actions has been confirmed, some groups failed to verify it and instead, showed that ERα acts as mER (Pedram et al., 2002; Otto et al., 2009). It seems that both classical estrogen receptor and GPCR30 may have a role in membrane-initiated actions of E2 and there may also exist interactions between them in certain cell types, which increases the complexity of estrogen signaling. Importantly, this presents a major challenge in experimental design when determining the contributions of the various ERs to the control of cellular function.

1.3.2 Signaling pathways initiated by mER

Several signaling pathways are activated by binding of E2 to membrane ERs. These include Ca^{2+} flux (Improta-Brears et al., 1999), cAMP (Nakhla et al., 1994; Watters and Dorsa, 1998), MAPK (Watters et al., 1997; Wade and Dorsa, 2003), PKA (Lagrange et al., 1997), PKC (Qiu et al., 2003), PI3K (Simoncini et al., 2000; Alexaki et al., 2004), and Src kinase (Kennedy et al., 2005). Though there are many potential interactions among these pathways, the activation events can be classified into three main signaling pathways: the Ras-Raf-MEK-MAPK, Src-PI3K-Akt-eNOS, and PLC-PKC-cAMP-PKA modules (Figure 1).

MAPK activation upon E2 or E2-BSA exposure has been described in breast cancer cells (Migliaccio et al., 1996), endothelial cells (Russell et al., 2000; Klinge et al., 2005) and adipocytes (Dos Santos et al., 2002). MAPK is activated by phosphorylation on specific threonine and tyrosine residues by MAPK kinases (MEKs). MEKs are themselves activated by several kinases such as the Raf proteins, which in turn are regulated by Ras family members. The binding of mER with E2 can induce the activation of receptor tyrosine kinases (RTK) such as EGF and IGF-1 receptors, which transmit signals through protein complexes including adaptor protein Shc, Grb2, and the guanine nucleotide exchange protein Sos, leading to Ras activation (Driggers and Segars, 2002; Zhang et al., 2004b). MAPK activation can also be modulated by either G protein through a signaling pathway involving non-receptor tyrosine kinase Src and PI3K (Dos Santos et al., 2002), or intracellular calcium which can modulate Ras activation (Improta-Brears et al., 1999; Kupzig et al., 2005).

The Src-PI3K-Akt-eNOS module is a pathway in endothelial cells mediating rapid E2-dependent release of nitric oxide (NO). In this process, the signal from mER is transmitted to PI3K through a G-protein and Src complex. In turn, PI3K activates Akt, which stimulates eNOS activation (Segars and Driggers, 2002; Haynes et al., 2003). A PLC-PKC-AC-cAMP-PKA module mediates the effect of E2 on the change of K^+ fluxes in neurons (Gu and Moss, 1996; Malyala et al., 2005). Early evidence showed that E2 can attenuate the ability of μ-opioid and GABA receptors mediated activation of G protein-gated inwardly rectifying K^+ channels (GIRKs) (Kelly et al., 1992). PKA is also involved because E2 action can be mimicked by the PKA activators forskolin and cAMP, whereas PKA antagonist can block this effect (Lagrange et al., 1997). Using a peptide inhibitor that interferes with G-protein receptor signaling and several PKC inhibitors, Qiu et al. (Qiu et al., 2003) illustrated that the E2 effects are mediated by G-protein and PKC. Activation of PKC is likely to be upstream of PKA. Selective PKC inhibition using bisindolymaleimide blocked the E2 effects, however forskolin can still mimic the effect of E2 even in the presence of bisindolymaleimide (Qiu et al., 2003). The PLC-PKC-AC-cAMP-PKA pathway is also activated by E2 in intestinal cells and this leads to changes in intracellular Ca^{2+} (Picotto et al., 1996; Picotto et al., 1999). Note that a change of Ca^{2+} fluxes can also be induced by PLC through IP_3 in the hypothalamus (Malyala et al., 2005).

The activation of signaling pathways by E2 is cell type-specific. For example, the effect of E2 on PKC catalytic activity has been observed in the preoptic area (POA) of female rat brain slices, but not in the remaining portion of the hypothalamus posterior to the POA (Ansonoff and Etgen, 1998). The activation of the G-protein/Src/PI3K/MAPK pathway by E2 was evident in late, but not early, differentiated rat preadipocytes (Dos Santos et al., 2002). The differential requirement of Src/PI3K or intracellular calcium for MAPK activation is also observed in diverse cell types (Improta-Brears et al., 1999; Dos Santos et al., 2002; Kupzig et al., 2005). It appears that differential expression patterns of some proteins, which are involved in signal transduction or the membrane structural organization, account for this diversity (Dos Santos et al., 2002). The differential utilization of signal pathways in different cells may contribute to the cell type-specific E2 action.

1.4 mER signaling to genomic transcriptional regulation

It is well known that activated signaling pathways involving MAPK and PKA can influence the activities of downstream proteins including chromatin remodeling factors, transcription factors (TFs) and also nER and its coactivitors through diverse posttranslational modifications (e.g. phosphorylation, methylation, or acylation). The change of states in phosphorylation and other modifications is necessary for the transcriptional activities of these transcription factors, which ultimately leads to changes in transcription of their target genes. Therefore, activation of kinase cascades via the mER is an alternative mechanism for E2 to regulate gene expression.

1.4.1 mER action can modulate gene expression through non-ER transcription factors

Many transcription factors have been shown to be regulated by mER-dependent signaling pathways. CREB (cAMP-response element-binding protein) is the most studied target. In a hippocampal cell line (Wade and Dorsa, 2003), adipocyte cells (Dos Santos et al., 2002) and colonic carcinoma cells (Hennessy et al., 2005), CREB transcriptional activity can be induced by E2 or E2-BSA through MAPK pathway, independently of PKA pathway. Such activation of CREB induces expression of several genes including c-fos and UCP-2. In contrast, in neuroblastoma cells activation of CREB by mER is mediated through a cAMP-

PKA pathway, leading to neurotensin gene expression (Watters and Dorsa, 1998). This may reflect cell-specific activation of the various signal pathways by mER.

Serum response factor (SRF) and ELK1 are also mER-responsive transcription factors, which are involved in regulating c-fos expression (Duan et al., 2001; Hennessy et al., 2005). It should be noted that in human MCF-7 breast cancer cells, SRF can be regulated by either MAPK pathway (Duan et al., 2001) or PI3K pathway (Duan et al., 2002), implying that the different pathways can cooperate to exert actions.

The AP-1 protein is another effector of mER action. Both DNA binding activity and transcriptional activity of AP-1 can be increased by E2 through the MAPK pathway (Dos Santos et al., 2002; Bjornstrom and Sjoberg, 2004), which further contributes to regulating expression of the cyclin D1 and collagenase genes (Marino et al., 2002; Bjornstrom and Sjoberg, 2004). Other targets include several members of the Stat family such as Stat 1, Stat3 and Stat5 (Bjornstrom and Sjoberg, 2002; Kennedy et al., 2005). In endothelial cells, activation of both Stat3 and Stat5 by E2 was mediated through signaling pathways involving MAPK, PI3K and Src, and function to regulate β-Casein expression (Bjornstrom and Sjoberg, 2002).

Furthermore, a global gene expression group was identified in human vascular endothelial cells responding to the mER-initiated PI3K signaling pathway (Pedram et al., 2002). E2 exposure for 40 minutes rapidly up-regulated approximately 250 genes, which was prevented by inhibiting the PI3K pathway with LY294002. With the development of new selective mER agonists, the mER-specific regulated genes have been profiled recently. For example, in MCF-7 breast cancer cells, over 300 genes are differentially expressed upon the treatment with estrogen-dendrimer conjugates, which only have mER action because of their charge and size (Madak-Erdogan et al., 2008). Additionally, a group of 241 genes in pig arcuate nucleus was identified after treatment with STX, which is a selective mER agonist (Roepke et al., 2008).

It should be noted that several typical genes responsible for mER actions, such as c-fos and cyclin D1, were also shown to be regulated by direct interactions between nER and other transcription factors, including Sp1 and AP-1 (Duan et al., 1998; Sabbah et al., 1999; Castro-Rivera et al., 2001; Liu et al., 2002). In future research it will be necessary to consider

both contributions of direct interactions of nERs with other transcription factors, as well as mER signaling, in the regulation of gene expression.

1.4.2 Membrane actions of E2 signaling to nER

mER–mediated signaling events may also have important modulatory effects on classical nERs and their coactivators (Vasudevan and Pfaff, 2008). It has been long known that E2 treatment can increase the phosphorylation state of nERs, and mutation of important phosphorylation sites reduces the transcription activity of nERs (Ali et al., 1993; Le Goff et al., 1994). Also AIB1, one nER coactivator, has been reported to be phosphorylated following E2 exposure in human HEK293 cells and MCF-7 cells (Wu et al., 2004). Multiple cellular signaling pathways, including p38, JNK, and ERK1/2, are involved. RNAi-mediated knockdown of these kinases diminishes the nER-induced expression of endogenous pS2, which implies a critical contribution of mER signaling in nER actions through AIB1. Furthermore, induction of MAPK and PI3K pathways by mER are both necessary and sufficient for the transcription of non-nER regulated genes, but also for the transcription of nER-regulated genes (Acconcia and Marino, 2003). Using a two-pulse paradigm of hormone administration, Vasudevan et al. (2001) demonstrated that rapid E2 action can potentiate slower nER-mediated transcriptional processes in neuroblastoma cells. Intracellular signaling cascades were shown to be important in such potentiation; inhibition of PKA, PKC, MAPK or PI3K in the first pulse decreased this effect (Kow and Pfaff, 2004). Besides modifying phosphorylation state, mER-initiated signaling may also regulate nER actions through other types of modifications. For example, nER binding to ERE is inhibited by an S-nitrosylation modification induced by a NO (Garban et al., 2005), which is a major product in the mER-initiated signaling pathway of the Src-PI3K-Akt-eNOS module. Moreover, the effect of mER action on nER action has been further verified using global gene expression profiling (Madak-Erdogan et al., 2008). There are about 243 genes in MCF-7 breast cancer cells whose expression is commonly induced by both E2 and endocrine disrupting chemicals (EDCs). The inductive effect of both E2 and EDCs on target genes was prevented by a treatment with inhibitors of MAPK kinase and c-Src.

1.5 E2-induced transcriptional regulatory cascades via third messengers

Besides mER-initiated signaling to nER, recent studies have illustrated another regulatory cascade; that is, early induction of some TFs via mER or nER can modulate subsequent nER actions. Global gene expression analysis by microarray or CHIP-chip combined with functional analysis and genome transcriptional factor binding site analysis has revealed many so-called third messengers (Kovacs, 1998) such as cyclin D1 (Yang et al., 2006), E2F members (Bourdeau et al., 2008), FoxA1 (Carroll et al., 2005; Laganiere et al., 2005; Eeckhoute et al., 2006), ANCCA (Zou et al., 2007), c-MYC (Cheng et al., 2006; Musgrove et al., 2008) and NF1C (Eeckhoute et al., 2006) (Figure 2).

In this context, the best studied transcription factor is FoxA1, whose expression is induced by nER action. Interestingly, this induction of FoxA1 was found to be necessary for subsequent nER action in human MCF-7 cancer cells (Carroll et al., 2005; Laganiere et al., 2005). The binding sites for FoxA1 are enriched in the nER binding regions of promoters of many genes. Small interfering RNA-mediated knockdown of FoxA1 blocks the transcription of other estrogen-regulated genes, namely TFF-1 and vitellogenin-B1. Therefore, early induction of FoxA1 by E2 action defines a specific gene repertoire for later nER action (Carroll et al., 2005; Laganiere et al., 2005). In addition to these third messengers induced by early nER action, those TFs that can be rapidly induced by mER signaling can also be third messengers. For example, the above-mentioned c-fos and cyclin D1 are known coregulators for nER action (Kovacs, 1998; Fu et al., 2004; Burd et al., 2005). Therefore, induction of these coregulators by mER action might be another generalized mechanism for early-to-late regulation of E2 action.

Utilization of third messengers by early E2 action may also be cell type-specific. Differential sets of third messengers may determine specific regulated gene repertoire of E2 action. Moreover, there may exist more complex transcriptional regulatory networks in which diverse TFs can coordinate or interfere in a series of cascades induced by E2 action. For example, the fine-tuning of cyclin D1 by E2 action is achieved by induction of FoxA1 to increase expression or induction of NF1C to inhibit expression of cyclin D1 (Eeckhoute et al., 2006).

1.6 An integrative model for mER and nER signaling

Based on the evidence described above, we propose a regulatory model for E2 action in which both membrane-to-nuclear signaling and early induction of third messanger-to-late modulation on nER action are integrated (Figure 1 and 2). Considering the controversy on the nature of mER, we used the generic "mER" to represent either GPCR30 or classical ER at the membrane in Figure 1. For cellular location, only one type of membrane is shown to represent either plasma membrane or endoplasmic reticulum membranes. Specifically, E2 initiates membrane actions and nuclear actions through its membrane receptors and nuclear receptors, respectively. In nuclear action, estrogen receptors binding E2 are activated to bind to EREs in promoter sites, thereby regulating expression of target genes. In contrast, E2 binding to mER rapidly induce the activation of the signaling pathways including the Ras-Raf-MEK-MAPK (Driggers and Segars, 2002), Src-PI3K-Akt-eNOS (Segars and Driggers, 2002) and PLC-PKC-cAMP-PKA modules (Malyala et al., 2005). These signaling pathways can influence the post-translational modifications of a series of transcription factors (e.g. CREB and AP1), which then modulate expression of some genes that do not contain the consensus ERE in the promoter region. At the same time, mER-initiated signaling has modulatory effect on nER action through targeting nER and its coactivators. Moreover, induced expression of third messengers (e.g. c-fos, cyclin D1, and FoxA1) is another important mechanism linking the earlier and later estrogen actions. Thus, all these regulatory cascades including membrane-to-nuclear signaling and early induction of third messanger-to-late modulation on nER action are combined to specify estrogen action in a cell-dependent manner.

1.7 Membrane receptor-mediated actions of other hormones

In addition to established nuclear receptor mechanisms, there is also evidence for membrane receptor-mediated processes for thyroid hormone (TH), progesterone (P4), androgens, glucocorticoids, vitamin D3 and aldosterone (Falkenstein et al., 2000). Common rapid effects include intracellular ion flux and activation of a series of protein kinases such as MAPK, PI3K, PKA. Membrane receptors for some of these hormones have been identified. For example, a classical nuclear receptor mediates the membrane actions of aldosterone in rat

mesenteric resistance vessels (Michea et al., 2005), while integrin αvβ3 may mediate the membrane actions of TH in an African green monkey CV-1fibroblast cell line (Bergh et al., 2005). The membrane receptor for progesterone in spotted sea trout ovaries was the first unequivocal steroid G-protein coupled receptor identified (Zhu et al., 2003). However, the classical nuclear progesterone receptor was also localized at the plasma membrane and contributes to rapid MAPK activation by progesterone in Xenopus laevis oocytes (Bayaa et al., 2000). Thus, both classical nuclear receptors and novel membrane-specific receptors mediate the membrane actions of both E2 and P4. It is reasonable to speculate that this is also true for the other hormones with identified "classical" receptors in the nuclear receptor superfamily.

The modulatory effects of membrane receptor activation on nuclear receptor actions observed for E2 likely exists for other hormones. Faivre et al. (2005) showed that membrane progesterone receptor-mediated rapid signaling can regulate the expression of genes important for breast cancer cell growth independently of its nuclear receptors, and can also enhance nuclear progesterone actions through phosphorylation events. In the case of aldosterone action, the nuclear mineralocorticoid receptor can be rapidly phosphorylated on serine and threonine residues following treatments with aldosterone or aldosterone-BSA, while its nuclear transcriptional activity can be inhibited through blocking the PKC pathway as demonstrated by suppression of aldosterone-induced Na+/K+/ATPase mRNA in the RCCD(2) rat cortical collecting duct cell line (Le Moellic et al., 2004). Thyroid hormone exposure of 293T cells leads to nuclear TR acetylation via a membrane receptor initiated MAPK pathway (Lin et al., 2005a). Thus, the modulatory effects of rapid membrane actions on slower nuclear actions of hormones via signaling pathways are common to several hormones.

Induction of cyclin D1 and c-fos may also be common to membrane steroid receptor actions. Cyclin D1 is increased by progesterone exposure via the MAPK pathway in several human breast cancer cell lines (Faivre et al., 2005), and also following thyroid hormone triiodothyronine (T3) exposure in mouse hepatocytes (Pibiri et al., 2001). Because of their distinctive function in regulating nuclear receptor action of hormones and their inducible expression in a non-hormone-specific manner, these immediate genes are expected to be third messengers to create extended circuitries of hormone action. Collectively, these data

suggest that like E2 (Figure 1), other hormone may have a similar mode of action involving an integration of rapid membrane and slower nuclear hormone receptor actions through kinases and induced third messengers (i.e. cyclin D1).

1.8 Crosstalk between various hormones through induced signal pathways

Given that signal transduction pathways such as MAPK, PKA are commonly utilized by different steroid hormones to link membrane actions and downstream nuclear actions, it is possible that one hormone-responsive transcriptional complex could be modulated by signaling pathways initiated from membrane receptors for a different hormone.

One study has shown that the Src/Shc/ERK signal pathway can be activated by either estrogen or androgen (DHT, 5α-dihydrotestosterone) in mammal osteoblasts, osteocytes, embryonic fibroblasts and Hela cells (Kousteni et al., 2001). Furthermore, several transcription factors, including Elk-1, CCAAT enhancer binding protein-beta (C/EBPβ), and CREB, or c-Jun/c-Fos can be activated by the Src/Shc/ERK pathway which is initiated from extranuclear actions of the ER or AR (Kousteni et al., 2003). This is one possible crosstalk model that illustrates how different hormones can regulate the same downstream TFs via the same signal pathway (Figure 3a).

Interaction between TH and E2 signaling represents another possible crosstalk model (Figure 3b). Thyroid hormone (T3 and T4) has an estrogen-like effect in breast cancer cells, inducing cell proliferation through p53 and hyperphosphorylation of the TF pRb (Dinda et al., 2002). Co-treatment with an ER antagonist prevented this effect of TH, suggesting ER involvement. Application of either TH or membrane impermeable agarose-TH leads to phosphorylation of serines 167 and 118 of ERα, implicating the involvement of a putative membrane TH receptor (mTR) (Dinda et al., 2002); the MAPK pathway may mediate this crosstalk event since an inhibitor of ERK1/2 phosphorylation restrained this process (Tang et al., 2004; Zhao et al., 2005). Thus, TH can affect nER action via a mTR-mediated pathway. This model is similar to the crosstalk between epidermal growth factor (EGF) and E2 in breast cancer cells. In addition to targeting nER, signaling from the EGF receptor can also modulate nER action by activating the ER coactivator protein AIB1 (Osborne and Schiff, 2003) (Figure 3c).

The regulatory effect of gonadotropin-releasing hormone (GnRH) on nER action in the mouse LbT2 gonadotrope cells is another example of crosstalk (Luo et al., 2005) (Figure 3d). In this process, the GnRH signaling pathway does not regulate nER directly. Rather, GnRH induces the expression of ubiquitin-conjugating enzyme 4 (ubc4), which subsequently promotes nER ubiquitylation. These examples all demonstrate that nER actions can be modulated by signaling pathways originating from both steroid and peptide hormone-membrane receptor binding.

1.9 Perspectives

The discovery of mER-mediated actions greatly expands our understanding of E2 action. The classical mechanism in which E2 regulates gene expression is through interactions with co-activators/co-repressors, dimerization of nERs and subsequent binding to specific EREs in target genes (Gruber et al., 2004). In contrast to this mechanism, membrane receptor binding of E2 initiates various signaling pathways and activates other transcription factors that can lead to transcription of some genes without EREs. Also membrane ER-mediated actions of E2 exert a modulatory effect on nER actions through signaling pathways targeting nER and coactivators, and increasing expression of immediately inducible genes (i.e, cyclin D1 or c-fos). This pattern of membrane receptor modulation of nuclear receptor action is likely to exist for other hormones. Furthermore, steroid hormones and other hormones acting through other members of the nuclear steroid hormone receptor superfamily (i.e. TH) also activate many of the same intracellular signaling cascades which provide an extensive crosstalk network between diverse hormones. This model integrating membrane receptor and nuclear receptor pathways (Figure 1) provides a framework to investigate the diverse actions of E2 and EDCs. For example, xenoestrogens, the structurally diverse group of compounds which can mimic or antagonize the physiological effects of E2 through the nER (Petit et al., 1997), may also activate signaling pathways initiated at the plasma membrane (Bulayeva and Watson, 2004; Canesi et al., 2004). Moreover, as I will discuss in the main content, numberous genes appears to be regulated by E2-mER pathway (Chapter 5). There may also exist a signaling crosstalk in regulating gene expression between E2 and other neurotransmitter such as gamma-aminobutyric acid (GABA) (Chapter 5).

Figure 1. An integrative model for E2 action involving both membrane and nuclear estrogen receptors

Abbreviations: AC, adenylyl cyclase; Akt, protein kinase Akt; Ca^{2+}, calcium; eNOS, endothelial nitric oxide (NO) synthase; CREB, cAMP-response element-binding protein; E, estrogen; G, G protein; GABAR, GABA receptor; GIRK, G protein-gated inwardly rectifying K^+ channels; GRB2, growth factor receptor-bound protein 2; MAPK, mitogen activated protein kinase; MEK, mitogen/extracellular signal protein kinase; mER, membrane estrogen receptor (ER or GPCR30); nER, nuclear estrogen receptor; NO, nitric oxide; P, phosphorylation modification; PI3K, phosphatidylinositol 3-OH kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; Src, protein kinase Src; Raf, v-raf-1 murine leukemia viral oncogene; Ras, related RAS viral (r-ras) oncogene homolog; RTK, receptor tyrosine kinase; Shc, Src-homology and collagen homology; SOS, son of sevenless; μ, μ-Opioid receptor.

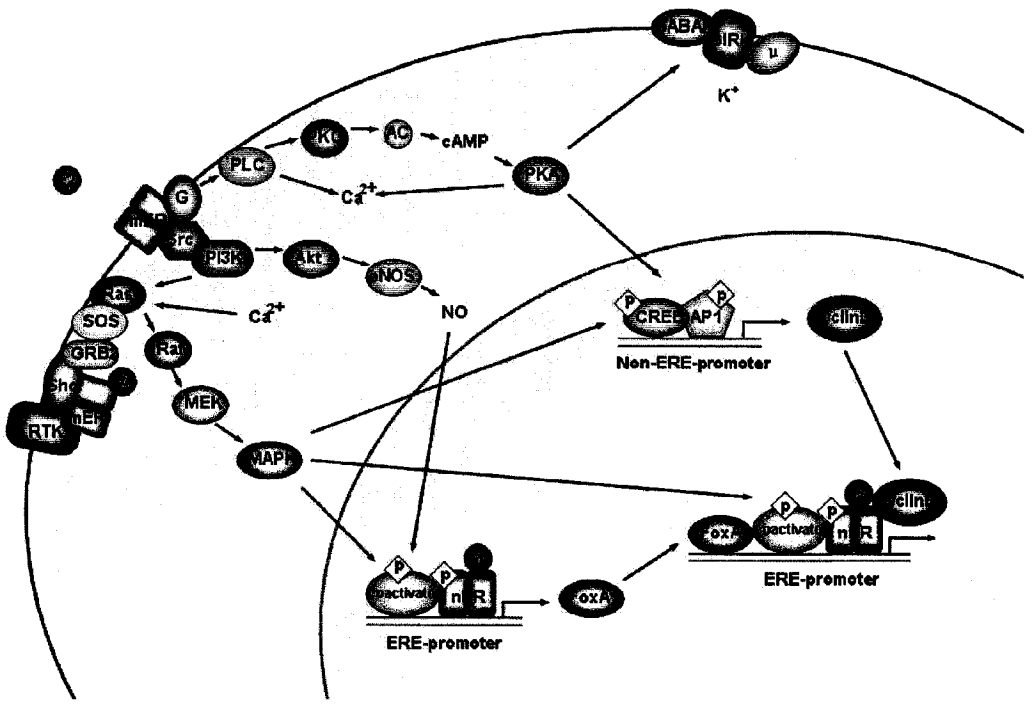


Figure 2. E2-induced transcriptional regulatory cascades via third messengers

A series of transcription factors can be induced by early E2 (mER or nER) actions and then further modulate late nER actions in regulating target gene expression.

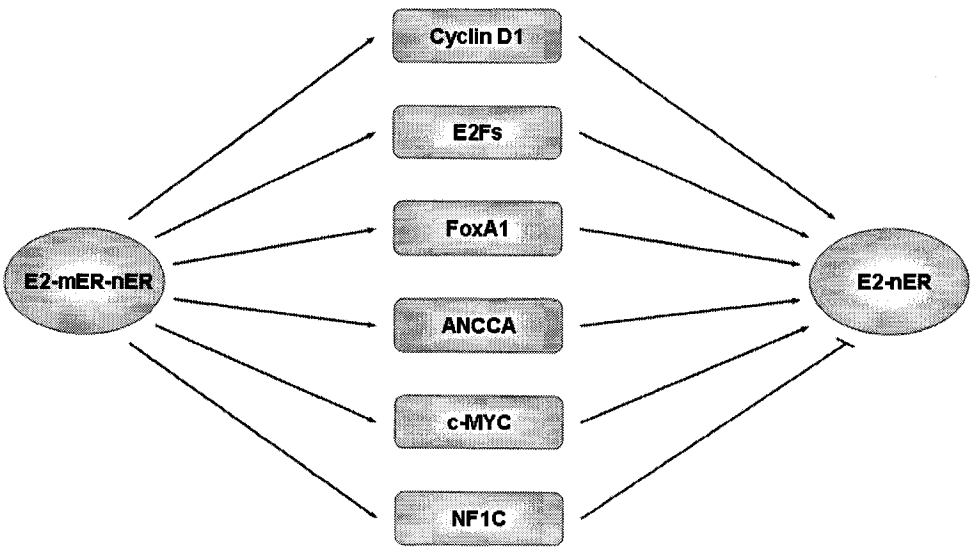
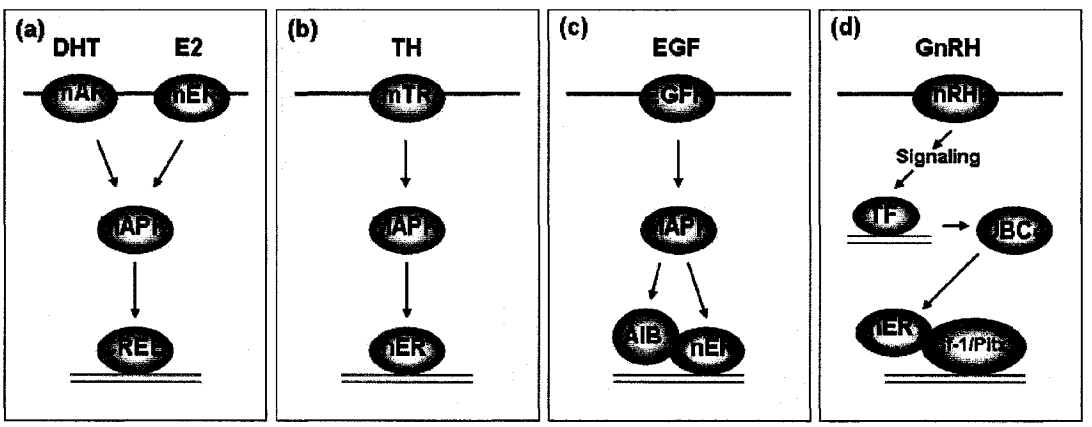


Figure 3. Crosstalk models between E2 and other hormones

(a) The same signaling pathway (MAPK) is utilized by E2 or androgen to activate CREB protein in mammal osteoblasts, osteocytes, embryonic fibroblasts and Hela cells (Kousteni *et al.*, 2001; Kousteni *et al.*, 2003). (b) Similar to E2, thyroid hormone regulates nER action through MAPK pathway in breast cancer cells (Dinda *et al.*, 2002; Tang *et al.*, 2004; Zhao *et al.*, 2005). (c) Membrane EGF receptor can regulate nER action through targeting both nER and its coactivator, AIB1 in vreast cancer cells (Osborne and Schiff, 2003). (d) GnRH regulate nER action indirectly through inducing UBC4 expression in the mouse LbT2 gonadotrope cells (Luo *et al.*, 2005). In the figure the upper line represents membrane structure, while the lower double line represents a gene promoter target for binding of the transcriptional complex. For details refer to main text and references. Abbreviations: mAR, membrane receptor for DHT; GnRHR, membrane receptor GnRH; Sf-1, steroidogenic factor 1; Pitx1, Pituitary homeobox 1; For other abbreviations not defined here refer to main text.



CHAPTER 2: PROFILING NEUROENDOCRINE GENE EXPRESSION CHANGES FOLLOWING FADROZOLE-INDUCED ESTROGEN WITHDRAWAL IN THE FEMALE GOLDFISH

Abstract

Fish represent unique models to study the role of neuroestrogen because of the extremely high aromatase activity in neuroendocrine regions. The B-subtype of aromatase (AroB; with active estrogen-response elements in the promoter) is localized exclusively in radial glial cells and locally converts testosterone (T) to 17β-estradiol (E2). Inhibition of aromatase activity by the specific inhibitor fadrozole has been shown to impair E2 production and influence neuroendocrine and reproductive functions in fish, frogs, and laboratory rodents. However, very few studies have identified the global transcriptomic effect of decreased E2 level as a result of fadrozole treatment in a physiological context. In our study, sexually mature female goldfish were exposed to fadrozole (50ug/L) in March and April when fish have the highest AroB activity and maximal gonadal size. This treatment caused significantly decreased serum E2 level (4.7 times lower, $p=0.027$) and depressed AroB expression (three times lower, p-value=0.006) in both the hypothalamus (Hyp) and telencephalon (Tel). Histological examination showed that in contrast to control fish, no mature oocytes and no visible vesicles (cortical alveolus) were observed in treated fish, suggesting that the development of the female ovary was delayed by fadrozole. Microarray expression profiling of the Tel further identified 98 genes and 85 unknown ESTs which are differentially expressed after fadrozole treatments ($q<0.05$). Some of these genes have shown previously to be estrogen-responsive in either fish or other species, including rat, mouse and human. GO analysis together with functional annotations revealed several regulatory themes for physiological E2 action in fish brain which include the regulation of calcium signaling pathway and auto-regulation of nER action. The expression change of three genes, activin βA, calmodulin, and ornithine decarboxylase 1, was verified using real-time PCR. Thus I assessed the effect of fadrazole-induced E2 reduction on goldfish neuroendocrine brain and

reproductive development based on physiological data and genomic transcriptional information.

2.1 Introduction

Estradiol-17β (E2), the main estrogenic hormone, plays fundamental regulatory roles in neuroendocrine and reproductive systems. Through binding to its nuclear estrogen receptors ERα and ER β, E2 regulates transcriptional process of target genes whose promoters contain estrogen-responsive elements (ERE) (Tsai and O'Malley, 1994). In addition to this classical nuclear action, E2 also has membrane actions in which a unique membrane receptor is utilized to rapidly activate a series of signaling pathways (Thomas et al., 2005; Zhang and Trudeau, 2006). Since the signaling pathway activation will ultimately influence the transcriptional activities of downstream transcription factors including nERs, membrane actions of E2 provide another mode to regulate genomic gene expression (Zhang and Trudeau, 2006). Fish are unique models to study E2 action because there is an extremely high activity of aromatase, the enzyme responsible for synthesizing E2 from testosterone (T), in the neuroendocrine regions (Pasmanik and Callard, 1988). Moreover, in contrast to mammals where aromatase and nER are commonly expressed in neuron cells, in fish AroB is only expressed in radial glial cells (Forlano et al., 2001) whereas nER is mostly expressed in neuron cells (Linard et al., 1996; Menuet et al., 2003). Thus, the local production of glial E2 and signaling to neurons is an important interaction between these cell types which has implications for neurogenesis, neuronal regeneration, neuroprotection, and neuroendocrine function (Garcia-Ovejero et al., 2005; Forlano et al., 2006; Mouriec et al., 2008).

Profiling the physiological effects of fadrozole, a reversible competitive inhibitor of aromatase, has been shown to be a good strategy to study E2 action in neuroendocrine and reproductive systems, as illustrated in different fish (Kwon et al., 2000; Ankley et al., 2002; Zerulla et al., 2002; McAllister and Kime, 2003; Bhandari et al., 2004a; Fenske and Segner, 2004; Navarro-Martin et al., 2009), frog (Olmstead et al., 2008) and rodent (Huddleston et al., 2006) models. By inhibiting activities of two aromatase subtypes (aromatase A in gonad and AroB in brain), fadrozole causes a decreased E2 serum level (Ankley et al., 2002; Bhandari et al., 2004a), impairs gonadal development (Ankley et al., 2002) and can even

induce sex reversal in fish (Kwon et al., 2000; Zerulla et al., 2002; Bhandari et al., 2004a; Fenske and Segner, 2004). However, the associated gene expression changes caused by fadrozole-induced E2 withdrawal in neuoendocrine systems are still largely unknown. Several studies have utilized real-time PCR of a few genes (Villeneuve et al., 2007; Zhang et al., 2008) and low-density microarrays (Villeneuve et al., 2008) to profile the fadrozole-induced gene expression change on a small scale. Recently, the zebrafish (*Danio rerio*) model was used to identify the relationship of the global transcriptomic changes between brain, testis and ovary after fadrozole treatments (Wang et al., 2008b). However, in their studies there were no attempts to link the gene expression changes with physiological outcome.

In the present study, we assessed the effects of fadrozole on neuroendocrine and reproductive systems in the goldfish model (Popesku et al., 2008) using both physiological data and transcriptomic information. In goldfish, E2 serum level exhibits a seasonal profile with an increase around April and May, corresponding to the prespawning period (Trudeau et al., 1991). Accordingly, a similar profile for aromatase activity (Pasmanik and Callard, 1988) and AroB expression (see Chapter 3) has been observed in goldfish brain. All these data indicate that the prespawning period from April to May is a critical period for physiological E2 action in neuroendocrine systems. This allows us to target this particular period and reverse E2 action by treating goldfish with fadrozole between March and April. We then evaluated the effect of fadrozole-induced E2 withdrawal by gonadal histology, serum E2 level, global gene expression profiling and gene ontology analysis.

2.2 Materials and methods

2.2.1 Experimental animals and design

Common goldfish *(C. auratus)* were purchased in January 2005 from Aleong's International Inc. (Mississauga, ON, Canada), and were maintained at 18 °C under a natural simulated photoperiod typical of Ottawa. Fish were fed with the standard goldfish food. The experiment was conducted from March to April. Female goldfish (n=64) were separated from males based on the absence of male-specific breeding tubercles on the operculum and pectoral fins and housed in 70L round tanks (n=16 per tank) supplied with constant aeration. Fadrozole was provided by Novartis Pharma AG (Switzerland) and diluted in water at a final

concentration of 50ug/L. The water was changed every three days. After the exposure period (40 days), goldfish were anesthetized with 3-aminobenzoic acid ethylester for all handling and dissection procedures. Care was taken to standardize all handling and sample protocols. Samples were rapidly dissected and immediately frozen on dry ice. Two or three hypothalami and telencephali were pooled for a total number of 12 samples in each group. At the time of sampling, body weights and gonadal weights were recorded and gonadosomatic index (GSI; body weight/gonad weight x 100) was calculated. Another independent treatment was conducted in 2006 as well.

2.2.2 E2 Radioimmunoassay (RIA)

E2 was measured by RIA according to an established protocol (McMaster et al., 1992). Sex steroids were extracted from 100 µl of serum from female goldfish. Since data were not normally distributed, a non-parametric test (Mann-Whitney U) was used to test for significant differences ($p<0.05$).

2.2.3 Gonadal histology

Ovaries (three females) were fixed and decalcified in Cal-Ex II (Fisher Scientific) for three days. They were stored in 70% ethanol until routine paraffin embedding. Embedded samples were sectioned at 5µm and transverse sections were stained by hematoxylin and eosin. Images of the sections were captured using a Micro Publisher 3.3 Digital microscope camera (Qimaging Corp., Surrey, BC, Canada).

2.2.4 RNA isolation and cDNA snythesis

Homogenization and disruption of the samples were done using an MM301 Mixer Mill (Retsch, Newton, PA, USA) at 20 Hz for 2 min. The RNeasy Plus Mini kit (Qiagen) was then used for RNA isolation as described in the manufacturer's protocol. Concentrations of RNA were determined using GeneQuant spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Total cDNA was prepared from 2µg total RNA and 200 ng random hexamer primers (Invitrogen) using Superscript II RNase H- reverse transcriptase (SSII) as described by the manufacturer (Invitrogen).

2.2.5 Microarray hybridization and data analysis

The description, validation and use of our goldfish-carp microarrays has been published previously (Martyniuk et al., 2006; Marlatt et al., 2008; Mennigen et al., 2008; Popesku et al., 2008; Williams et al., 2008). Version 1.1 of the cDNA array was used here and contains 8664 clones printed in duplicate (GEO Platform: GPL7056). For the microarray hybridizations, a common reference pool was made from all control Tel RNA samples. The reference pool and five separate pools of Tel RNA from fadrozole-treated fish were analyzed. We used the Genisphere Array 900MPX cDNA microarray labeling kit (Genisphere, Hatfield, PA, USA) for all microarray hybridizations. This indirect labeling kit uses Cyanine 3 (Cy3) and 5 (Cy5) as the fluorescent dyes. The complete hybridization protocol is found at (http://www.genisphere.com/pdf/array900mpx_protocol_v06-22-04.pdf). We used 2 μg total RNA for the first strand synthesis. A 2x formamide-based hybridization buffer was used for the microarray prehybridization step and the 2x SDS-based hybridization buffer was used for the light capture reaction (3DNA hybridization step).

Microarrays were scanned at full-speed 10-μm resolution on a ScanArray 5000 XL system (Packard Biosciences/Perkin-Elmer) using both red and blue lasers. Images were obtained with ScanArray Express software using automatic calibration sensitivity varying photomultiplier tube (PMT) gain (PMT starting at 65% for Cy5 and 70% for Cy3) with fixed laser power at 80% and the target intensity set for 90%. Microarray images were opened using QuantArray (Packard Biosciences/Perkin-Elmer) and raw signal intensity values obtained for duplicate spots of genes. Data filtering were applied prior to data normalization. Firstly, in each slide, spots whose estimated fluorescence intensities were below or equal to the estimated background signal intensity and spots with poor hybridization were removed. Secondly, in all slides, those spots whose intensities cannot be deected in more than 20% replicates will be removed. Detailed analysis and descriptions about microarray quality can be found in Xiong (2008) and Martyniuk et al. (2006).

Lowess normalization (Yang et al., 2002) was used to decrease within-slide bias. For between slide normalization, either the Scale method (Yang et al., 2002) or a novel GPA method (Xiong et al., 2008) were used. Significance analysis of microarray (SAM) (Tusher et al., 2001) was performed to assess the significance of differential expression of the genes. The genes with false discovery rate (FDR) lower than 0.05 are considered as differentially

expressed ones. We finally compared the performance of GPA and Scale normalizations based on positive identification of known E2-responsive genes which were retrieved by PubMed searching (see below). Compared to the Scale normalization, GPA normalized data generated fewer differentially expressed genes, however it retrieved more known E2 regulated genes. For example, both somatostatin type 2 and somatostatin type 5, which are differentially regulated by E2 in rat pituitary (Kimura et al., 1998), were identified in the GPA normalized data but not in the Scale normalized data. This result provides a practical support for reliable performance of GPA method in microarray data analysis.

2.2.6 Functional annotations

The Gene Ontology (GO) analysis for differentially expressed genes was carried out by using the Blast2Go program (http://www.blast2go.de/). The specific GO terms were selected with the confluence score higher than cutoff values (9 for biological process category; 5 for molecular function category). This Blast2Go score takes into account the number of sequences converging at one GO term and penalizes by the distance to the term where each sequence actually was annotated (Gotz et al., 2008).

Gene function was also checked by retrieving and comparing homologous sequences from the GenBank database and profiling domain information in the Pfam database (Finn et al., 2008). To illustrate the regulatory relationship between E2 and the identified gene candidates, we retrieved research papers by searching key words of "estrogen" and an individual gene name in PubMed database.

2.2.7 Real-time RT-PCR assay

SYBR green real-time RT-PCR assay was conducted to measure the relative gene expression of AroB, ODC1, activin βA, and calmodulin, aldolase c, EF1α, 18s rRNA in both control and treatment groups. Primers (Table 1) were designed using Primer3 (You et al., 2008) and purchased from Invitrogen. The Mx4000 Quantitative PCR System (Stratagene, La Jolla, CA) was used to amplify and detect the transcripts of interest. Each PCR reaction contained the following final concentrations: 25 ng first strand cDNA template, 1× QPCR buffer, 3.5mM $MgCl_2$, optimized concentrations (150nM-300nM) of gene-specific primers, 0.25× SYBRGreen (Invitrogen), 200 μM dNTPs, 1.25U HotStarTaq (Invitrogen), and 100nM

ROX reference dye, in a 25μL reaction volume. The thermal cycling parameters were an initial 1 cycle Taq activation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15s, optimized annealing temperature (58–60 °C) for 5s, 72 °C for 30 s, and a detection step at 80 °C for 8s. Dilutions of cDNA (1:10 to 1:31,250) from all samples were used to construct a relative standard curve for each primer set. After the reaction was complete, a dissociation curve was produced starting from 55 °C (+1 °C /30 s) to 95 °C. For each PCR reaction, negative controls were also introduced including a no template control (NTC) where RNase-free water was added to the reaction instead of the template (cDNA) and a no reverse transcriptase control (NRT) where RNase-free water was added to the cDNA synthesis reaction (previously described) instead of the SSII enzyme. The SYBR green assay for every target gene was optimized for primer concentration and annealing temperature to obtain a dRn value higher than 0.98, an amplification efficiency between 90%-110% and a single sequence-specific peak in the denaturation curve.

Data were analyzed using the MxPro software package. The relative standard curve method was used to calculate the relative mRNA level of target and reference genes between samples. The gene copy numbers of target genes were normalized to those of EF1α, an internal reference gene whose expression did not significantly change with the treatment. The final normalized data from biological replicates (10-12 RNA samples per group) are presented as means + SEM of fold changes relative to control sample, which was set at 1. For statistical analysis, significant changes in gene expression in normally distributed data were evaluated using an Independent Samples T-test ($p<0.05$; SigmaStat3.5), and non-normally distributed data were evaluated using Mann-Whitney U test ($p<0.05$).

2.3 Results

2.3.1 Gonadosomatic index (GSI) and histological examinations

Fadrozole exposure decreased ovarian GSI by 43% (Figure 4), however it was not significantly changed ($p=0.251$ by the Mann-Whitney U test).

Histology was used to determine the effect of fadrozole on female goldfish since effects on gonad development has been observed in other fish and frog species (refer to

Chapter 4). In contrast to the female control group, there were no mature oocytes and no visible vesicles (cortical alveolus) in the female treatment group, suggesting that reduction of E2 delayed development of the female ovary (Figure 5). These conclusions are tentative because they are based on histological data from only 3 animals. More ovaries should be examined to increase confidence in the conclusion.

2.3.2 Fadrozole treatment decreases serum E2 level and aromatase b expression

Considering the histological differences in female ovary between control and treated groups, we next measured serum E2 level in female goldfish. Compared to control group, significantly lower (-4.7 times) levels of circulating serum E2 were found in treated fish ($p=0.027$; Figure 6). This indicates that fadrozole treatment effectively reduced E2 production in goldfish.

We want to know whether E2 reduction by fadrozole affected expression of AroB, which is known to be directly regulated by E2 in fish (Callard et al., 2001). Indeed, real-time PCR indicated that fadrozole treatment was effective and in both Hyp and Tel, AroB expression was three times lower in treated than control groups ($p=0.006$; Figure 7). This indicates that the E2 reduction by fadrozole is achieved not only by inhibiting aromatase activity, but also by further decreasing AroB expression in the brain.

2.3.3 Microarray analysis of gene expression in Tel

Since AroB is one typical target of E2 transcriptional action (Marlatt et al., 2008), its expression change suggests that E2 reduction may have a global effect on expression of other E2-regulated genes. The goldfish-carp cDNA microarray was then used to identify these potential E2-regulated genes in Tel, which including 98 known genes and 85 unknow EST clones ($q<0.05$). Among the 98 known differentially expressed genes, 68 were up-regulated (Table 2) and 30 were down-regulated (Table 3) after fadrozole treatment.

Some of the identified genes have been previously shown to be estrogen-responsive in fish or other species (Table 2 and 3). For instance, in our previous microarray analysis of the Hyp (Marlatt et al., 2008), matrix metalloproteinase 9 was decreased in male goldfish after exposure to E2, while it is up-regulated in the present study. There is also evidence showing that some identified gene candidates have regulatory relationships to estrogen in

other tissues of other species. These include SNAP25, somatostatin-14, somatostatin receptor 2 and 5, neuropeptide Y, lactate dehydrogenase a, ubiquitin carboxy-terminal hydrolase L1, parvalbumin, matrix metalloproteinase-13. We list the PMID of the retrieved references for this information (Table 2 and 3).

2.3.4 Functional significance of the differentially expressed genes

We used gene ontology analysis to explore the potential biological processes or molecular functions for these differentially expressed genes (Figure 8). When the cutoff value of classification is 9, the GO biological processes include the terms such as transport, multicellular organismal process, signal transduction, response to stress, cellular metabolic process, response to chemical stimulus, negative regulation of cellular process and calcium ion transport (Figure 8A). The specific GO molecular functions (cutoff value=5) identified include terms such as catalytic activity, ATP binding, zinc ion binding, DNA binding, receptor binding, calcium ion binding, calcium channel activity, and calcium channel inhibitor activity (Figure 8B). Interestingly, in both categories, GO terms related to calcium signaling are retrieved with very high confluence scores. This indicates that fadrozole treatment, and by extension, estrogen withdrawal, affects calcium signaling in the brain by changing the expression of the calcium binding proteins. This hypothesis is further supported by functional annotations with domain information for these proteins. From the analysis, there are 12 proteins shown to have calcium binding activity including calmodulin, SNAP25, EPD I, EPD II, neurogranin, visinin-like 1, similar to testican 3, EF hand domain containing 2, secretogranin II precursor, S100 calcium binding protein β, parvalbumin α and fish-egg lectin (functional theme C in Table 2 and 3).

Reference reviewing also retrieved several genes whose homologues are somehow functionally related to E2 actions (functional theme E in Table 2 and 3). These genes fall into two types of regulation of E2 actions: one is direct regulation of nER activity which includes calmodulin (Biswas et al., 1998), HSP90b, O-GlcNAc transferase (Bowe et al., 2006), ubiquitin-conjugating enzyme E2 (UCE2) variant 1 (Nawaz et al., 1999), activin βA (Burdette and Woodruff, 2007) and the dynein light chain (Rayala et al., 2005; Hu et al., 2008); Another type is regulation of nER mRNA expression which includes PACAP (Bryant et al., 2006) and activin βA (Kipp et al., 2007a). Their expression change by fadrozole

treatment suggests that in fish brain, E2 can affect its own actions through regulating expression of its coactivitors or inducers. Other gene groups also include the ubiquitin–proteasome pathway related genes, ubiquitin thiolesterase, ubiquitin-conjugating enzyme E2, ubiquitin c-terminal hydrolase L1, and cdc23 and SMT3 suppressor of MIF two 3 homolog 3 (functional theme U), and three somatostatin-related genes, preprosomatostatin-14, somatostatin receptor type 2 and somatostatin receptor type 5 subtype B (functional theme S), which are known E2 targets (Kimura et al., 1998; Xu et al., 1998; Scanlan et al., 2003).

2.3.5 Real-time RT PCR verifications

Real-time PCR was further used to examine the expression change of four genes in Tel as detected in the microarray analysis. Expression changes in the Hyp, another major neuroendocrine region, were also examined. Except for aldolase c, three other genes including calmodulin, activin βA, ornithine decarboxyase 1 (ODC1) showed significant changes in both Tel and Hyp (Figure 9). Calmodulin is a calcium binding protein as identified by GO and domain analysis. After fadrozole treatment, its mRNA level increased by 1.32 times ($p=0.031$) in Tel and 1.41 times ($p=0.004$) in Hyp. Activin βA is a member of the transforming growth factor β (TGFβ) superfamily (Keah and Hearn, 2005). It was chosen for its functional relevance to ER action (E2 function theme in Table 2 and 3) (Burdette and Woodruff, 2007). After fadrozole treatment, activin βA expression was decreased by 1.43 times ($p=0.009$) in Tel and 1.36 times ($p=0.018$) in Hyp. ODC1 is the key enzyme responsible for catalyzing the reaction of ornithine to putrescine in the ODC/polyamine system (Slotkin and Bartolome, 1986). Putrescine has been shown to be an alternative precursor source of the neurotransmitter gamma-aminobutyric acid (GABA) (Tillakaratne et al., 1995). Real-time PCR assays showed an increase in ODC1 mRNA level in both Tel (1.98 times; $p=0.002$) and Hyp (1.68 times; $p=0.008$) after treatments. Aldolase c expression was not significantly changed ($p=0.087$); however, it showed a small increase of 1.23 fold in Tel which is consistent with the microarray analysis. It should be noted that fold changes ranged from 1.32 to 1.98, which is typical and highly similar to previous observations in fish brain (Martyniuk et al., 2006).

2.4 Discussion and conclusions

In the present study, we used the goldfish model to evaluate the effects of fadrozole on neuroendocrine and reproductive functions. In contrast to other studies, we treated the female goldfish from March to April, the seasonal period when the goldfish has maximal brain aromatase activity and serum E2 levels (Pasmanik and Callard, 1988; Trudeau et al., 1991). We intended to produce an E2 "knockdown phenotype" in fadrozole-treated goldfish. Thus, when compared to control fish which have been exposed to the normal seasonal E2 profile and physiological expression changes of E2-regulated genes, fadrozole-treated fish may exhibit opposite and contrasting effects on physiology and gene expression.

Indeed, there was a significant decrease in serum E2 level for the female goldfish treated with fadrozole. We further observed that such fadrozole-induced E2 reduction has delayed the ovarian development of the treated goldfish. Their ovaries have no mature oocytes and no cortical alveolus (Figure 2). These E2 effects can be partially achieved by down-regulating vitellogenin, an egg yolk precursor protein for forming cortical alveolus, whose expression is extremely sensitive to E2 level (Pakdel et al., 1991; Teo et al., 1998). Our results are comparable to the histological observations in fadrozoled-treated adult fathead minnow (*Pimephales promelas*) (Ankley et al., 2002). As expected, for adult fish, we did not observe any sex reversal or intersex phenotype typical of those reported in sexually undifferentiated European sea bass (*Dicentrarchus labras*) (Navarro-Martin et al., 2009) and the developing zebrafish (*Danio retio*) (Fenske and Segner, 2004) following fadrozole exposures. Further discussion on the possible mechanism of E2 regulation on gonadal development will be covered in Chapter 4.

We also found that AroB mRNA is significantly decreased in both Tel and Hyp after fadrozole treatments. Aromatase B is a well-characterized, validated E2 target in the fish brain. Its decreased expression here indicates an effective inhibition of E2 production in radial glial cells and most probably depressed nER action in brain after fadrozole treatments. We then used cDNA microarrays to profile global gene expression changes in Tel that may be associated with fadrozole-induced E2 reduction. We identified numerous candidate E2-responsive genes and confirmed the expression change of three genes including calmodulin, activin βA, ODC1 by using real-time PCR. Some other genes, for example MMP9 (Marlatt et al., 2008) and preprosomatostatin-14 (Canosa et al., 2002), have also been shown to be E2-

regulated in the goldfish brain. By reviewing published papers, we further identified another set of genes whose homologues are subjected to E2-regulation in other species (Table 2 and 3). The evidence strongly supports the idea that fadrozole treatment can reverse E2 action in regulating gene expression. Moreover, the conserved transcriptional regulation by estrogen of those genes in different species may indicate an ancestral regulatory mechanism which is most likely located at some evolutionarily conserved EREs in promoter regions. Further comparative genomic analysis and functional characterization are needed to substantiate this hypothesis.

Gene expression changes associated with E2 reduction provide functional insights into physiological E2 actions in the fish brain. Firstly, GO analysis together with domain annotations identified twelve gene products, including calmodulin, that possess calcium binding activity, which indicates that estrogen deprivation significantly impacted calcium signaling via the classical E2 transcriptional pathways. It has been extensively documented that in neurons of mammals (rat, pig, and monkey), E2 can regulate cellular calcium concentration (Joels and Karst, 1995; Qiu et al., 2006) or oscillations (Abe et al., 2008; Noel et al., 2009). This E2 effect has been shown to be mediated by a membrane-initiated signaling pathway of E2 involving GPCR30 (Abe et al., 2008; Noel et al., 2009). Moreover, in rat pituitary cells, E2 can also regulate calcium signaling by affecting the expression of some calcium-binding proteins, such as SNAP25 (Weiss et al., 2007) and parvalbumin (Fujimoto et al., 2004). In teleost fish, E2 is known to induce an increase in circulating levels of vitellogenin (Bradley and Grizzle, 1989; Mosconi et al., 1998), a calcium binding protein from the liver, and a parallel increase in blood plasma calcium (Persson et al., 1994; Guerreiro et al., 2002). However, the molecular mechanisms involved in this process and their physiological effects in fish are still largely unknown. Here our findings suggest that E2 can regulate calcium signaling pathway in fish brain and this can be achieved by the classical regulatory mode via influencing gene expression.

Numerous differentially regulated genes have been implicated in estrogen receptor-mediated actions (functional theme E in Table 2 and 3), which are typified by calmodulin (Li et al., 2006; Gallo et al., 2008), hsp90b (Lee et al., 2002; Gougelet et al., 2005), O-GlcNAc transferase (Bowe et al., 2006), UCE2 variant 1 (Nawaz et al., 1999; Verma et al., 2004), and dynein light chain (Rayala et al., 2005; Hu et al., 2008). The expression change of

calmodulin was verified by real-time PCR in the current study. Calmodulin, hsp90b and dynein light chain are required for the transcriptional activity of ERα. For example, by directly interacting with ERα (Castoria et al., 1988), calmodulin modulates the ability of ERα to bind to the ligand E2 (Bouhoute and Leclercq, 1992) or the DNA target ERE (Biswas et al., 1998) and promotes its stability by preventing ubiquitin-proteasome (UP) degradation (Li et al., 2006). Hsp90b also contributes to protecting ER stability against the UP pathway (Lee et al., 2002) whereas dynein light chain has been recently shown to unite nER to other chromatin remodeling complexes (Hu et al., 2008). In contrast, O-GlcNAc transferase and UCE2 variant 1 and other proteins including ubiquitin thiolesterase, ubiquitin c-terminal hydrolase L1, cdc23 and SMT3 suppressor of MIF two 3 homolog 3 (functional theme U in Table 2) are all functionally related to the UP degradation pathway, which regulates nER stability (Nawaz et al., 1999; Verma et al., 2004; Bowe et al., 2006). Therefore, the differential expression of these modulatory factors after fadrozole treatments suggests an auto-regulation mechanism of E2 action in the neuroendocrine brain. In fact, in another study where male goldfish were treated with E2, a decreased expression of another UCE2 enzyme was observed in the Hyp (Marlatt et al., 2008), further substantiating estrogen regulation of these pathways.

The differential expression of activin βA provides another regulatory mechanism for the crosstalk between E2 and activin signaling in fish. Activin βA is one subunit of activin which structurally belongs to the TGF β superfamily, and contains three other subunits activin βB, αA and αB. A dimeric growth factor, activin, can be formed by two subunits and bind the membrane serine/threonine kinase receptors (Mathews, 1994), which then induce downstream signaling pathway activation and affect activities of transcriptional factors such as Smad (Shi and Massague, 2003). Very recently, activin signaling was shown to regulate ERα activity in the human breast cancer cells (Burdette and Woodruff, 2007) and induce expression of ERβ in the mouse granulosa cells (Kipp et al., 2007a). Moreover, the regulation of E2 on mRNA expression of either activin beta subunit in the mouse neonatal ovary (Kipp et al., 2007b) or type II activin receptor in rat hypothalamus (Trudeau et al., 1996) was also observed. Therefore, there is a crosstalk between E2 and activin signaling in mammals. Here decreased E2 results in decreased activin βA expression, which indicates a regulatory

crosstalk between activin and estrogen signaling, and also an involvement of activin signaling in E2 action in fish brain.

ODC1 is another gene whose expression was increased after fadrozole treatment. It catalyzes the formation of putrescine from ornithine, a rate-limiting step during the biosnythesis of the polyamines spermidine and spermine (Slotkin and Bartolome, 1986). This so-called ODC/polyamine system is important in neuronal cell development (Slotkin and Bartolome, 1986) and modulates the ion channel and calcium-dependent transmitter release (Bernstein and Muller, 1999). Moreover, there is emerging recognition that putrescine is an alternative precursor source of GABA (Tillakaratne et al., 1995; Yamasaki et al., 1999; Sequerra et al., 2007), an major neurotransmitter which has an important role in reproduction because of stimulatory actions on luteinizing hormone release in goldfish (Popesku et al., 2008). The increased ODC1 expression after fadrozole treatment may indicate an enhancement of ODC/polyamine system and/or suggest a new pathway for E2 to regulate GABA production.

The candidate E2-responsive genes may serve as novel gene indicators of environmental pollutants which have estrogenic or anti-estrogenic activities or have effects on neuroendocrine functions (Rotchell and Ostrander, 2003). For example, the expression of death associated protein 1a (dap1a), an identified E2-responsive gene here, was increased after waterborne 17 α-ethinylestradiol (EE2) treatment of zebrafish, as shown by microarray profiling of the Tel (Martyniuk et al., 2007b). Another gene of interest is interferon-related developmental regulator 1, whose expression is induced by EE2 (Martyniuk et al., 2006), but decreased in our study.

In conclusion, in the present study, we used fadrozole to severely impair estrogen production in the female goldfish and evaluated the associated physiological effects on neuroendocrine and reproductive systems. We profiled gene expression changes in brain upon fadrozole-induced E2 reduction that was further verified by the real-time PCR. Functional annotations and GO analysis revealed several regulatory themes for physiological E2 actions in fish brain, including the regulation of calcium signaling pathway and auto-regulation of nER action by influencing global gene expression. Our results may serve as a basis for understanding melecular mechanisms of E2 action in brain and its link to physiological effects.

Table 1. Oligonucleotide primers used for real-time RT-PCR assays

Gene target	GenBank ID	Sequence 5'-3'	Amplicon size
EF1 α	AB056104	F: GATTGTTGCTGGTGGTGTTG	227 bp
		R: GCAGGGTTGTAGCCGATTT	
18s rRNA	AF047349	F: AAACGGCTACCACATCCAAG	166 bp
		R: CACCAGATTTGCCCTCCA	
Activin β A	AF169032	F: TTTAAGGACATCGGGTGGAG	237 bp
		R: TGATTGATGACGGTGGAATG	
Ornithine decarboxyase 1	AY640230	F: TTGACATTGGAGGAGGCTTT	194 bp
		R: GATGACCTTTTTGGCGATG	
Calmodulin	AY656699	F: CATTTCCATCAGCGTCCA	108 bp
		R: GCACCATCACGACCAAAGA	
Aromatase b	AB009335	F: TGCTGACATAAGGGCAATGA	153 bp
		R: GGAAGTAAAATGGGTTGTGGA	
Aldolase c	U36777	F: GGCACGTCCATAAGAGAAGG	100 bp
		R: GGAGGTCAGAGTGAGGAGGA	

Table 2. List of up-regulated genes identified by microarray analysis in female goldfish after fadrozole treatment (q value <5%)

Gene name (up, 58)	GenBank ID	Fold	PMID	Function
Calmodulin	AAT73046	+1.58		C, E
SNAP25	P36978	+1.41	17293448(rat) 9576603(rat)	C
EPD I	P13506	+1.45		C
EPD II	P12958	+1.39		C
Neurogranin	ACJ64077	+1.38		C
Visinin-like 1	XP_689658	+1.32		C
Similar to testican 3	AAH96871	+1.37		C
EF hand domain containing 2	NP_077305	+1.42		C
Secretogranin II precursor	AAC94994	+1.36	8321414(rat)	C
S100 calcium binding protein, beta (neural)	AAH76251	+1.32		C
Synuclein beta	ACA96673	+1.43		
Rho guanine nucleotide exchange factor 9	XP_689415	+1.36		
Receptor-type tyrosine phosphatase N polypeptide 2	AAI63485	+1.56		
Fatty acid desaturase 2	NP_571720	+1.31		
Ornithine decarboxyase 1	AAV34288	+1.48		
GABA A receptor beta 2	NP_001019558	+1.37		
Solute carrier family 6 (GABA transporter), member 1	AAT58230	+1.28		
Glula (glutamine synthase a)	NP_853537	+1.29		
Glutamate-cysteine ligase, catalytic subunit	NP_954971	+1.31		
Delta-aminolevulinic acid dehydratase	NP_001017645	+1.46		
Gamma-glutamyl cyclotransferase	NP_998170	+1.47		
Hematological and neurological expressed 1	NP_991176	+1.47		
MAP-kinase activating death domain-containing	CAF99926	+1.47		
Hsp 90b	O57521	+1.43		E
Hsp binding protein 1	NP_957048	+1.33		
Mdkb (midkine-related growth factor b)	NP_571791	+1.42		
Na,K-ATPase alpha subunit 3	BAB60722	+1.37		
ATPase, H+ transporting, lysosomal, V0 subunit c	NP_001098606	+1.22		
O-GlcNAc transferase	XP_694454	+1.48		E
Ubiquitin thiolesterase	NP_958885	+1.48	14667807(human)	U
Ubiquitin-conjugating enzyme E2 variant 1	AAI07636	+1.37		U, E
Ubiquitin c-terminal hydrolase l1	NP_958885	+1.30	16734731(human)	U
Cdc23	NP_957227	+1.35		U
SMT3 suppressor of MIF two 3 homolog 3	AAH68341	+1.30		U
Beta actin	P83750	+1.45		
Alpha actin	BAA08755	+1.27		
F-actin capping protein beta	ACH70650	+1.28		
FKBP12-rapamycin associated protein	AAC94996	+1.37		
Apolipoprotein Eb	NP_571173	+1.33		
Aldolase C	NP_919365	+1.43		
Pyruvate kinase	AAH67143	+1.27		
Enolase alpha non-neuron	AAH59511	+1.30		
Ckb (creatine kinase, brain)	NP_775329	+1.40		
Puromycin sensitive aminopeptidase	XP_684042	+1.37		
Myelin basic protein	AAW52552	+1.35		
GAPDH	NP_998259	+1.35		
Isotocin neurophysin	NP_840076	+1.34		

Neuropeptide Y	AAA49186	+1.26	16675543(mouse)	
PACAP	NP_999880	+1.32		E
Stathmin	NP_001017850	+1.29		
Gpia (glucose phosphate isomerase a)	NP_658909	+1.35		
Protein tyrosine phosphatase, receptor D isoform 3	XP_685643	+1.35		
Jumonji containing 3, histone lysine demethylase	NP_001025349	+1.28		
HMP19; neuron specific gene family member 2	AAI52653	+1.32		
Lactate dehydrogenase-a	AAD40736	+1.40	17010207(rat)	
Interferon-related developmental regulator 1 (ifrd1)	NP_001070023	+1.68		
Hypothetical protein LOC447815	NP_001004554	+1.31		
Meiotic recombination 11 homolog A	NP_001001407	+1.48		
Integral membrane protein 2B	NP_955940	+1.31		
DAZ associated protein 1	AAI53535	+1.52		
High-mobility group box 2	AAH81415	+1.26		
High mobility group-T protein	NP_001092721	+1.24		
zgc:110141	NP_001017856	+1.32		
wu:fj40g07	XP_695097	+1.28		
Similar to phospholysine phosphohistidine inorganic	NP_001092251	+1.64		
Matrix metalloproteinase 9	NP_998288	+1.40	11245078(rat)	
Somatostatin receptor type five, subtype B	AAN76495	+1.46	9528936(rat)	S
Glucagon receptor precursor	AAS93685	+1.20		

GenBank ID: Protein ID for homologues sequences from fish except NP_077305 which we could not find sequence entry for fish homologue.

Fold: Gene expression fold change from microarray data analysis

PMID: PubMed ID for reference that shows regulation of E2 on the candidate gene in specific species

Function: Functional themes which candidate genes may have. These include the effect on E2 action via either regulating nER expression or activity (E), calcium binding activity (C), ubiquitin-proteasome pathway related (U) and somatostatin-related (S)

Table 3. List of down-regulated genes identified by microarray analysis in female goldfish after fadrozole treatment (q value <5%)

Gene name (down, 30)	GenBank ID	Fold	PMID	Function
Parvalbumin alpha	NP_991137	-1.32	15276619 (rat)	C
Fish-egg lectin	AAK01373	-1.31		C
Preprosomatostatin-14	NP_898893	-1.87	12097812 (goldfish)	S
Somatostatin receptor type 2	XP_694885	-1.51	9528936 (rat) 9705075 (human)	S
Fatty acid binding protein 2, intestinal	AAH75970	-1.41		
zgc:55455	AAI64355	-1.45		
Activin beta A	AAD50448	-1.94		E
RD RNA binding protein	NP_001002375	-1.40		
Kinectin 1	AAH44389	-1.43		
Hypothetical protein LOC567341	NP_001020721	-1.33		
Solute carrier family 7 (cationic amino acid transporter) member 3	NP_001007330	-1.55		
Ribosomal protein S4, X-linked (rps4x)	NP_001005589	-1.34		
U6 snRNA-associated Sm-like protein LSm7	AAI33958	-1.30		
BolA-like 3	AAH78189	-1.29		
Death associated protein 1a (dap1a)	ACI70034	-1.31		
Farnesyltransferase, CAAX box, beta	NP_001002128	-1.34		
Matrix metallopeptidase 13 (Collagenase 3)	XP_685843	-1.31	12893436 (rat) 16919424 (rabbit)	
Cadherin 1, epithelial (cdh1)	NP_571895	-1.28		
Pecanex-like protein 3	XP_690089	-1.30		
sc:d0375	XP_001923590	-1.27		
Small Hsp B15	ABQ57502	-1.30		
cL41a ribosomal protein L41 mRNA	AAM77969	-1.26		
PRP6 pre-mRNA processing factor 6	NP_997820	-1.27		
Pyridoxine (pyridoxine, vitamin B6) kinase	NP_001119921	-1.28		
Muscle-specific beta 1 integrin binding protein	AAI52227	-1.31		
Titin-like (ttnl)	NP_997066	-1.34		
Cytokine receptor-like factor 3	CAJ80719	-1.28		
Dynein, light chain, LC8-type 2	NP_998189	-1.25		E
Heterogeneous nuclear ribonucleoprotein U	NP_001028767	-1.28		
wu:fc14a08	XP_696080	-1.28		

Figure 4. GSI values of the female goldfish in control and fadrozole treated groups

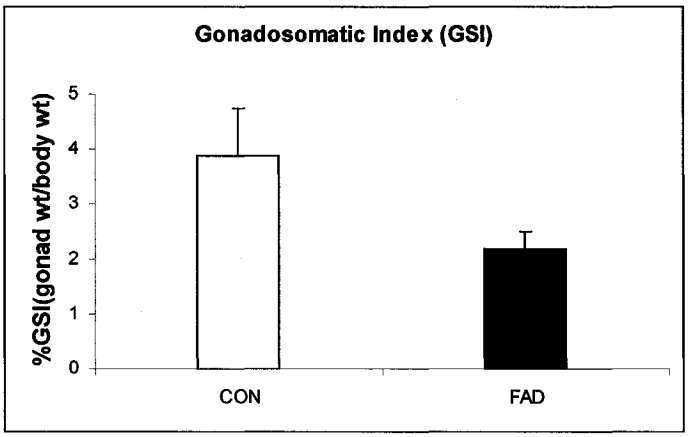


Figure 5. Histological photos of ovary from control (A, B) and fadrozole treated (C, D) female goldfish

GSI values of sampled goldfish are indicated below the A, B, C, D. Abbreviations: MO, mature oocyte; YO, young oocyte; V, vesicle or cortical alveolus.

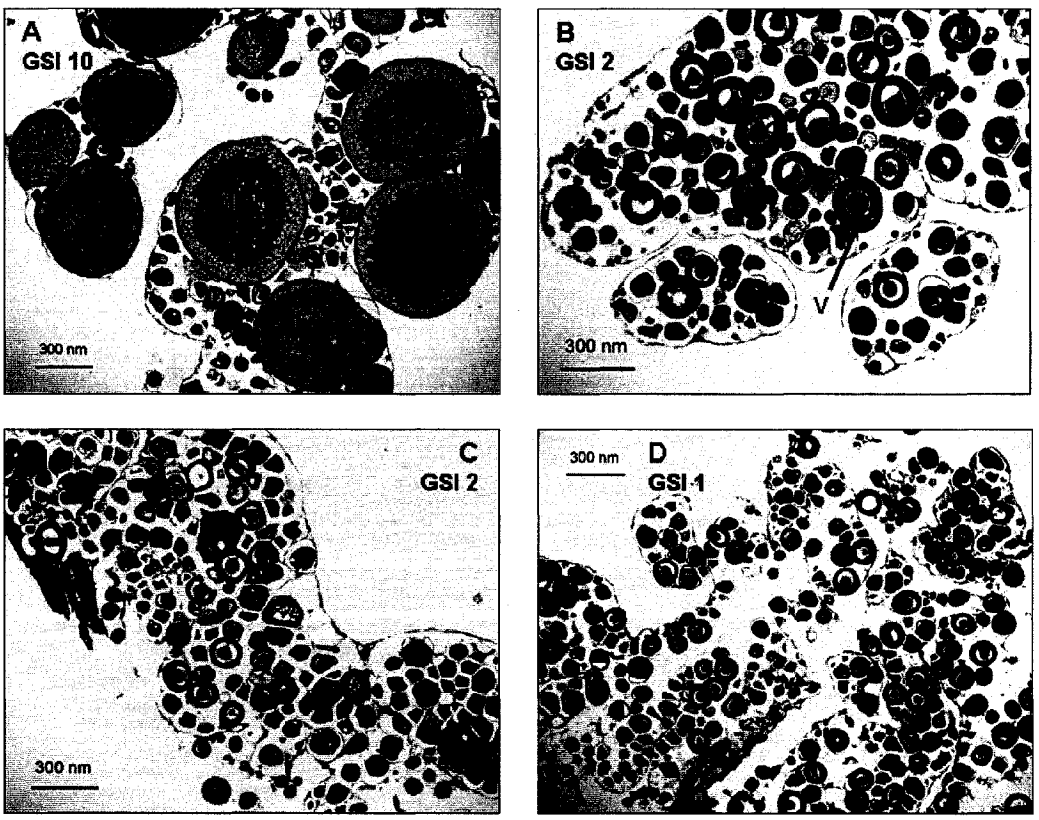


Figure 6. Serum E2 level change in female goldfish after fadrozole treatment

Asterisks (*) indicate significant differences between control and treatment groups ($p<0.05$ by Mann-Whitney U test)

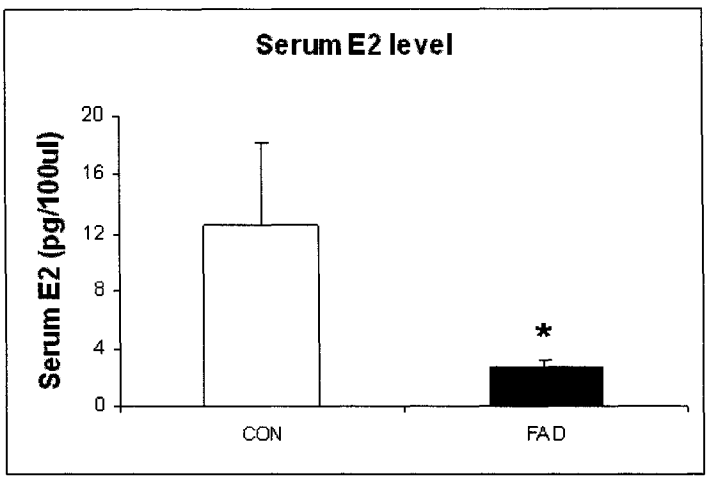


Figure 7. Relative gene expression of AroB in Tel and Hyp after fadrozole treatment

The expression of AroB was nomalized to EF1alpha. Mean+SEM values are shown for each group (n=10–12). Asterisks (*) indicate significant differences between control and treatment groups ($p<0.05$ by Mann-Whitney U test)

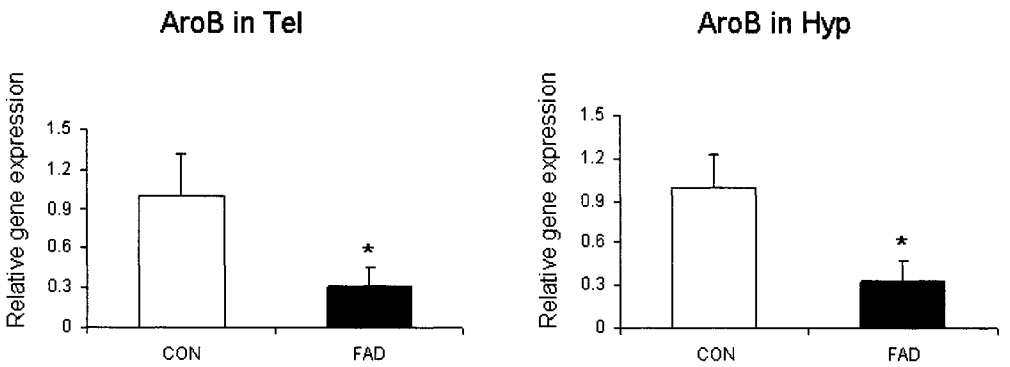


Figure 8. Gene Ontology (GO) term analysis of differentially expressed genes after fadrozole treatment

The GO terms whose confluence scores are higher than cutoff values (9 for biological process category; 5 for molecular function category) are shown. The specific score (S) and the number of genes (N) for the individual GO term are indicated. The GO terms related to calcium signal pathway are shown in red.

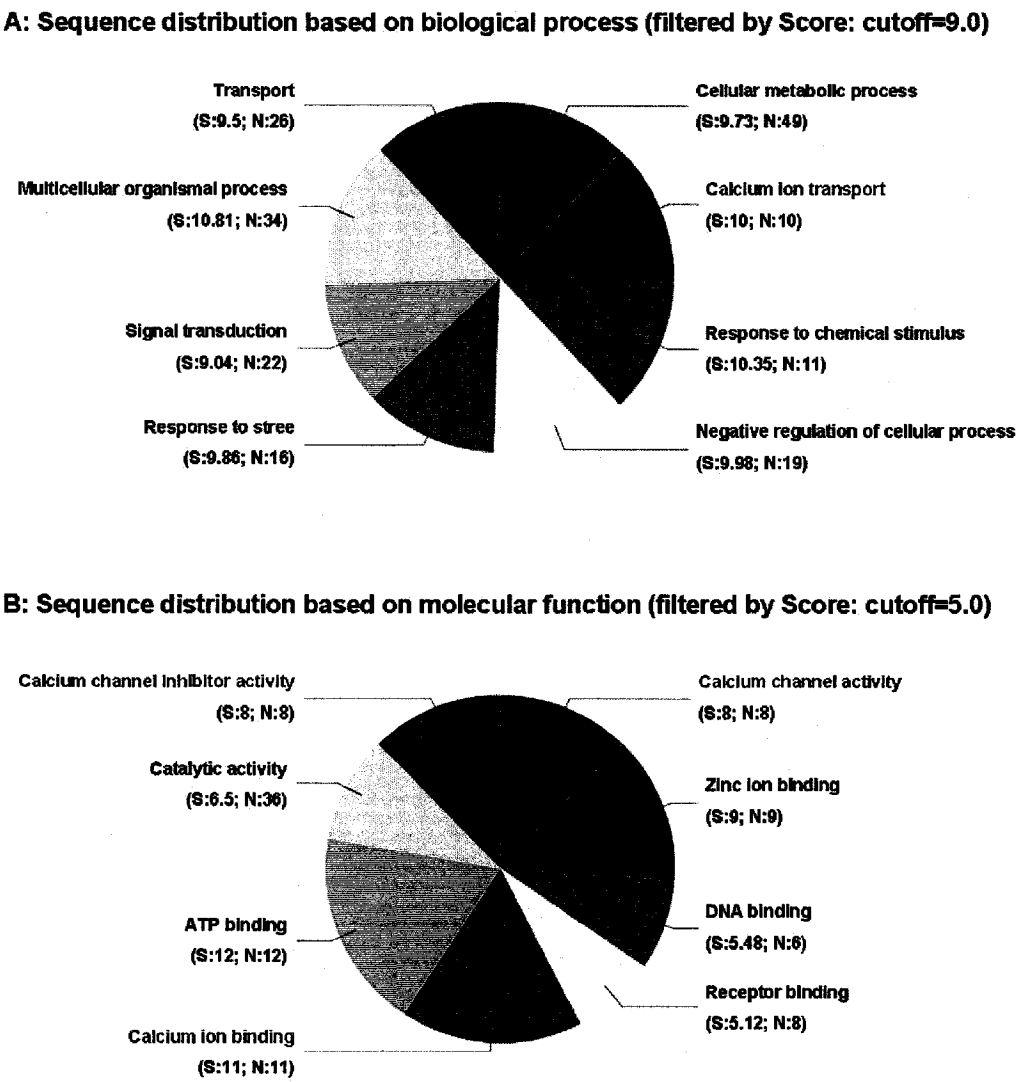
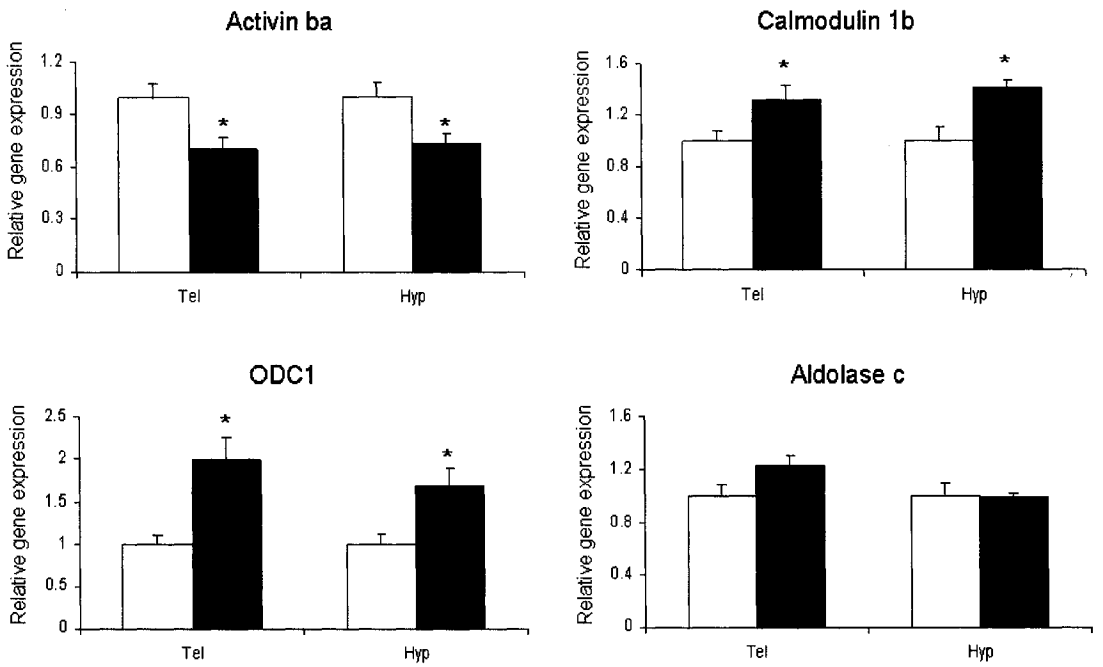


Figure 9. Relative gene expression change in both Tel and Hyp after fadrozole treatment

Control samples are shown as blank bars whereas fadrozole treated samples are shown as black bars. Mean+SEM values are shown for each group (n=10–12). Asterisks (*) indicate significant differences between control and treatment groups ($p<0.05$ by T-test)



CHAPTER 3: DEFINING NEUROENDOCRINE GENE EXPRESSION PATTERNS ASSOCIATED WITH REPRODUCTIVE SEASONALITY IN FISH

Abstract

Many vertebrates, including the goldfish, exhibit seasonal reproductive rhythms, which are a result of interactions between external environmental stimuli and internal endocrine systems in the hypothalamo-pituitary-gonadal axis. While it is known that some neuroendocrine genes are seasonally differentially expressed, no biological systems-level investigation of transcriptomic change has yet been conducted. In the present study, I attempted to characterize the seasonal gene expression patterns in goldfish brain obtained from microarray data analysis and experimental strategies. I have identified a core set of genes (883 genes) in the hypothalamus (Hyp) which are differentially expressed between May, August and December, corresponding to physiologically distinct stages that are sexually mature prespawning, sexual regression, and early gonadal redevelopment. By examining one dataset obtained from fish in October that were kept under long-daylength photoperiod (16h) typical of the springtime breeding season (May), I observed that the expression of identified genes appears regulated by photoperiod, a major factor controlling vertebrate reproductive cyclicity. Gene ontology analysis revealed that some hormone genes and other genes functionally involved in G-protein coupled receptor signaling pathway and transmission of nerve impulses are significantly enriched in an expression pattern, whose transition is located between prespawning and sexually regressed stages. The existence of seasonal expression patterns was verified for several genes including ependymin II, GABA-A gamma2 receptor, calmodulin, and aromatase b by independent samplings of goldfish brains from six seasonal time points and real-time PCR. Therefore, using both theoretical and experimental strategies, I defined global gene expression patterns throughout a breeding season which may account for dynamic neuroendocrine regulation on fish seasonal reproductive development.

3.1 Introduction

Almost all organisms, be they yeast, plants, fish or mammals, are characterized by their biological rhythms with a periodic (daily, monthly or annual) change in behaviour or physiology (Ripperger, 2007). The daily circadian rhythm is exemplified by opening/closing of flowers or the daily awake/sleep cycle in humans. The menstrual cycle of women is a typical monthly rhythm whereas circannual rhythms include bird migrations, hibernation in frogs and mammals, and marked reproductive seasonality in numerous vertebrate classes including fish. How diverse organisms exhibit a biological rhythm still remains elusive. It has long been accepted that external environmental influences such as photoperiod (Davis and Millar, 2001; Bayarri et al., 2004) and temperature (Blazquez et al., 1998; Koger et al., 1999) exert dominant roles in biological rhythms, and internal neuroendocrine systems such as the pineal gland, hypothalamus and pituitary coordinate these signals (Trudeau, 1997; Lincoln and Richardson, 1998; Thiery et al., 2002; Ruan et al., 2008). In this study, we use theoretical and experimental approaches to better understand global genomic regulation of the neuroendocrine system during seasonal reproduction. The bony fishes or teleosts represent more than half of all vertebrates. Numerous characteristics of goldfish (*Carassius auratus*), a member of the Cyprinidae, one of the largest vertebrate classes, make this species an excellent model for studying neuroendocrine signaling and the control of seasonal reproduction (Callard et al., 1995; Popesku et al., 2008). Similar to many teleosts, goldfish employ a seasonal reproductive strategy characterized by distinct stages: gonadal regression (June-October), recrudescence or redevelopment (October-March), prespawning (April-May) and spawning or breeding season (May or early June) (Blazquez et al., 1998).

Seasonal reproductive cyclicity including annual redevelopment of gonadal tissues is mediated by a hormone regulatory pathway primarily involving gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), growth hormone (GH), melatonin and sex steroid hormones (Trudeau, 1997; Lincoln and Richardson, 1998; Thiery et al., 2002). The main hypophysiotrophic systems controlling LH and GH are located in the preoptic region of the telencephalon and the hypothalamus, which have been well-described and characterized in goldfish (Peter et al., 1986; Trudeau, 1997). Transduction of photoperiodic signals via retino-hypothalamo-pineal pathways results in melatonin secretion patterns typically reflecting the length of the dark period (Darrow and Goldman, 1985; Davies et al., 1994;

Barrell et al., 2000). The released melatonin influences the neuroendocrine system via its receptors which are widely distributed in teleost brains (Ekstrom and Vanecek, 1992; Vernadakis et al., 1998; Mazurais et al., 1999). Multiple GnRH forms (Somoza et al., 2002) activate anterior pituitary receptors to stimulate the expression and release of LH, follicle-stimulating hormone (FSH) and GH, which are all involved in controlling seasonal gonadal development and sex steroid synthesis. Moreover, synchronization of fish reproductive behaviours in the breeding season involves a well-defined sex pheromone response system culminating in a surge release of LH that induces ovulation in females and sperm release in males (Sorensen et al., 1998). Testosterone (T) and estrogen (E2) exert a primary role in gonadal development locally, and have profound positive and negative feedback actions at the levels of the brain and pituitary (Trudeau, 1997). In addition to these core members of hypothalamo-pituitary-gonadal (HPG) axis, there is a complex and interactive regulation of GnRH and LH/FSH by a multitude of neuroendocrine factors including classical neurotransmitters and other neuropeptides (Trudeau, 1997; Lincoln and Richardson, 1998; Trudeau et al., 2000; Thiery et al., 2002). Glutamate, gamma-aminobutyric acid (GABA), and dopamine are the most important neurotransmitters, in which glutamate and GABA have predominantly stimulatory effects whereas dopamine is the single most potent inhibitor of LH release in teleosts (Trudeau, 1997; Trudeau et al., 2000). Neuropeptides include isoctocin, growth hormone, neuropeptide Y (NPY), cholecystokinin (CCK), most of which potentially have stimulatory effects on GnRH/LH/FSH release in teleosts.

It has been extensively described that reproductive seasonality of teleosts is characterized by their seasonal profiles or changes in serum hormone levels (Pasmanik and Callard, 1988; Trudeau et al., 1991; Gonzalez and Piferrer, 2003), brain hormone content (Joy et al., 1999), brain enzyme activities (Pasmanik and Callard, 1988), morphological (McNulty, 1982) and histological (Munkittrick and Leatherland, 1983) phenotypes. Moreover, recently there is recognition that seasonal expression of the neuroendocrine genes including neurohypophysial hormones, synthesis enzymes and related signaling pathway proteins may be utilized to link the external environmental effluences and the establishment of fish reproductive cyclicity. It has been observed that in teleosts many neuroendocrine genes including aromatase b (Gelinas et al., 1998), GnRH receptors (Jodo et al., 2005), glutamic acid decarboxylase (GAD) (Lariviere et al., 2005), CCK (Peyon et al., 1999),

preprotachykinin (Peyon et al., 2000) and secretogranin-II (Samia et al., 2004) have seasonal expression profiles, correlating with seasonal changes in gonadal size and serum sex steroid levels (Pasmanik and Callard, 1988; Trudeau et al., 1991; Sohn et al., 1999; Orlando et al., 2007). However, no study at the transcriptomic level has been conducted to investigate the global gene expression changes in neuroendocrine systems throughout an annual reproductive cycle. We have previously utilized microarrays to identify hormone or endocrine disrupting chemical-regulated genes in the goldfish neuroendocrine system (Martyniuk et al., 2006; Marlatt et al., 2008; Mennigen et al., 2008). All experiments were based on goldfish sampled at different seasonal time points; thus, these microarray datasets potentially have seasonal gene expression information. In the present study, on the basis of multiple goldfish microarray datasets, we used comprehensive normalizations, differential gene expression identification, multivariate and gene ontology (GO) analyses to characterize the season-related expression patterns in the female goldfish brain. A core gene set was identified whose expression exhibits a seasonal change with fish reproductive cycle. We illustrate that the gene expression change is regulated by photoperiod. Gene ontology (GO) analysis further revealed that neuroendocrine-related genes are significantly enriched in an expression pattern, which follows a high-low-low along the spring-summer-winter. This pattern was confirmed by a series of seasonal brain sampling and real-time PCR verification on four important neuroendocrine genes. We hypothesize that the seasonal expression patterns may account for dynamic neuroendocrine regulation of reproductive cyclicity.

3.2 Materials and methods

3.2.1 Microarray datasets and data analysis

A total of 48 cDNA microarray slides representing 6119 unique gene sequences and 2329 EST sequences printed in duplicate on each slide were used for this analysis. Microarray datasets (Table 4) of gene expression in female goldfish hypothalamus and telencephalon have been collected to represent four seasonal time points. The hybridization, scanning procedures and validation of microarrays have been described previously by our group (Martyniuk et al., 2006). The detailed data transformation and normalization procedures were described in the thesis of Xiong (Xiong, 2008). Briefly, Lowess

normalization (Yang et al., 2002) with span 0.4 was used to decrease the intensity dependent biases within slide, followed by Scale normalization (Yang et al., 2002). Thereafter, control sample intensities were recovered and extracted to build a new series of expression data for all the slides. Following this, Quantile normalization (Bolstad et al., 2003) was used for across-slide normalization to minimize biases among different experiments. An optimal discovery procedure (ODP) method implemented in the Extraction of Differential Gene Expression (EDGE) program (Storey et al., 2007) was used to assess the significance of differential expression of the genes between different seasonal time points.

3.2.2 Multivariate data analysis

Two multivariate methods including principal component analysis (PCA) and hierarchical clustering analysis (HCA) were utilized in this study. PCA was used to examine the transcriptome similarity relationship among slides that are corresponding to different seasonal time points. HCA was used to classify/cluster the gene expression patterns. Briefly, PCA combines two or more correlated factors (i.e. transcripts) into one new variable, a principal component (PC) (Pearson, 1901; Basilevsky, 1994; Sherlock, 2001). In PCA the dimensionality of the dataset is reduced by replacing the original variables by a smaller number of newly formed variables that are linear combinations of the original variables and that explain the majority of the information (variability) from the experiment. A hierarchical clustering tree (Heyer et al., 1999) generates a binary dendrogram representing the association structure of pairs of arrays or genes, which was measured in terms of the Pearson correlation of the standardized intensity measurements. The algorithm identifies the gene pair with the smallest distance and groups them with a link, where distance is defined to be one-minus the correlation. The algorithm proceeds in a recursive manner to build the tree structure step by step. PCA and HCA were conducted by the MultiExperiment Viewer (MeV) (Saeed et al., 2003).

3.2.3 Gene Ontology analysis

The resulting differentially expressed genes were submitted to the blast2go web server (Conesa et al., 2005). Enrichment of multiple-level GO functional categories was determined using the Gossip/Fisher Exact test package (Bluthgen et al., 2005), through a

comparison of the categories found in our list of genes compared to the list of all genes found on the goldfish cDNA microarray. FDR controlled *p*-values (FDR < 0.05) were used for the assessment of functional significance.

3.2.4 Animal husbandry and tissue sampling

Adult female goldfish (*Carassius auratus*) were purchased from a commercial supplier, acclimated for 3 weeks to 18 °C under a natural seasonally variable simulated photoperiod except for the animals sampled in October. In this case, animals were acclimated to 18 °C and 16h daylength that is typical of springtime. Fish were fed and maintained on standard flaked goldfish food. For the seasonal profile, a total of 18 female fish were collected every two months starting in January, for a total of six time points. Goldfish were anesthetized using 3-aminobenzoic acid methylester for all handling and dissection procedures. Care was taken to standardize all handling and sample protocols. Goldfish were sacrificed by spinal transection and 3 hypothalami were pooled for a total n=6. Samples were rapidly dissected and immediately frozen on dry ice. At the time of sampling, body weights and gonadal weights were recorded and the gonadosomatic index (GSI; body weight/gonad weight x 100) was calculated.

3.2.5 RNA extraction, quality assessment and cDNA synthesis

RNA was isolated using the RNeasy Plus Mini kit (Qiagen) as described in the manufacturer's protocol. Briefly, brain samples were homogenized using Tungsten beads before using the RNeasy Plus Mini kit. Upon purification, concentration and quality of all samples was assessed using the 2100 Bioanalyzer (Agilent). RIN values of RNA samples ranged from 7 to 9.5, which is above the recommended cut-off value of 5 for quantitative real-time RT PCR applications (Fleige and Pfaffl, 2006). Total cDNA was prepared from 1μg total RNA and 200 ng random hexamer primers (Invitrogen) using Superscript II RNase H- reverse transcriptase (SSII) as described by the manufacturer (Invitrogen). Each 20μl reaction was diluted 10-fold in nuclease-free water and used as the template for the real-time RT-PCR assays. All brain cDNA samples from different seasonal time points were synthesized in parallel.

3.2.6 Real-time RT-PCR verification

SYBR green real-time RT-PCR assay was used to validate relative expression of four genes along the reproductive cycle. Primers (Table 5) were designed using Primer3 (You et al., 2008) and ordered from Invitrogen. Each real-time RT-PCR reaction contained the following final concentrations: 25 ng first strand cDNA template, 1× QPCR buffer, 3.5mM $MgCl_2$, optimized concentrations (150nM-300nM) of gene-specific primers, 0.25× SYBRGreen (Invitrogen), 200 μM dNTPs, 1.25U HotStarTaq (Invitrogen), and 100nM ROX reference dye, in a 25μL reaction volume. The thermal cycling parameters were an initial 1 cycle Taq activation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, optimized annealing temperature (58–60 °C) for 5 s, 72 °C for 30 s, and a detection step at 80 °C for 8 s. Dilutions of cDNA (1:10 to 1:31,250) from all samples were used to construct a relative standard curve for each primer set. After the reaction was complete, a dissociation curve was produced starting from 55 °C (+1 °C /30 s) to 95 °C. For each PCR reaction, negative controls were also introduced including a no template control (NTC) where RNase-free water was added to the reaction instead of the template (cDNA) and a no reverse transcriptase control (NRT) where RNase-free water was added to the cDNA synthesis reaction (previously described) instead of the SSII enzyme. The SYBR green assay for every target gene was optimized for primer concentration and annealing temperature to obtain a dRn value higher than 0.98, an amplification efficiency between 90%-110% and a single sequence-specific peak in the denaturation curve.

Data were analyzed using the MxPro software package. The relative standard curve method was used to calculate relative mRNA abundance between samples, which was then presented as means +SEM of gene expression from six biological replicates for each time point. One way ANOVA and Holm-Sidak post-hoc test in SigmaStat3.5 (SPSS Inc.) were used to evaluate significant changes in gene expression between different time points. All data were first tested for normality and those data with non-normal distribution were subjected to square root transformation previous to statistic analysis.

3.3 Results

3.3.1 Seasonal gene expression information can be retrieved from multiple female goldfish microarray datasets

Experimental microarray data from the AURATUS goldfish environmental genomics project were used to define seasonal gene expression information (Table 4). Datasets can be divided into three seasonal time points (May, August, and December), which correspond to the distinct physiological periods of sexually mature prespawning, sexually regressed, and early redevelopment of goldfish ovary, respectively.

The global transcriptomic relationships among slides were evaluated by principal component analysis (PCA). The results of the PCA are visualized in a two-dimensional plot. This sub-space captures the highest amount of total variability. The points in the plots represent the global transcriptomes of the different slides. The transcriptomes that are clustered together are considered overall to be similar, while more dissimilar transcriptomes are further apart. As shown in Figure 10, although from different experiments, global transcriptomes of the Hyp slides from a given season are closely clustered together. This indicates that our normalization procedure was effective in identifying the season-dependent gene expression information in these microarray datasets.

The normalized data sets were further used to identify differentially expressed genes in female hypothalamus across the reproductive seasonal cycle. A total of 883 unique genes were identified, including 662 genes whose function is at least partially characterized and 221 unidentified EST sequences. In summary, the identified differentially expressed genes fall into four gene expression clusters comprising H-L-L, H-H-L, L-H-H and L-L-H (L, relatively low expression and H, relatively high expression) patterns along the seasonal cycle (May-August-December) (Appendix B). Most of the identified genes belong to H-L-L and L-H-H expression clusters.

3.3.2 The identified gene expression patterns are regulated by photoperiod

Photoperiod is a major environmental factor that governs fish reproductive seasonality (Duston and Bromage, 1987; Blazquez et al., 1998; Bayarri et al., 2004). Photoperiodic manipulations have been used to control the egg production and fertilization success of diverse fish such as goldfish (Hontela and Peter, 1983; Razani et al., 1987), rainbow trout (*Salmo gairdneri*) (Bromage et al., 1982), Japanese medaka (*Oryzias latipes*)

(Koger et al., 1999) and European sea bass (*Dicentrarchus labrax L.*) (Bayarri et al., 2004). Therefore, it can be hypothesized that photoperiod regulates the identified gene signature between seasons. One dataset provides a unique opportunity to clarify this question in which Hyp brain samples were taken from fish in October but acclimated to a long-daylength photoperiod (16h), typical of the springtime breeding season in May (Figure 11a). The prediction is that if the identified gene expression signature is photo-responsive, these genes in 16h-October fish should have similar expression patterns to fish in May acclimated for 3 weeks under our seasonally varying daylength protocol and sampled at 15.8h light-length.

We first examined the relationship of the transcriptomes between the 16h-October Hyp samples and the other three normal Hyp samples using PCA. As shown in Figure 11b, the points of 16h-October slides are closely clustered with May slides, indicating similar global transcriptome status between May and 16h-October. We also compared the gene expression relationship of the identified differentially expressed genes in these four cases. As shown in Figure 11c, the expression of these 883 genes highly resembles that observed in May. Moreover, the gene expression patterns are reversed in 16h-October compared to other seasons August and December. When we combined this dataset for a re-analysis to identify differential genes between seasons, we obtained a very similar gene list and gene patterns (data not shown). Therefore, we concluded that the identified differential expression patterns of these genes are likely regulated by photoperiod.

3.3.3 Gene ontology analysis of differentially expressed genes

We have shown that a core set of genes were differentially expressed between seasons in both Hyp and Tel regions and they are photoperiod-responsive. We hypothesize that those genes should be related to neuroendocrine regulation of the fish reproductive cyclicity. We utilized GO term analysis to explore such potential functional significance among these genes. A series of GO categories implicated in neuroendocrine function are significantly over-represented for the genes of the H-L-L pattern. Figure 12 shows the primary GO categories with member genes, some of which can be found in multiple categories. Importantly, many genes have been functionally assigned to hormone activity, including two GnRH forms (salmon GnRH and chicken GnRH-II), growth hormone (GH), isotocin, LH, tachykinin, NPY, pituitary adenlyate cyclase activating polypeptide 1 beta

(PACAP 1b). Most of them, especially the GnRHs, are well-known and central to neuroendocrine control of reproduction and growth. Another identified molecular function is GABA-A receptor activity. Four subunits, GABA-A alpha1, beta4, gamma1 and gamma2, were highly expressed in May. The GABA-A receptor is a pentameric protein that is ligand-gated Cl^- ion channel whose activation by GABA or the specific GABA-A agonist muscimol results in rapid and sustained LH release in goldfish (Trudeau et al., 1993). Many other genes identified are involved in several biological processes including: transmission of nerve impulse, glutamine family amino acid metabolic process, G-protein coupled receptor (GPCR) signaling pathway and neuron differentiation. Some genes are shared between these biological process and molecular function categories, among which transmission of nerve impulse is a central core linking other categories. Successful identification of a series of neuroendocrine function-related genes by GO enrichment analysis supports the biological implication of gene expression change of the H-L-L gene pattern (May-August-December) in reproductive seasonality. Moreover, since the timing of major expression change corresponds to the transition from prespawning/spawning to sexually regressed female goldfish, the high expression status of these genes around May, therefore, should be required for the fish spawning period.

3.3.4 Seasonal goldfish brain sampling and real-time RT-PCR verifications

Following bioinformatics analysis, we collected goldfish brain samples at six seasonal time points for experimental verification of gene expression patterns. Gonadosomatic index (GSI), an indication of the sexual maturity of the fish, shows variation along the reproductive season and reaches a peak around May (Figure 13). The observed changes are comparable to previous studies in Japan (Sohn et al., 1999) and Canada (Trudeau et al., 1991). We used real-time RT-PCR to examine the seasonal expression profiles of four genes, GABA-A gamma2, calmodulin, ependymin II and aromatase b. Among these genes, GABA-A gamma2 and calmodulin are enriched in GO term analysis (Figure 12). GABA-A gamma2 is one subunit of GABA-A receptor complex; it is a representative of GABA system genes. Calmodulin is a calcium ion-binding protein and has diverse regulatory roles in L-type calcium channel, signal transduction, synthesis and release of neurotransmitters (Zhang et al., 2004a), and transcriptional action of estrogen receptors (Li et al., 2003). Ependymin II is a

brain glycoprotein and is implicated in neuronal regeneration (Shashoua, 1991). Aromatase b is the brain type aromatase, a P450 family enzyme expressed exclusively in teleost radial glial cells (Forlano et al., 2001), and contributes to catalyzing the aromatization of testosterone (T) to 17beta-estradiol (E2). Although aromatase b is not identified by our meta-analysis, we are interested in it since both T and E2 have regulatory effect on the neuroendocrine system and the seasonal activity of aromatase b in fish brain is known to correlate significantly with seasonal reproductive cycles (Pasmanik and Callard, 1988).

As shown in Figure 14, seasonal expression profiles of these four genes in Hyp exhibit a clear increased expression in May. Significant differences ($P<0.05$; Holm-Sidak multiple test) can also be observed for the expression of most genes between May and other seasons. Therefore, these results strongly support the patterns found in our meta-analysis of microarray data with an apparent expression peak in May representing the prespawning season of the goldfish.

3.4 Discussion and conclusion

An emerging concept about neuroendocrine regulation of biological rhythms is the recognized importance of seasonal gene expression profiles in the neuroendocrine brain. Although several previous studies have attempted to define gene expression change associated with hibernation in whole squirrel brain (Williams et al., 2005), migration in sparrow telencephalon (Jones et al., 2008) or photo-response in hamster hypothalamus (Prendergast et al., 2002), no system-level study has been conducted to profile the global gene expression changes along a whole reproductive cycle. The purpose of this study was to extract and evaluate such seasonal gene expression characteristics from existing microarray datasets using the goldfish model. A core set of genes were differentially expressed between three examined seasons (May, August, and December). Moreover, I illustrated that artificially increasing daylength in October (16h) resulted in an expression profile in Hyp very similar to that of female goldfish in May (15.8h). This supports the hypothesis that photoperiod is a major environmental cue for vertebrate reproduction because gene expression profiles in the neuroendocrine brain were photoperiodically-regulated to some degree.

Most differential genes showed expression changes between May and August in H-L-L and L-H-H patterns. May and August correspond to fish reproductive status of the prespawning and sexual regression respectively, and histological (ovary) and serum hormone changes have been extensively described between these two stages (Munkittrick and Leatherland, 1983; Pasmanik and Callard, 1988; Trudeau et al., 1991). Thus, expression transition of this core gene set in the neuroendocrine brain may provide molecular basis for phenotype changes in serum hormone level and gonad status between seasons. Indeed, a series of GO categories related to neuroendocrine function and process are significantly overrepresented for the genes of the H-L-L pattern (Figure 12). Many associated genes have been shown directly involved in regulating brain function and seasonal reproduction, as exemplified by the neuropeptides GnRH, LH, isotocin, NPY, and CCK. Moreover, other biological processes have also been identified including glutamate/GABA systems, GPCR signaling pathway and transmission of nerve impulse. Although no GO category is significantly enriched for the genes of L-H-H pattern, we speculate that they may include some inhibitory factors for neuroendocrine function such as the dopamine system related genes. Other potential functions of these L-H-H genes may be involved in the growth phase of goldfish, which has shown different from reproductive phase (Trudeau, 1997). We further verified the seasonal expression profiles for four genes which represent GABA system (GABA-A gamma2), E2 system (aromatase b), calcium binding proteins (ependymin II and calmodulin) and GPCR signaling pathway (calmodulin). Their seasonal expression profiles all exhibit similar predicted patterns with the apparent peak around May, thereby suggesting that a seasonal expression change is employed by their represented systems (*e.g.* E2 and GABA) to coincide with fish reproductive development.

Our study may serve as a basis for understanding reproductive seasonality of other fish species. Seasonal patterns in serum levels of reproductive hormones has been documented in many teleost species (*e.g.*, Florida gar (*Lepisosteus platyrhincus*) (Orlando et al., 2007), masu salmon (*Onchorhynchus masou*) (Westring et al., 2008), and European sea bass (Gonzalez and Piferrer, 2003)), including the goldfish (Sohn et al., 1999). It can be hypothesized that although different fish species have their own reproductive strategies and particular seasonal pattern, they may share one core set of genes with an increased expression prior to spawning period. Indeed, similar increased expression pattern of several

neuroendocrine genes has been observed in other fish species. For instance, Okuzawa and colleagues (Okuzawa et al., 2003) have shown that GnRHs exhibit a similar seasonal expression pattern in the brain of female red seabream (*Pagrus major*) with an expression peak around April, correlating with high steroid level and mature gonad state. Increased expression of GnRHs can also be found in the turbot (*Scophthalmus maximus*) (Andersson et al., 2001), in parallel with an increase in oocyte diameter. Aromatase is another example; in the midshipman fish (*Porichthys notatus*), aromatase b in the magnocellular preoptic area of brain showed higher expression in the pre-nesting period when compared to nesting and non-reproductive periods (Forlano and Bass, 2005). Moreover, in both red-spotted grouper (*Epinephelus akaara*) (Li et al., 2007) and in the European sea bass (Gonzalez and Piferrer, 2003), aromatase b showed enhanced activity during their own prespawning periods, which are corresponding to April and January, respectively.

Overall, using both bioinformatic and experimental strategies, we defined a core gene set whose expression exhibits a seasonal change along fish reproductive cycle. We illustrated that the gene expression change is primarily regulated by photoperiod. Moreover, most genes undergo an expression transition between May and August, which corresponds to the transition from prespawning to sexual regression. Thus the gene expression patterns may account for dynamic neuroendocrine regulation on fish seasonal reproductive development and the increased expression of these neuroendocrine related genes in May is crucial for the ensuing spawning period. This is all the more critical in species with a short breeding period or where seasonally timed spawning occurs only once in the year.

Table 4. Detailed information about the experimental design, brain tissue, treatment seasonal time and other information

Month	Sex	Brain	Chemical	Sampling time	Gonad state
May	Female	Tel	MPTP-MPT	22-May-04	Prespawning
May	Female	Hyp	MPTP-MPT	23-May-04	Prespawning
May	Female	Hyp	D1R agonist, SKF 38393	May 10, 2006	Prespawning
May	Female	Tel	D1R agonist, SKF 38393	May 10, 2006	Prespawning
May	Female	Tel	D2R agonist, quinpirole	May 10, 2006	Prespawning
May	Female	Hyp	D2R agonist, quinpirole	May 10, 2006	Prespawning
August	Female	Tel	GABAA agonist, muscimol	late Aug. 2004	Intact, sexually regressed
August	Female	Hyp	GABAA agonist, muscimol	late Aug. 2004	Intact, sexually regressed
August	Female	Hyp	GABAB agonist, Baclofen	early Sep, 2004	Intact, sexually regressed
August	Female	Tel	GABAB agonist, Baclofen	early Sep, 2004	Intact, sexually regressed
October	Female	Hyp	MeHg	Oct, 2004	Early redevelopment
December	Female	Hyp	Fluoxetine	12-Dec-04	Early redevelopment

Table 5. Oligonucleotide primers used for real-time RT-PCR assays

Gene target	GeneBank ID	Sequence 5'-3'	Amplicon size
Ependymin II	J04986	F: TGATGCGGAACAATGAAAGTG	258 bp
		R: TCAGACTCGTGAGTGGCATA	
GABA-A gamma2	AY640227	F: AGCTGGCACTCTGCATCAA	173 bp
		R: CTGCGTCTCAACAGCAACA	
Calmodulin	AY656699	F: CATTTCCATCAGCGTCCA	108 bp
		R: GCACCATCACGACCAAAGA	
Aromatase b	AB009335	F: TGCTGACATAAGGGCAATGA	153 bp
		R: GGAAGTAAAATGGGTTGTGGA	

Figure 10. Two-dimensional PCA plot for transcriptomes from Hyp slides in three seasonal time points (May, August, and December)

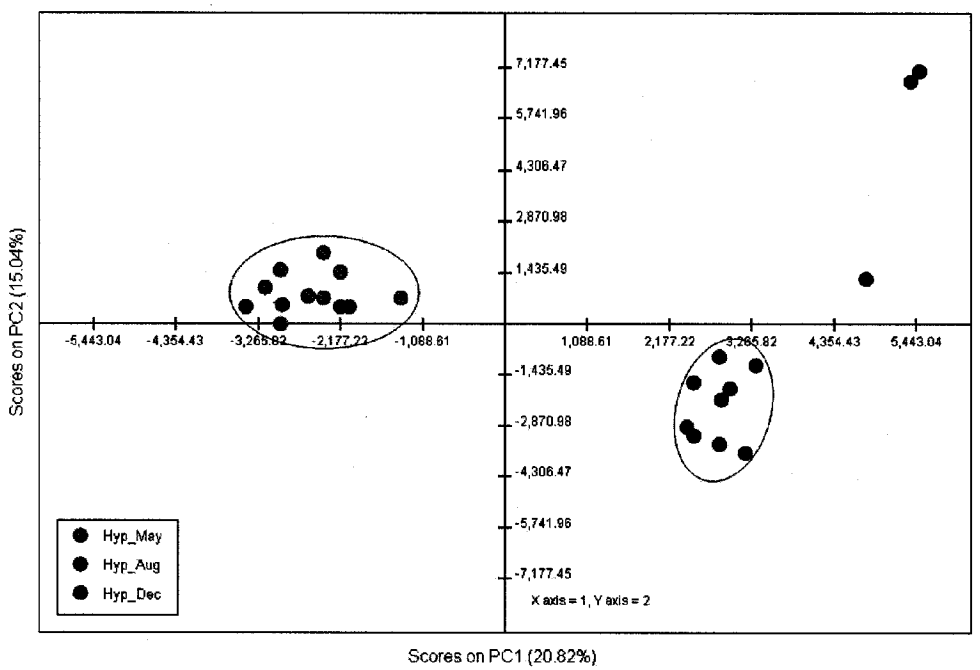


Figure 11. Regulation of brain gene expression patterns by photoperiod

(a) Seasonal profile of the daylength in Ottawa, Canada (45°27'N, 75°42'W). Pink, blue, grey and red boxes indicate daylength in May, August, October and December, respectively. The light blue box indicates a 16-hour daylength that we used for one fish group in October. Green bar indicates breeding season for goldfish typical in temperate North America. (b) Two-dimensional PCA plot for transcriptomes from Hyp slides in four seasonal time points (May, August, 16h-October, and December). (c) Hierarchical clustering of expression profiles of significantly differentially expressed genes in Hyp slides along four seasonal time points.

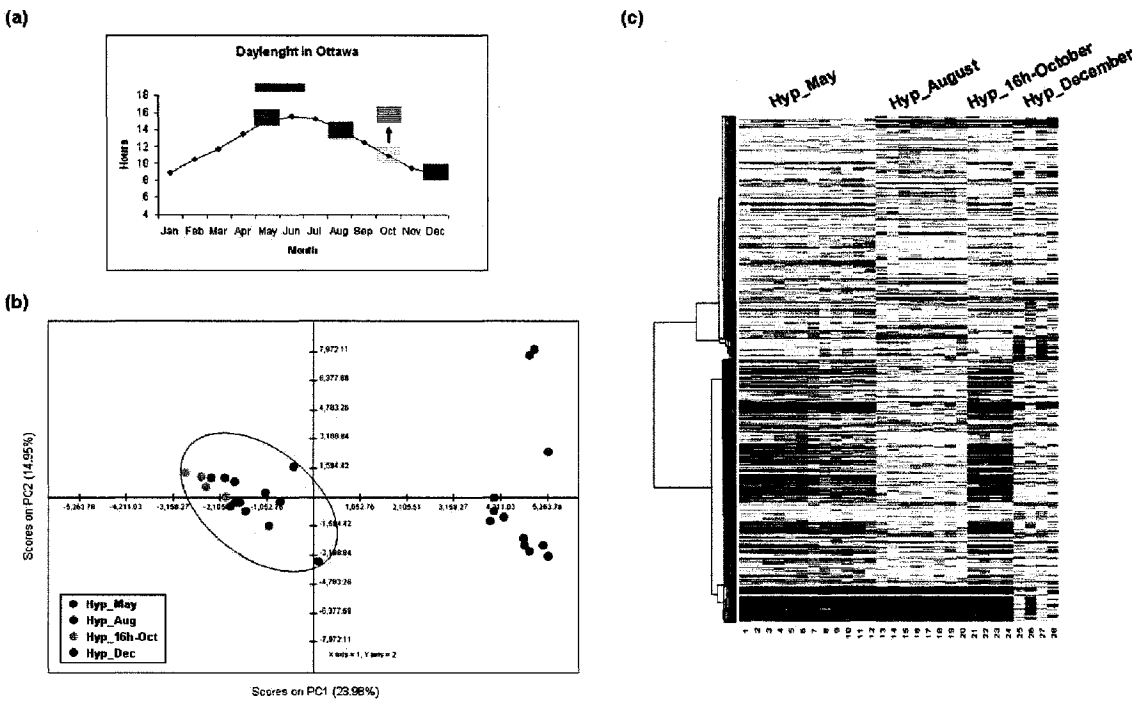


Figure 12. The neuroendocrine genes which are significantly enriched in the H-L-L expression pattern and their associated Gene Ontology categories

Gene abbreviations: Calm, calmodulin; Camk2d, calcium/calmodulin-dependent protein kinase (CaM kinase) II delta; Car-D, carnosine dipeptidase 1; CCK, cholecystokinin; cGnRH-II, chicken gonadotropin-releasing hormone II; Ckb, creatine kinase, brain; GABA-A a1, b4, g1 and g2, GABA-A receptor alpha1, beta4, gamma1 and gamma2; GABA:Na, GABA:Na symporter; GAD3, glutamate decarboxylase isoform 3; GH, growth hormone; GLP1R, Glucagon-like peptide-1 receptor; GluL a, glutamate-ammonia ligase alpha; GluR2, glutamate receptor, metabotropic 2; HMP19, neuron specific gene family member 2 (protein p19); LH, luteinizing hormone; NPY, Neuropeptide Y; NSF, n-ethylmaleimide-sensitive factor; PACAP 1b, pituitary adenlyate cyclase activating polypeptide 1 beta; PACAP 1R, PACAP type 1 receptor; Pro-D, Proline dehydrogenase; Rab14, member 14 of RAS oncogene family; sGnRH, salmon gonadotropin-releasing hormone; Synuclein b, synuclein beta; TAU, microtubule-associated protein tau; UchL1, ubiquitin carboxyl-terminal esterase L1 (also called ubiquitin thiolesterase); Vdac1, voltage-dependent anion channel 1; YWHAG, 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide 2.

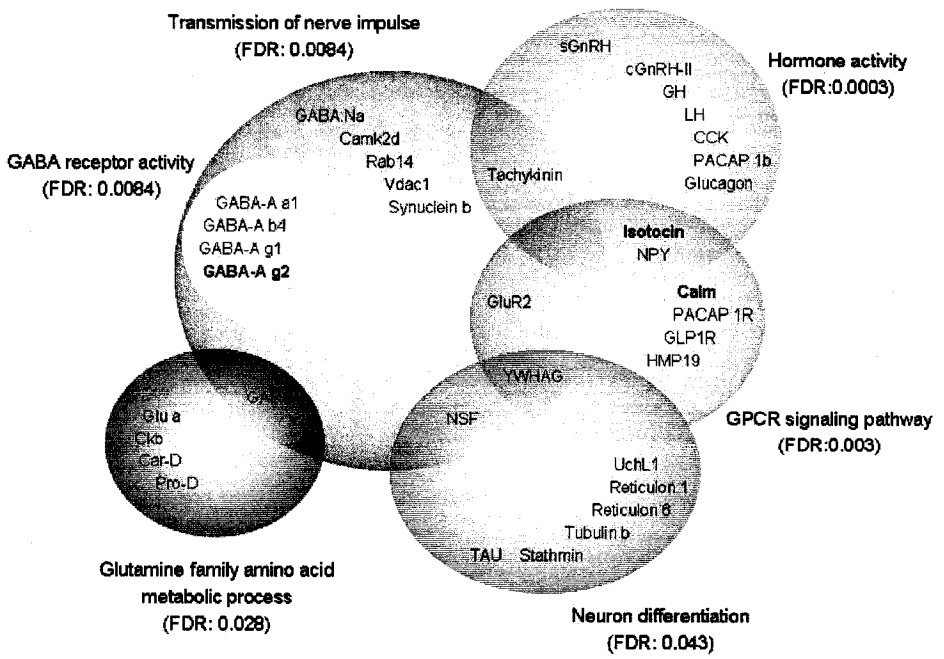


Figure 13. Seasonal GSI values of female goldfish

The grey bar indicates the typical breeding period for goldfish in temperate North America.

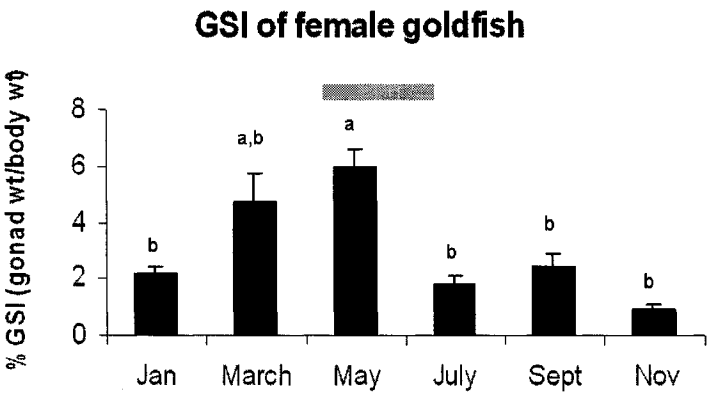
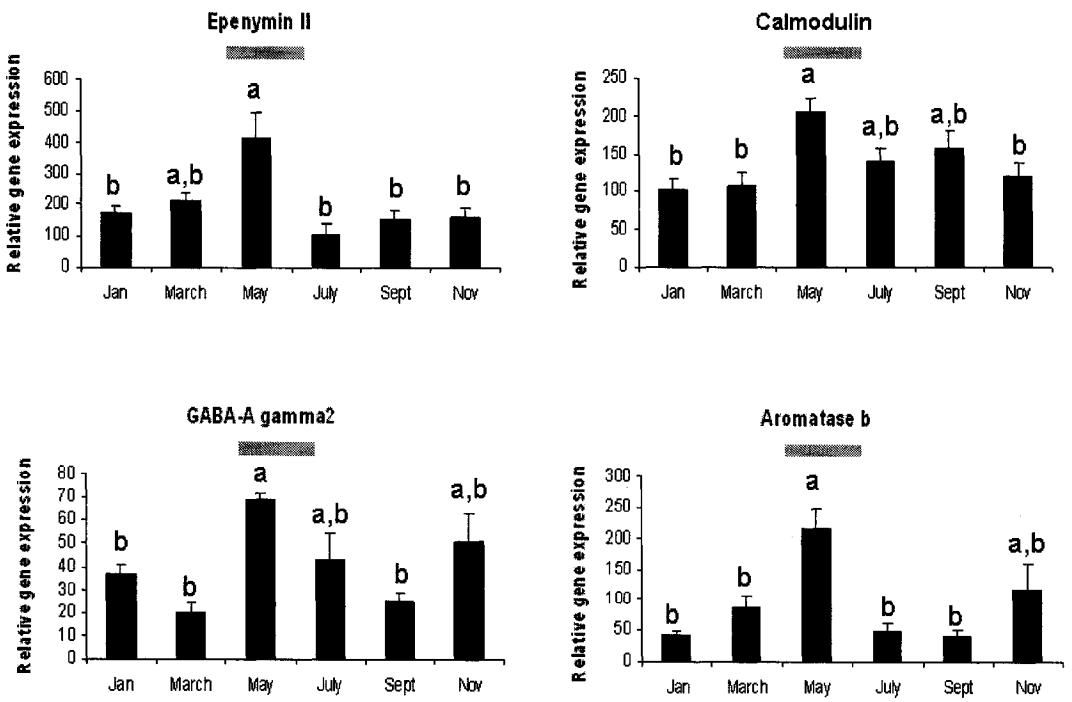


Figure 14. Relative gene expression of neuroendocrine genes in female goldfish hypothalamus along a whole seasonal cycle

Mean+SEM values are shown for each group (n=3–6). The grey bar indicates the typical breeding period for goldfish in temperate North America.



CHAPTER 4: HORMONE REGULATION OF GONADAL DEVELOPMENT VIA GERMLINE PIRNA PATHWAY AND BIOINFORMATICS ANALYSIS OF MAELSTROM FUNCTION

Abstract

This chapter is driven by the evidence that ovary maturation of female goldfish was delayed by fadrozole treatment (Chapter 2). Similar effects have been extensively documented in both amphibians and teleost fish, where exposure to exogenous steroid hormones like estrogen, androgen, xenoestrogen or steroid-biosynthesis inhibitors can impair gonadal development or induce sex reversal against genotypic sex. However, the molecular mechanism is still unknown. Here I hypothesize that these chemicals affect gonadal development and sex differentiation by regulating germline cell development. Maelstrom (MAEL) is one key protein functionally involved in the piRNA pathway, which has been recently illustrated to be the determinant of the development of germline cells. However, we could not retrieve any MAEL fish homologue from any published teleost fish genome. Thus we utilized the frog model to investigate whether fadrozole and finasteride treatments can affect MAEL expression (Part 1). Moreover, the loss of MAEL in the fish lineage promotes us to infer about the unique evolutionary history of MAEL and its putative function (Part 2). We found that in contrast to the loss in fish species, MAEL exhibits ancient lineage-specific expansions in some insects, Ciona and some protists. Further fold recognition analysis in conjunction with structure modeling defined a distant similarity of MAEL domains to a DnaQ-H exonuclease family in which ancestral protist homologues bear a DnaQ-H active site with the DnaQ-H characteristic residues, DEDHD. This evolutionary link suggests that MAEL domains may have a potential nuclease activity or RNA-binding ability that may be implicated in piRNA biogenesis.

Part 4.1: Fadrozole and finasteride exposures affect the mRNA expression of Maelstrom, a germline piRNA pathway protein, during the pipid frog (*Xenopus tropicalis*) embryogenesis

Abstract

It has been extensively documented that exposure to exogenous steroid hormones like estrogen, androgen, xenoestrogen or steroid-biosynthesis inhibitors in amphibians or teleost fish can impair their gonadal development or induce sex reversal against genotypic sex. However, molecular pathways underlying sexual development and effects of steroids or endocrine disrupting chemicals in these aquatic animals remain elusive. Recently, a novel small RNA pathway was discovered in gonad which utilizes a set of piwi-interacting RNAs (piRNAs) and a series of germ plasm-specific proteins including Maelstrom (MAEL) to regulate genome stability via silencing certain endogenous genetic elements such as retrotransposons and repetitive sequences. This piRNA pathway has been found to be the determinant of the development of germline cells, the precursors of either sperms or eggs. The aim of the present study is to examine whether sex steroid hormones can regulate gonadal sex differentiation via this pathway. We exposed embryos of the pipid frog (*Silurana (Xenopus) tropicalis*) to two steroid-biosynthesis inhibitors fadrozole (0.5, 1.0, 2.0 μM) and finasteride (25, 50, 100 μM). Fadrozole specifically inhibits the aromatase enzyme which catalyzes aromatization of testosterone (T) to 17beta-estradiol, whereas finasteride inhibits both 5-alpha and 5-beta reductases that are responsible for converting T to 5αDHT, and metabolizing T to the relatively inactive form 5βDHT (Figure 15). Both treatments result in male-biased or female-biased phenotypes for tadpoles. The MAEL mRNA expression change was evaluated using real-time RT-PCR and we found that MAEL was significantly increased by either fadrozole or finasteride treatments at high concentrations. These results indicate that the piRNA pathway is regulated by sex steroids and represents a mechanism by which exogenous sex steroids and endocrine disrupting chemicals (EDCs) disrupt gonadal development or induce sex reversal.

4.1.1 Introduction

Gonadal development involves the differentiation of both gonad somatic cells and germline cells. In this process, germline cells specifically differentiate into sperm (spermatogenesis) and eggs (oogenesis) in the gonad whereas somatic cells differentiate into interstitial gonadal tissue (Hayes, 1998; Nakamura et al., 1998). Sex steroid hormones including estrogen (e.g. 17beta-estradiol, E2) and androgen (e.g. testosterone, T and 5-alpha dihydrotestosterone, 5αDHT) exert critical roles in regulating gonadal sex differentiation, which has been illustrated in both teleost fish and amphibian models (Hayes, 1998; Nakamura et al., 1998). In different fish species, an increase in sex steroid hormones before and during spawning is commonly observed (see Chapter 3). Abnormal disruptions of endogenous steroid production using inhibitors of the synthesizing enzymes have been found to induce a delay in gonad development (Ankley et al., 2002), gonadal intersex phenotype (Bhandari et al., 2004a) or even sex reversal (masculinisation or feminization) in different fish species (Guiguen et al., 1999; Kwon et al., 2000; Zerulla et al., 2002; Bhandari et al., 2004b; Fenske and Segner, 2004). Exposure to exogenous steroid hormones (E2, T) and estrogenic or androgenic chemicals (17alpha-methyltestosterone, MT; 17alpha-ethinylestradiol, EE2) which are found in the environment can also result in similar sexual disruptions (Zerulla et al., 2002; Brion et al., 2004; Fenske and Segner, 2004; Lee et al., 2004; Nash et al., 2004; Navarro-Martin et al., 2009). Studies using amphibian models provide important support for the effects of sex steroid hormones on sexual differentiation. It has been extensively demonstrated that exposure of different amphibians with steroids E2, T (Saidapur et al., 2001), estrogenic or androgenic chemicals (Levy et al., 2004; Pettersson et al., 2006; Hogan et al., 2008) or aromatase inhibitors (Hayes et al., 2006; Olmstead et al., 2008) can alter gonadal differentiation and result in the presence of intersex phenotypes and female or male-biased sex ratio.

Although hormone regulation of gonadal development and sex reversal by exogenous hormones have been well-recognized, the underlying molecular mechanisms still remain elusive. Considering that sex reversal requires the regeneration or reprogramming of germline cells which differentiate into either sperm or eggs, we hypothesize that steroid hormones including E2 could also affect their development. Several lines of evidence indicate the potential action of E2 in germline cells. In aromatase deficient mouse, E2

depletion causes severe disruption of early spermatogenesis (Murata et al., 2002). Both aromatase and estrogen receptors are expressed in human germline cells (Lambard et al., 2004). Moreover, a recent paper showed that E2 significantly promoted DNA synthesis in ovarian germ cells in two teleost fish, Japanese huchen (*Hucho perryi*) and common carp (*Cyprinus carpio*) (Miura et al., 2007). However, less is known about the direct targets of E2 in germline cells.

One common characteristic of germline cells among different species is the presence of a morphologically unique organelle called the germ plasm (Ikenishi, 1998; Parvinen, 2005). Depending on the developmental stage and the species, this organelle is also referred to as nuage, pole plasm, or polar granules in *Drosophila*, perinuclear P granules in *Caenorhabditis elegans*, dense bodies, mitochondrial cloud or germinal granules in *Xenopus*, and chromatoid body in mammals. This organelle has been considered the determinant of germline development. Only cells inheriting the germ plasm in the early stage of embryogenesis can develop into primordial germ cells (PGC) or the progenitors of germ cells (Ikenishi, 1998). Transplantation of the germ plasm to the presumptive somatic region results in PGC formation (Illmensee and Mahowald, 1974). Very recently, a germ plasm-specific small RNA pathway has been identified, in which a new type of PIWI-interacting RNAs (piRNAs) and a series of germline specific proteins including Vasa, Spindle-E, Maelstrom (MAEL) are utilized to regulate germline genomic stability of germline cells by silencing certain endogenous genetic elements such as retrotransposons and repetitive sequences (Vagin et al., 2006; Klattenhoff and Theurkauf, 2008). Given the importance of this piRNA pathway and the germ plasm in germline development, we hypothesize that the piRNA pathway and its associated proteins may be direct targets of sex steroid hormones. In the present study we tested this hypothesis by examining the expression of one piRNA pathway protein MAEL in embryos of the pipid frog *Silurana (Xenopus) tropicalis* exposed to two steroid-biosynthesis inhibitors fadrozole (0.5, 1.0, 2.0 μM) and finasteride (25, 50, 100 μM) (Langlois et al., 2009). The reason why I chose MAEL as a target is that based on sequence analysis and phylogenetic analysis, there is only one copy for MAEL in most species, especially in vertebrates (Chapter 4.2). This indicates that MAEL may be a unique representative for piRNA pathway.

4.1.2 Materials and methods

4.1.2.1 Animals, chemical exposure and RNA samples

The mRNA samples used in this study are from another study in our lab (Langlois et al., 2009) in which embryos of *Silurana (Xenopus) tropicalis* were exposed to fadrozole (0.5, 1.0, 2.0 μM; Novartis) dissolved in water and finasteride (25, 50, 100 μM; Sigma) delivered in ethanol (0.05 %) from Nieuwkoop Faber (NF) stage 12 (11 hours post fertilization (hpf), gastrulation) until NF 46 (72 hpf, beginning of gonadal development) (Nieuwkoop and Faber, 1994). Whole NF 46 larvae were sampled from each treatment by pooling 10 larvae per sample. Total RNA was isolated by using RNase Mini Kit (Qiagen, Mississauga, ON, Canada) as described in the manufacturer's protocol. Total cDNA was prepared from 2μg total RNA and 200 ng random hexamer primers (Invitrogen) using Superscript II RNase H-reverse transcriptase (SSII) as described by the manufacturer (Invitrogen). The detailed description of the experimental design and exposure can be found in the method section of (Langlois et al., 2009).

4.1.2.2 Real-time RT-PCR

SYBR green real-time RT-PCR assay was used to measure the relative expression of MAEL and the ribosomal protein L8 in the different treatment groups. Primers of MAEL (F: ATCCCCATCTCTGGCTTTG; R: GGTTTTCCATTCCTGCTTTG) and L8 (F: CCCTCAACCATCAGGAGAGA; R: TCTTTGTACCACGCAGACGA) were designed using Primer3 (You et al., 2008) and optimized to get reaction efficiencies between 90-110%. Each real-time RT-PCR reaction contained the following final concentrations: 25 ng first strand cDNA template, 1× QPCR buffer, 3.5mM $MgCl_2$, 150nM gene-specific primer (Invitrogen), 0.25× SYBRGreen (Invitrogen), 200 μM dNTPs, 1.25U HotStarTaq (Invitrogen), and 100nM ROX reference dye, in a 25μL reaction volume. The thermal cycling parameters were an initial 1 cycle Taq activation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15s, 59 °C (MAEL) or 62 °C (L8) for 5s, 72 °C for 30s, and a detection step at 80 °C for 8s. Dilutions of cDNA (1:10 to 1:31,250) from a mix of all samples were used to construct a relative standard curve for each primer set. After the reaction was complete, a dissociation curve was produced starting from 55 °C (+1 °C /30s) to 95 °C to

confirm the presence of a single amplicon. Data were analyzed using the MxPro software package. The standard curve was used to calculate the mRNA level in between samples.

4.1.2.3 Data analysis

The relative mRNA level of MAEL was normalized to that of L8. Data are presented as means +SEM of fold changes in mRNA relative to control which is set to 1. One-way ANOVA and Holm-Sidak post-hoc test in SigmaStat3.5 were used to evaluate significant differences in gene expression. All data was first tested for normality and those data with non-normal distribution were subjected to square root transformation prior to statistical analysis.

4.1.3 Result and discussion

4.1.3.1 Both fadrozole and finasteride treatments increase the mRNA expression of MAEL during embryogenesis

Figure 16 shows the relative mRNA expression of MAEL in embryos following fadrozole or finasteride exposures. There is no significant difference between control samples and treated samples with low (0.5 μM) or moderate (1.0 μM) concentrations of fadozole (Figure 16a). However, the highest concentration (2.0 μM) of fadrozole caused a 1.9-fold increase in mRNA level of MAEL ($p<0.05$). A similar gene expression profile can also be observed in the finasteride treated samples. In contrast to the low (25 μM) and moderate (50 μM) concentrations of finasteride, the high (100 μM) treatment induced a 3.2-fold increase in MAEL mRNA level ($p<0.05$) (Figure 16b).

4.1.3.2 A piRNA pathway may mediate steroid hormone regulation of gonadal sex differentiation

Fadrozole is a pharmaceutical compound that competitively inhibits the activity of aromatase which catalyzes the aromatization of testosterone to 17beta-estradiol. Fadrozole exposure results in decreased serum E2 level, partial sexual reversal or complete masculinization in frogs (Olmstead et al., 2008) and teleost fish (Kwon et al., 2000; Ankley et al., 2002; Zerulla et al., 2002; Bhandari et al., 2004b; Fenske and Segner, 2004). A

disruption of gonadal development was also observed later in tadpole development after exposure to fadrozole in *S. tropicalis* (Langlois et al., 2009). Chronic exposure from NF 12 until NF 60 (metamorphic climax) to fadrozole (2.0 μM) shifted the sex ratio: increasing the number of males and the incidence of intersex individuals compared to the control group. The current study showed that inhibition of aromatase by fadrozole can increase the MAEL mRNA level. Interestingly, we also found that an increase in MAEL mRNA in embryo samples treated with finasteride. Finasteride inhibits both 5-alpha and 5-beta reductases that are responsible for converting T to 5αDHT, and metabolizing T to the relatively inactive form 5βDHT. Histological study has shown that chronic exposure to finasteride in *S. tropicalis* resulted in a partial sex reversal toward the female phenotype and the presence of intersex frogs (Langlois et al., 2009).

MAEL is one piRNA pathway protein and our bioinformatics analysis has predicted it to be a 3'-5' exonuclease or RNA-binding protein whose activity may be implicated in piRNA synthesis (Part 2 of this chapter). In addition to MAEL (Clegg et al., 1997; Findley et al., 2003; Lim and Kai, 2007), the piRNA pathway proteins include VASA (Lim and Kai, 2007), SPN-E (Clegg et al., 1997), Armitage (Cook et al., 2004), Krimper (Lim and Kai, 2007), Dead end (Weidinger et al., 2003) and Zucchini and Squash (Pane et al., 2007). Including MAEL, all of these proteins are believed to functionally cooperate in piRNA synthesis and function since loss-of-function mutations of one protein causes a common effect with a huge reduction in the amount of piRNAs or rasiRNAs and an increase in transcript level of transposable elements in the germline cells (Lim and Kai, 2007). Moreover, these proteins have a hierarchical relationship during germ plasm assembly. Their germ plasm-specific locations strictly follow a SPN-E-VASA-Aubergine/Tudor-Krimper-MAEL-Dicer/Argonaute2 chain, in which the correct location of an upstream protein in germ plasm facilitates the location of the downstream proteins in the cascade (Findley et al., 2003; Lim and Kai, 2007). Therefore, the increase in the expression of MAEL in our study may indicate an effective activation of the piRNA pathway. Furthermore, similar induction of MAEL by both fadrozole and finaseride, which induce opposite sex phenotypes, indicates that the piRNA activation is necessary not only for estrogenic hormone-induced change, but also for androgenic hormone-induced change. In other words, piRNA pathway is likely a common pathway by which different steroid hormones regulate gonadal sex differentiation.

Figure 15. A schematic showing the inhibiton of fadrozole and finasteride on the biosynthesis of estradiol and dihydrotestosterone from testosterone in vertebrates which are catalyzed by aromatase and 5-alpha and 5-beta reductases

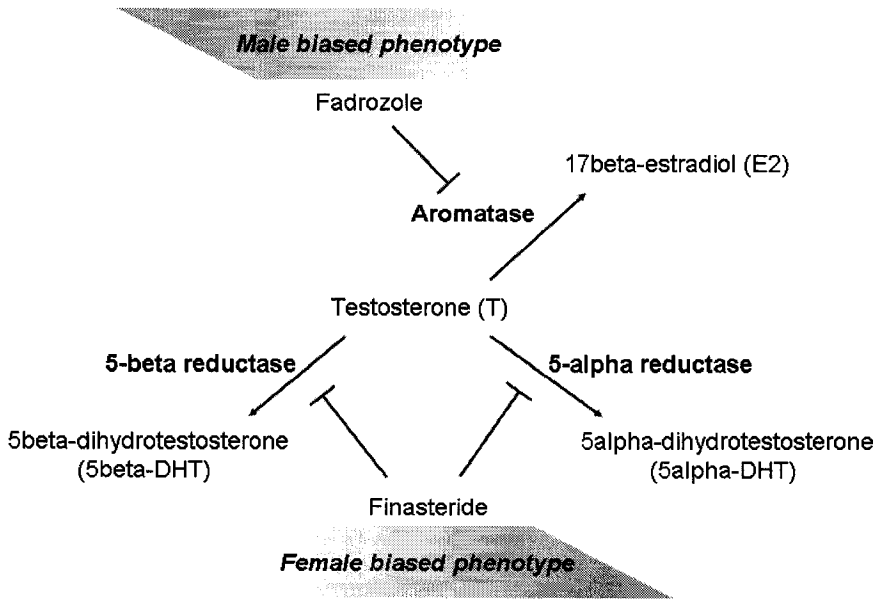
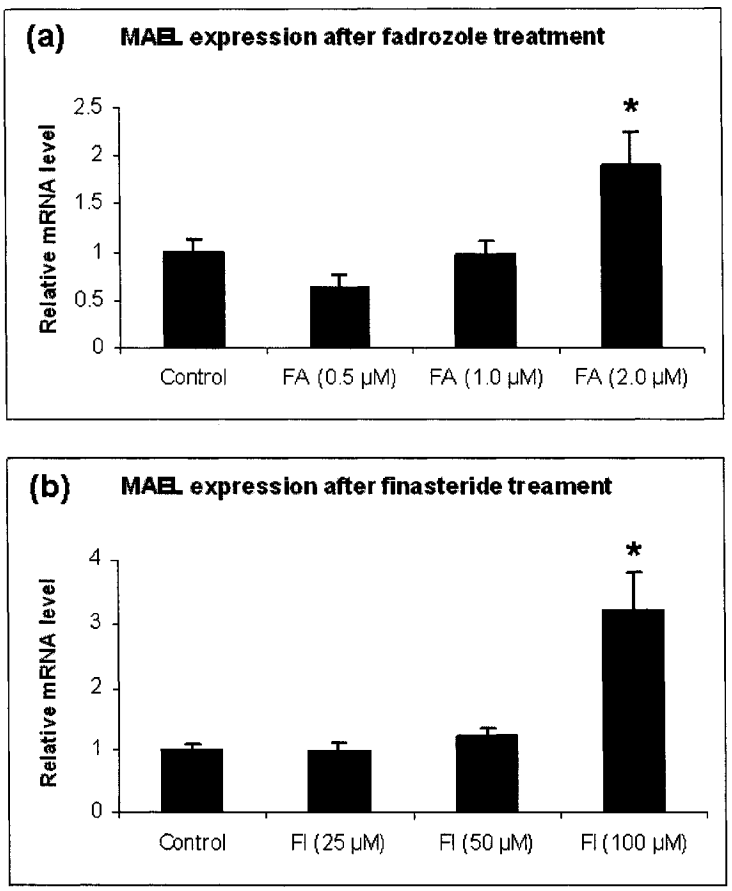


Figure 16. Relative mRNA level of MAEL in NF stage 46 embryos of the pipid frog exposed to fadrozole (FA) (a) and finasteride (FI) (b)

Bars represent the mean +SEM of 4-8 pools of 10 embryos by treatment. Asteriks (*) indicate statistically significant differences between treatments and control (one-way ANOVA, Holm-Sidak post-hoc test, $p<0.05$).



Part 4.2: Bioinfomatics analysis reveals new insight into Maelstrom function in the germline piRNA pathway

Abstract

Maelstrom (MAEL) plays a crucial role in a recently-discovered piRNA pathway; its expression has been shown to be regulated by both estrogen and testosterone. However its specific function remains unknown. Here a novel MAEL-specific domain characterized by a set of conserved residues (Glu-His-His-Cys-His-Cys, EHHCHC) was identified in a broad range of species including vertebrates, sea squirts, insects, nematodes, and protists. It exhibits ancient lineage-specific expansions in several species, however, appears to be lost in all examined teleost fish species. Functional involvement of MAEL domains in DNA- and RNA-related processes was further revealed by its association with HMG, SR-25-like and HDAC_interact domains. A distant similarity to the DnaQ-H 3'-5' exonuclease family with the RNase H fold was discovered based on the evidence that all MAEL domains adopt the canonical RNase H fold; and several protist MAEL domains contain the conserved 3'-5' exonuclease active site residues (Asp-Glu-Asp-His-Asp, DEDHD). This evolutionary link together with structural examinations leads to a hypothesis that MAEL domains may possess a potential nuclease activity or RNA-binding ability that may be implicated in piRNA biogenesis. The observed transition of two sets of characteristic residues between the ancestral DnaQ-H and the descendent MAEL domains suggests a new mode for protein function evolution called "active site switch", in which the protist MAEL homologues are the likely evolutionary intermediates due to harboring the specific characteristics of both 3'-5' exonuclease and MAEL domains.

4.2.1 Introduction

Germline cells among different species are characterized by the presence of a morphologically unique organelle called the germ plasm (also referred to as nuage, polar granules or mitochondrial cloud) (Ikenishi, 1998; Kotaja and Sassone-Corsi, 2007). This organelle has been considered the determinant of germline development. Very recently a

germ plasm-specific small RNA pathway has been identified, in which a new type of small RNAs called PIWI-interacting RNAs (piRNAs) or repeat-associated small interfering RNAs (rasiRNAs) play a role in ensuring the genomic stability of germline cells by silencing certain endogenous genetic elements such as retrotransposons and repetitive sequences (Aravin et al., 2003; Lau et al., 2006; Vagin et al., 2006; Aravin et al., 2007; Hartig et al., 2007; Klattenhoff and Theurkauf, 2008). Different from short interfering RNAs (siRNAs) and microRNAs which are usually 21-22nt long, piRNAs or rasiRNAs have longer nucleotide composition (26-31nt) and 2'O-methyl modification in 3' end. Many germ plasm proteins are functionally important in piRNAs synthesis and function, including PIWI proteins (PIWI, Aubergine and AGO3) (Vagin et al., 2006; Carmell et al., 2007; Houwing et al., 2007), VASA (Lim and Kai, 2007), MAEL (Lim and Kai, 2007), SPN-E (Clegg et al., 1997; Cook et al., 2004), Oskar (Jones and Macdonald, 2007), Tudor domain proteins (Chuma et al., 2006), Armitage (Cook et al., 2004), Krimper (Lim and Kai, 2007), Cutoff (Chen et al., 2007), Dead end (Weidinger et al., 2003) and Zucchini and Squash (Pane et al., 2007). Their loss-of-function mutations commonly cause a huge reduction in the amount of piRNAs or rasiRNAs and an increase in transcript level of transposable elements in the germline cells (Klenov et al., 2007; Lim and Kai, 2007; Nishida et al., 2007) as well as the spindle-class gene phenotypes: failure in establishing anterior/posterior polarity in early oocytes, disrupted asymmetric subcellular mRNA localization of Oskar, Gurken and Biocoid, ectopic expression of Oskar and Gurken, failure to proceed to the karyosome stage (Findley et al., 2003; Cook et al., 2004; Lim and Kai, 2007; Klattenhoff and Theurkauf, 2008).

The molecular functions of most germ plasm proteins in the piRNA pathway have been assigned based on domain examination, biochemical and genetic characterizations. For instance, PIWI proteins contain the PAZ and PIWI domains, which contribute to recognition of single-stranded RNA (Song et al., 2003) and sequence-specific endonucleolytic cleavage of target nucleotide (Parker et al., 2004; Song et al., 2004a), respectively. VASA, SPN-E and Armitage share DEAD RNA helicase domains, which provide helicase activities for piRNA production or retrotransposon silencing (Cook et al., 2004; Sengoku et al., 2006). Zucchini and Squash are putative nucleases, which are believed to be involved in piRNA maturation (Pane et al., 2007). Other Dead end, Krimper and Tudor proteins, contain RNA binding domains RRM (Maris et al., 2005) or Tudor (Ponting, 1997) which may facilitate the

assembly of multiprotein RNA-induced silencing complex (RISC) and targeting substrate RNA recognition during cleavage. In contrast, although many studies including specific knockouts, protein interaction and cellular distribution experiments have been conducted, the definitive function of MAEL in piRNA pathway remains unknown. MAEL was initially identified in a genetic loss-of-function *Drosophila* mutant, whose germline cells exhibit incorrect posterior localizations of several transcripts (*i.e.*, Gurken, Oskar and Bicoid) (Clegg et al., 1997). It is a germ plasm-specific protein with all spindle-class gene phenotypes (Clegg et al., 1997; Clegg et al., 2001; Findley et al., 2003; Cook et al., 2004) and directly involved in the piRNA pathway (Lim and Kai, 2007; Soper et al., 2008). The correct location of either SPN-E, VASA, Aubergine, Tudor or Krimper in germ plasm determines the location of MAEL (Lim and Kai, 2007), which in turn delineates the location of Dicer and Argonaute2 (Findley et al., 2003). MAEL can shuttle between germ plasm and the nucleus (Findley et al., 2003). Direct interaction between MAEL and chromatin remodeling proteins SNF5/INI1 and SIN3B during heterochromatin formation has also been demonstrated (Costa et al., 2006). Therefore, MAEL is the only known protein connecting germ plasm and the piRNA pathway to chromatin remodeling, a process required for piRNA-initiated genome transposon silencing (Robert et al., 2005). In the present study, I am motivated to understand the putative function of MAEL using combined bioinformatic strategies including extensive homologous sequence mining, phylogenetic analysis, domain architecture, protein fold recognition, and structure modeling.

4.2.2 Materials and methods

4.2.2.1 Database searching

The C-terminal sequences of MAEL from several species were used as queries for PSI-BLAST searches (Altschul et al., 1997) against the protein non-redundant (NR) database at National Center for Biotechnology Information (NCBI) with a profile inclusion expectation (*E*) value threshold of 0.005. A substitution matrix of BLOSUM62 and the gap penalty (existence: 11 and extension: 1) were utilized for scoring. The searches were iterated until convergence. Other homologous sequences were also identified through BLAST

searching in Ensembl database (Flicek et al., 2008) and TBLASTN searching in NCBI nucleotide/EST database with default parameters. Furthermore, for other protist homologues, we searched the database of the GeneDB project (Hertz-Fowler et al., 2004). A total of 47 homologous sequences have been collected in these searches by May 1, 2008. Additional homologous sequences can be retrieved from the NCBI database on July 2008 when we prepare this manuscript. However, they are not included in the current analysis since new sequences do not influence our result.

4.2.2.2 Sequence analysis

In order to construct the multiple alignment of MAEL domain sequences, we first utilized the Muscle (Edgar, 2004) and Promals (Pei et al., 2007) programs. The logomat-p program (Schuster-Bockler and Bateman, 2005) was also used to align sequence profiles of vertebrates, insects, nematodes, sea squirts and protists. Careful manual adjustments were conducted to avoid introducing gaps into the sequences where consensus secondary structures are occupied. The final alignment was colored using Chroma (Goodstadt and Ponting, 2001). The putative secondary structures for most MAEL domains were predicted by PSIPred program (McGuffin et al., 2000). A consensus-deriving secondary structure prediction program, SYMPred, was also utilized for some specific predictions (http://zeus.cs.vu.nl/programs/sympredwww/). In the SYMPred prediction, PSIPred (McGuffin et al., 2000), SSPro (Pollastri et al., 2002), YASPIN (Lin et al., 2005b), and PROFsec (Rost, unpublished) programs were considered and dynamic programming was used as a consensus deriving scheme. Domain composition was deduced by searching protein domain databases Pfam (Finn et al., 2008) and SMART (Letunic et al., 2006).

4.2.2.3 Phylogenetic inference

Based on the final multiple sequence alignment of MAEL domains, an unrooted phylogenetic tree was constructed with maximum likelihood (ML) analysis implemented in PhyML program (Guindon and Gascuel, 2003) and Bayesian analysis implemented in MrBayes 2.1 program (Huelsenbeck and Ronquist, 2001). The ML tree was determined under a Jones-Taylor-Thornton (JTT) model for amino acids substitution with a discrete gamma distribution (four categories), a proportion of invariant and an initial BIONJ tree. A

bootstrap analysis with 100 repetitions was performed to assess the significance of phylogenetic grouping. For Bayesian phylogenetic inference, firstly we used ProtTest 1.3 (Abascal et al., 2005) to determine the best fitting model of amino acid substitution for the data under the maximum likelihood assumption. A WAG model with a gamma distribution (four rate categories), a proportion of invariable sites, and observed amino acid frequencies (WAG+G4+I+F) turned out to be the best model and was utilized in Bayesian analysis subsequently. The Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling approach was used to calculate posterior probabilities. Four Markov chains were run 10,000,000 times. The chain was sampled every 100th generation, and burn-in values were determined from the likelihood values. The final unrooted tree diagram was generated using MEGA Tree Explorer (Kumar et al., 2008).

4.2.2.4 Fold recognition and structure modeling

Protein fold assignment was conducted using *meta* Server (http://meta.bioinfo.pl/), which assembles different state-of-the-art fold recognition programs including meta-BASIC (Ginalski et al., 2004), ORFeus-2 (Ginalski et al., 2003b), and FFAS03 (Jaroszewski et al., 2005) and further evaluates the modeled three-dimensional structures based on a consensus scoring computed by a 3D-JURY system (Ginalski et al., 2003a). The domain and structural fold annotation was then assigned for all the candidate hits by checking Pfam domain database (Finn et al., 2008) and the Structural Classification of Proteins (SCOP) database (Andreeva et al., 2008). Structure-based multiple sequence alignment was built using CE-MC server (Guda et al., 2004). The final sequence and secondary structure alignment of MAEL domains with several DnaQ-H domains was established carefully by hand on the basis of CE-MC results, alignment in fold recognition, published literature information, and predicted secondary structures. The final alignment was then used for structural homology modeling, which was performed via Modeller9v1 program based on multiple templates (Marti-Renom et al., 2000). Structural alignment was conducted by MultiProt server (Shatsky et al., 2004). Non-homologous regions were predicted by Loopy (Xiang et al., 2002). Disulfide bond prediction was conducted by an artificial neural network method (Ferre and Clote, 2006). Structural visualization and manipulations were performed using VMD program (Humphrey et al., 1996).

4.2.3 Results

4.2.3.1 A conserved MAEL-specific domain and its unique lineage-specific evolutionary expansion and loss

Domain annotation showed that mouse MAEL protein contains a HMG domain in its N-terminal segment, which is a DNA-binding module in many non-histone components and transcriptional regulators (Bianchi and Agresti, 2005). However, no domain information could be assigned for the C-terminal segments of MAEL proteins (240 amino acids long). We conducted homologous sequence searching for this region using PSI-BLAST against the NCBI NR database. Many unique homologues were identified in a broad range of species from veterbrates, echinoderms, insects, nematodes, to the protists (*Entamoeba histolytica*, *Entamoeba dispar*, and *Trypanosoma brucei*). We also examined NCBI nucleotide and Ensembl genome databases and identified eight other homologues in insects and urochordates (*Ciona intestinalis* and *Ciona savignyi*). Three more protist homologues were obtained through searching GeneDB database. A multiple sequence alignment was built for all the retrieved sequences (not shown) and a condensed one is shown in Figure 17. Although the overall sequence identity is very low, the conservation is apparent across all these MAEL homologues. Six residues Glu-His-His-Cys-His-Cys (EHHCHC) are highly conserved, suggesting that they may contribute to MAEL-specific activity. Thus the C-terminal segment appears to define a novel MAEL-specific domain that we now refer to as the MAEL domain.

For the majority of species, only one copy of MAEL domain exists. However, there are multiple MAEL homologues in several other species; for instance, two copies are found in sea squirts (*C. intestinalis* and *C. savignyi*) and mosquito (*A. aegypti*), three copies in *Culex pipiens*, and five copies each in amoeba *E. dispar* and *E. histolytica*. Phylogenetic tree construction suggests that multiple MAEL copies are generated from a series of ancient lineage-specific duplication events (Figure 18A). Strikingly, no fish MAEL homologues could be identified. Its absence in teleost fish was confirmed by carefully examining the published whole genome databases in Ensembl for five different species (*Danio rerio, Gasterosteus aculeatus*, *Oryzias latipes*, *Takifugu rubripes* and *Tetraodon nigroviridis*). It

can be inferred that it is the ancestor of the fish lineage after the divergence of teleost and tetrapod lineages that underwent the loss of MAEL domain. The timing of this loss is probably related to the ancient fish-specific genome duplication (Hoegg and Meyer, 2005).

4.2.3.2 Functional insight from domain architectures

Three other domains are associated with MAEL domains, including HMG (SMART: SM00398), HDAC_interact (SMART: SM00761), and SR-25-like domain (DUF1777, Pfam: PF08648) (Figure 18B). HMG is a common DNA-binding module in a variety of chromatin-associated proteins and functionally involved in the nucleoprotein complex assembly during genome recombination, initiation of transcription, and DNA repair (Bianchi and Agresti, 2005). The association between MAEL and HMG domains in most species suggests that the MAEL domain may somehow function in a DNA-related process. This functional assignment is also suggested by the association of MAEL domain with HDAC_interact domain in two homologues from mosquitoes (*A. aegypti* and *A. gambiae*). The HDAC_interact domain is known to bind to histone deacetylases (HDACs), core enzymes for removing acetyl group from lysine residue of histones during chromatin remodeling process (Khochbin et al., 2001). It has been observed that pairs of interacting domains in one organism may have a fusion homologue composing of these two domains in another organism, known as the rosetta stone protein theory (Marcotte et al., 1999). Mosquito MAELs may be rosetta stone proteins and it can be hypothesized that there are interactions between other MAEL and some HDAC_interact-containing proteins in other species. Indeed, it has been illustrated that mouse MAEL can interact with the SIN3B protein which contains an HDAC_interact domain (Costa et al., 2006). The associated SR-25-like domain provides another link between the MAEL domain and RNA-related process. The SR-25-like domain is associated with RNA-binding modules, RNA recognition motif (RRM) (Maris et al., 2005) and PRP38 (Blanton et al., 1992), It is also distantly related to SR-25 domain which may be involved in RNA splicing, as revealed by the SCOOP program (Bateman and Finn, 2007). Therefore, domain architecture suggests a potential involvement of MAEL domains in DNA binding, RNA binding and chromatin remodeling.

4.2.3.3 A distant similarity between MAEL domains and the DnaQ-H 3'-5' exonuclease family with the RNase H fold

We applied a fold recognition strategy to identify remotely related homologues of MAEL domains. The rationale is that in the case of remote homology, conserved protein structural folds can be kept despite limited sequence identity (Orengo and Thornton, 2005). A *meta* server was utilized, which assembles various state-of-the-art fold recognition methods and further evaluates modeled structures based on a consensus score computed by a 3D-JURY system (Ginalski et al., 2003a). MAEL domains from human, *X. tropicalis*, *Ciona* and *Drosophila* were first used as queries and several structural hits were identified by MetaBasic, ORFeus and BasicDist with consensus scores from 21 to 46. Although these 3D-Jury scores are below the cutoff 50, which corresponds to correct assignment with statistical significance (Ginalski and Rychlewski, 2003), domain and fold examinations showed that all retrieved structures belong to the DnaQ-H 3'-5' exonuclease family with the RNase H fold (Zuo and Deutscher, 2001; Thore et al., 2003). We extended our search using an ancestral *E. histolytica* MAEL domain (GI: 67477376, residues 315-532) as a query. Eleven structural hits were identified with high scores around 58-69, and they all belong to the DnaQ-H 3'-5' exonuclease family. Structural fold similarities between DnaQ-H and MAEL domains encouraged us to re-examine this relationship using PSI-BLAST. We noticed that several DnaQ-H exonucleases can be retrieved as insignificant candidates in our initial PSI-BLAST searching with a profile inclusion expectation (*E*) value of 0.005. However, when we set inclusion *E* value at 0.05, significant similarity between the first 100aa segment of MAEL domains and several prokaryotic DnaQ-H exonucleases was achieved in the fourth iteration.

We next examined this homologous relationship by building structure-based multiple sequence alignments for MAEL domains and DnaQ-H 3'-5' exonucleases. Since sequence identity among different DnaQ-H domains is very low, their alignment was first generated based on structural information as assessed by a CE-MC server (Guda et al., 2004) followed by manual adjustment based on published literature. Thereafter, we combined this alignment with the aligned MAEL domains based on fold recognition results and predicted secondary structures. The final alignment showed the conserved residues among/between two domains and compositions of secondary structures (Figure 19A). It is to be noted (Figure 19A) that equivalents of beta sheet (β) 3 of the RNase H fold in most MAEL domains are predicted to

be alpha helix (α). We believe that this is a wrong prediction since in the canonical RNase H fold, β3 is an edge β strand, which can usually be misidentified as an α helix because of its solvent sequence property (Siepen et al., 2003). As shown in Figure 19A, the secondary structures of MAEL domains resemble those of DnaQ-H 3'-5' exonucleases; both have a β1- β2- β3- α1- α2- β4- α3- β5- α4- α5- α6 composition. More importantly, several ancestral protist MAEL domains also share all the critical DnaQ-H characteristic residues (Asp-Glu-Asp-His-Asp, DEDHD). These residues are commonly utilized by diverse DnaQ-H 3'-5' exonucleases and interact with two divalent metal ions to form an active site (Hamdan et al., 2002; Cheng and Patel, 2004; Perrino et al., 2005). Thus, in contrast to a very low sequence identity (<15%) between MAEL domains and DnaQ-H 3'-5' exonucleases, the similar structural fold and the notable existence of DEDHD residues in protist MAEL domains strongly support a distant evolutionary relationship.

4.2.3.4 Structural examinations on active sites by DEDHD and EHHCHC residues in MAEL domains

The tertiary structures of protist and chicken MAEL domains were further constructed by comparative modeling. Like DnaQ-H domains (Figure 19B, C), these MAEL domains adopt a similar RNase H structural fold which is characterized by a compact α/β fold with open anti-parallel β sheets in the middle and several α helices surrounded (Figure 19D, E). Moreover, the characteristic DEDHD residues in protist MAEL domains are clustered into a structural core, which resembles active sites of DnaQ-H domains (Figure 19A, B, C). In contrast, most other MAEL domains lack the DnaQ-H specific residues DEDHD. However, they are characterized by another conserved stretch of residues, EHHCHC. During evolution such conservation of MAEL-specific residues may reflect functional contributions most likely to a distinct active site. The spatial locations of EHHCHC residues were then examined in these modeled MAEL structures to check their possibility of forming an active site. Unexpectedly, we found that all MAEL-specific residues have very close spatial locations and they are clustered together at one side of the middle anti-parallel β sheets (Figure 19E). Four residues (EHHchC) can shape a structural core and other two residues may also potentially face down to it with slight structural rearrangements. Similar change of structural conformations of α5 and α6 comprising last

CHC residues has been observed in crystal structures of DnaQ-H domains (not shown). There may exist another possibility that a disulfide bond (-S-S-) is formed between two Cys residues (C178 and C189 in the chicken MAEL domain) because of their close proximity. This is also supported by disulfide bond predictions (Ferre and Clote, 2006). Formation of a disulfide bond may facilitate the last His to approach other EHH residues, thus forming an active site with EHHH residues. Therefore, structural examinations suggest that protist MAEL domains with DEDHD residues may form a DnaQ-H active site whereas other MAEL domains with EHHCHC residues may potentially form a new active site based on the canonical RNase H scaffold.

4.2.4 Discussions and conclusion

4.2.4.1 Functional insight into MAEL in germline piRNA pathway

The proposed evolutionary link of MAEL domains to DnaQ-H 3'-5' exonuclease with RNase H fold may provide functional clues for MAEL domains. The DnaQ-H 3'-5' exonuclease family, also known as DEDDh exonuclease family or Exonuc_X-T domain (Pfam ID: PF00929), is one member of RNase H fold superfamily (SCOP: 53098) which also includes RNase H, mu transposase, crossover junction resolvase RuvC, and PIWI domain families (Katayanagi et al., 1993; Ariyoshi et al., 1994; Rice and Mizuuchi, 1995; Aravind et al., 2000; Parker et al., 2004). They all share a canonical RNase H fold but contain different active site residues. The DnaQ-H family is characterized by five conserved residues, DEDHD, which form an active site in coordination with divalent metal ions (Figure 19A). Its members contribute to diverse nucleic acid metabolism processes such as replicative proofreading (1J53:A) (Hamdan et al., 2002), DNA repair or RNA degradation (exonuclease I and oligoribonuclease) (Cheng and Patel, 2004; Perrino et al., 2005), and RNA interference (ERI-1) (Kennedy et al., 2004). Although different nucleotide targets (DNA or RNA) or diverse metal ions (Zn^{2+}, Mg^{2+}, or Mn^{2+}) are involved (Hamdan et al., 2002; Cheng and Patel, 2004; Perrino et al., 2005), their active sites formed by the EDDHD residues delineates a common 3'-5' exonuclease activity. That is, the acidic DEDD together with two metal ions shape a negative pocket, which provides space for accommodating the 3' termini of

oligonucleotide (DNA or RNA) chains. Thereafter, the coordinated metal ions and another conserved H are in direct contact with the bound chain, which induces a break of the phosphodiester bond of nucleotide in the 3'-5' direction (Cheng and Patel, 2004). Therefore, protist MAEL domains, harboring DnaQ-H specific DEDHD residues and active sites, may also employ a 3'-5' exonuclease activity, although their associated metal ions and nucleotide targets are still unknown.

In contrast to the protist MAEL domains, most recent MAEL domains do not contain the DnaQ-H specific residues but are characterized by the EHHCHC residues. What is the functional contribution of these residues to MAEL domains? Structural observations showed that a structural core can be potentially formed by the MAEL-specific residues EHHCHC or EHHH. This may provide a structural basis for an active site. On the one hand, this active site may confer RNA-binding ability for MAEL domains because of the lack of DnaQ-H specific residues. In this way, MAEL may contribute to stabilizing or positioning the RNA substrate in piRNA pathway. On the other hand, MAEL-specific residues and its potential active site may define another nuclease activity. We noticed that although all related families with the RNase H fold have low sequence identities and contain different active site residues, they all have DNA/RNA 3' or 5' end-directed nuclease activities with metal ion coordination in their own active sites (Ariyoshi et al., 1994; Rice and Mizuuchi, 1995). For example, RNase H is a non-specific endonuclease whose catalytic activity requires divalent ions (Mg^{2+} or Mn^{2+}) and is responsible for the hydrolysis of the RNA in a DNA/RNA duplex (Katayanagi et al., 1993; Ding et al., 1995). In contrast, PIWI domains contribute to 5'-3' exonuclease catalytic activity for the Argonaute family proteins (Slicer) in all types of small RNA pathways (siRNA, miRNA, and piRNA). The activity is achieved by three PIWI active site residues, DDH, in coordination with one divalent ion and used to cleave single-stranded RNA substrate guided by complementary double-stranded small RNAs (piRNA or siRNA) (Parker et al., 2004; Song et al., 2004a; Ma et al., 2005; Parker et al., 2005; Rivas et al., 2005). It seems that the RNase H structural fold is an efficient scaffold from which diverse nuclease families have evolved distinct nuclease activities by developing their own active site residues with metal ion coordination. Therefore, being one member of RNase H superfamily, the MAEL domain may share this characteristic, thus the residues EHHCHC may form an active site with a new nuclease activity. It has been shown in diverse proteins that H, C and E

residues often interact with Zn^{2+} (Tamames et al., 2007). Moreover, the residue composition of EHHH is commonly utilized by several *Escherichia coli* proteins including ColE7 endonuclease (Doudeva et al., 2006), Zinc transport protein ZnuA (Li and Jogl, 2007), and Aldolase (1DOS) for their active sites, which also interact with metal ions, especially Zn^{2+} (Blom et al., 1996),

Experimental evidence suggests that MAEL may be involved in piRNA biogenesis since its loss-of-function mutant impairs the production of piRNAs or rasiRNAs and increases the transcript level of transposable elements (Lim and Kai, 2007). Different from siRNA and miRNA pathways, piRNAs biogenesis employs a Dicer-independent mechanism (Vagin et al., 2006; Houwing et al., 2007). A ping-pong model has been recently proposed for this process and it is hypothesized that AGO3 bound to the sense strand of piRNAs catalyzes cleavage of the antisense strand that generates 5' end of antisense piRNAs. The 3' end of the resulting antisense piRNAs is subjected to a 3' cleavage by an unknown endonuclease or exonuclease and a HEN1-processed 3' methylation. Thereafter, the produced antisense piRNAs associate with Aubergine or PIWI and direct cleavage of transposon sequences, which then generates the sense strand piRNAs after 5' cleavage, 3' cleavage and 3' methylation (Aravin et al., 2007; Hartig et al., 2007; Klattenhoff and Theurkauf, 2008). This cycling model is not complete since the exonuclease or endonuclease enzyme responsible for the 3' terminal maturation remains uncharacterized (Aravin et al., 2007; Hartig et al., 2007; Klattenhoff and Theurkauf, 2008). Thus, because of its evolutionary relationship to 3'-5' DnaQ-H exonuclease and the potential (3'-5' exo-) nuclease activities, MAEL may be the nuclease candidate implicated in the cleavage of the 3' termini. Recently, the nucleases Zucchini and Squash have been proposed as the 3' termini nuclease candidate based on the evidence that they are also located in germ plasm and have a similar mutation phenotype in a loss of transposon silencing (Pane et al., 2007). However, MAEL is distinct from those above two nucleases due to its translocation between germ plasm and nucleus and the direct interaction with chromatin remodeling proteins (Findley et al., 2003; Costa et al., 2006). We believe that multiple nucleases are involved in the diverse steps of piRNA pathway in a sequential manner, similar to PIWI family members targeting 5' cleavage of piRNAs (Yigit et al., 2006); and MAEL is involved in a genomic DNA-related piRNA step, which may include chromatin remodeling process and initial transcription of

transposons. In this way, the MAEL-associated HMG domain or other chromatin remodeling proteins facilitate access of piRNA complex to genomic regions where are enriched with transposon sequences. The transposon transcripts undergoing processing interact with the piRNA complex in which PIWI, one RNase H member, generates 5' end of transposon transcripts via piRNA-directed homologous cleavage whereas MAEL, another RNase H member, contributes to the 3' terminal cleavage of transposon transcripts.

4.2.4.2 Unique evolutionary characteristics for MAEL domains

Phylogenetic analysis has revealed several unique characteristics of MAEL domains including single-copy status in most species, ancient lineage-specific expansion and complete loss in the teleost fish lineage. It has been long recognized that during evolution eukaryotic species have high duplication rates (Wagner, 2001) and vertebrates have experienced two or three whole or regional genome duplications (Evans et al., 2005; Hoegg and Meyer, 2005; Panopoulou and Poustka, 2005), which led to expansions of some domain families. It is of great interest that the MAEL domain has escaped the usual duplication potential in most species, especially in vertebrates. It is also possible that the duplicated sequence was lost after duplication. However, it seems that this single-copy status is commonly inherited by several domains including the SANTA domain (Zhang et al., 2006); an evolutionary selection against domain duplication together with functional conservation, therefore, should account for the establishment of this status. We did observe MAEL domain expansion in several species. One or two duplication events occur at the ancestor of each lineage before its further divergence (Figure 18A). This ancient lineage-specific expansion may be caused by the release of evolutionary constraints in individual lineages. Thereafter, functional complexity may have arisen, as exemplified by diverse protist MAEL domains with either DEDHC+EHHCHC residues or EHHCHC residues (Figure 18A and legend).

We also observed loss of the MAEL domain in all examined teleost fish species. Gene loss in protein family evolution is well-recognized. The lost member may be functionally replaced by another member of the same family. However MAEL does not belong to this case because of its single-copy nature especially in the vertebrates. What happens in teleost fish germline cells without the MAEL protein? One possibility is that fish have a distinct but functionally similar counterpart, which remains to be characterized.

Another possibility is that MAEL loss results in a unique piRNA pathway or a unique developmental morphology in fish germline cells compared to mammals and flies. Indeed, a distinct cellular distribution of Vasa protein, a marker for germline cells, has been observed in fish (Houwing et al., 2007). Moreover, it seems that although RNAi is evolutionarily conserved among species, individual lineage tends to develop some unique steps for the RNAi pathway, as shown in plant-specific XS domain in post-transcriptional gene silencing (Zhang and Trudeau, 2008) and the worm-specific Argonaute subfamily (Yigit et al., 2006). Furthermore, although the evolutionary and functional implications of MAEL loss in the teleost lineage are not yet understood, a practical implication can be hypothesized that fish may be amenable natural MAEL knockout-like models where transgenic insertion of MAEL proteins could be used to as a strategy for studying its function and the germline piRNA pathway.

4.2.4.3 Active site switch, a novel path towards protein function change

How did MAEL domain evolve from the DnaQ-H domain? Considering the oldest identified MAEL domains are from *Protista* that represents the earliest eukaryotic branches (Eichinger and Noegel, 2005), we believe that the first generation of MAEL domains should be traced back to an ancestral eukaryotic or a prokaryotic DnaQ-H domain, from which the MAEL-specific characteristics might have originated. Indeed, the first three MAEL-specific residues EHH are more ancient than others and commonly found in different prokaryotic ε exonucleases (Figure 19A). Their spatial locations are also close as shown in 1J53:A (Hamdan et al., 2002) (Figure 20), thus providing a substrate for evolving to a mature active site. It can be hypothesized that the DnaQ-H ancestor underwent a gene duplication event (Figure 21) in early *Eukaryota* or during the divergence of the prokaryotes and eukaryotes, corresponding to the time when small RNA pathways emerged. Thereafter, the duplicated one (MAEL ancestor) obtained a protein motif comprising CHC residues, forming an evolutionary intermediate which has both DEDHD and EHHCHC residues. The original DnaQ-H activity was attained by some ancestral protist MAEL domains. However, driven by relaxed evolutionary constraint associated with functional specification, other MAEL domains generated by further lineage-specific duplications or species speciation (duplication 2) may have lost the original active site with DEDHD residues, but at the same time

developed a new active site with EHHCHC residues while keeping RNase H structural scaffold (Figure 22). The diversity of characteristic residues among three Eh MAEL domains (Eh67476664, Eh67477376-C, and Eh67477376-N) in an amoeba duplication branch (node value 89%/100%) supports this evolutionary path (Figure 18A). Compared to two other paralogs (Eh67476664, Eh67477376-C) which have both sets of DEDHD and EHHCHC residues, the Eh67477376-N has lost the DnaQ-H specific residues. Thus, MAEL domains have experienced a transition from DnaQ-H active site residues to MAEL active site residues which, we believe, may represent a novel mode for protein function evolution called the active site switch.

It has been long recognized that although protein superfamilies tend to preserve their structure during evolution, a divergent evolution with functional changes is permitted (Todd et al., 2002; Anantharaman et al., 2003; Orengo and Thornton, 2005; Glasner et al., 2006). Protein function changes involve diversity or variability in active sites, properties of related residues or their spatial locations, as reviewed by Todd et al. (Todd et al., 2002). Several possible mechanisms underlying protein function changes have been proposed including evolutionary optimization via functional residue hopping, independent recruitment of active sites in different lineages, circular permutation, and functional convergence after divergence (Todd et al., 2002). Here, MAEL domains undergoing the active site switch provide another mode for protein function change; that is, during evolution new activities can be developed by introducing new active sites based on a preexisting protein scaffold. This evolutionary mode has long been hypothesized based on many *in vitro* directed evolution studies (Aharoni et al., 2005; Park et al., 2006; Patrick and Matsumura, 2008). It has been shown that new activity can be introduced by simultaneous incorporation and adjustment of functional elements through insertion, deletion, and substitution of several active site loops, followed by point mutations to fine-tune the activity (Park et al., 2006). A similar process may have occurred in MAEL domain evolution. In addition, the ancestral protist MAEL domains which harbor the characteristics of both DnaQ-H and MAEL domains, for the first time, illustrate the existence of an evolutionary intermediate during protein function evolution. The identification of such an evolutionary intermediate may facilitate establishing real evolutionary links between protein superfamily members with different catalytic activities, or protein superfamilies which have overall similar structural folds but different functions.

Figure 17. Multiple sequence alignment of representatives of MAEL domain

The sequences are represented by an abbreviation of species name followed by database entry ID. The homologues of *C. savignyi* identified in Ensembl database are indicated by Cs1 and Cs2. The consensus in 80% of the sequences is shown below the alignment based on default amino acid classes in Chroma. The numbers in bracket are indicative of the excluded residues from sequences. Species name abbreviations: Aa, *Aedes aegypti*; Ag, *Anopheles gambiae*; Am, *Apis mellifera*; Cb, *Caenorhabditis briggsae*; Ce, *Caenorhabditis elegans*; Ci, *Ciona intestinalis*; Cs, *Ciona savignyi*; Dm, *Drosophila melanogaster*; Ed, *Entamoeba dispar SAW760*; Eh, *Entamoeba histolytica*; Gg, *Gallus gallus*; Gm, *Glossina morsitans*; Hs, *Homo sapiens*; Md, *Monodelphis domestica*; Lb, *Leishmania braziliensis*; Tb, *Trypanosoma brucei TREU927*; Tr, *Trypanosoma congolense*; Tv, *Trypanosoma vivax*; Xt, *Xenopus tropicalis*.

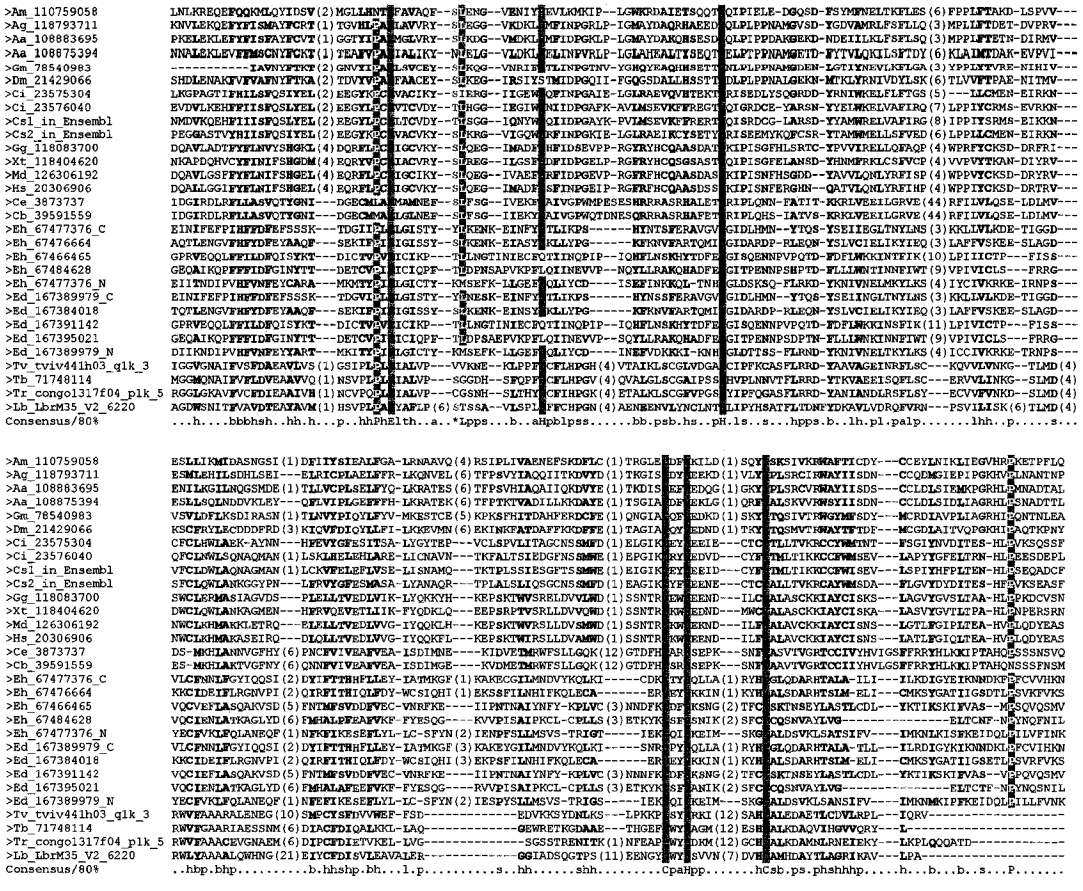


Figure 18. Phylogenetic relationship and domain architectures of MAEL proteins

(A) An unrooted phylogenetic tree was reconstructed using maximum likelihood (ML) analysis and Bayesian analysis. Single domain sequence is represented by species names. Duplicated MAEL domains in the species of Aa, Ci, Cp, Ed, Eh are represented by species names following Genbank ID, whereas the duplicated Cs MAEL domains are represented by Cs1 and Cs2. Branch length is proportional to estimated evolutionary change by PhyML program; the scale bar represents 0.5 substitution per site. Node supporting values greater than 75% from ML bootstrap analyses and Bayesian MCMCMC sampling are shown on the left and on the right of the slash, respectively. Lineage-specific expansions of MAEL domains in amoeba, mosquito and sea squirt are highlighted with different colors (red, turquoise blue and pink) and the ancient duplication events were indicated by circled numbers. The loss of MAEL domain in teleost fish is indicated in blue dashed. Asterisk labeled MAEL domains are the ones containing both conserved EHHCHC and EDDHD residues (see following main text). (B) Domain architectures of representatives of the MAEL proteins were deduced through searching against Pfam and SMART domain databases and drawn approximately to scale. The domains shown are: SR-25-like (DUF1777, Pfam: PF08648); HDAC_interact, named after Histone deacetylase (HDAC) interacting (SMART: SM00761); HMG, named after High Mobility Group (SMART: SM00398). Species name abbreviations: Aa, *Aedes aegypti*; Ag, *Anopheles gambiae*; Am, *Apis mellifera*; Bm, *Brugia malayi*; Bt, *Bos taurus*; Cb, *Caenorhabditis briggsae*; Ce, *Caenorhabditis elegans*; Cf, *Canis familiaris*; Ci, *Ciona intestinalis*; Cp, *Culex pipiens quinquefasciatus*; Cs, *Ciona savignyi*; Dm, *Drosophila melanogaster*; Dp, *Drosophila pseudoobscura*; Dy, *Drosophila yakuba*; Ec, *Equus caballus*; Ed, *Entamoeba dispar SAW760*; Eh, *Entamoeba histolytica*; Gg, *Gallus gallus*; Gm, *Glossina morsitans*; Hs, *Homo sapiens*; Lb, *Leishmania braziliensis*; Ll, *Lutzomyia longipalpis*; Md, *Monodelphis domestica*; Mm, *Mus musculus*; Mu, *Macaca mulatta*; Nv, *Nasonia vitripennis*; Lb, *Leishmania braziliensis*; Rn, *Rattus norvegicus*; Sp, *Strongylocentrotus purpuratus*; Ss, *Sus scrofa*; Tb, *Trypanosoma brucei TREU927*; Tc, *Tribolium castaneum*; Tr, *Trypanosoma congolense*; Tv, *Trypanosoma vivax*; Xt, *Xenopus tropicalis*.

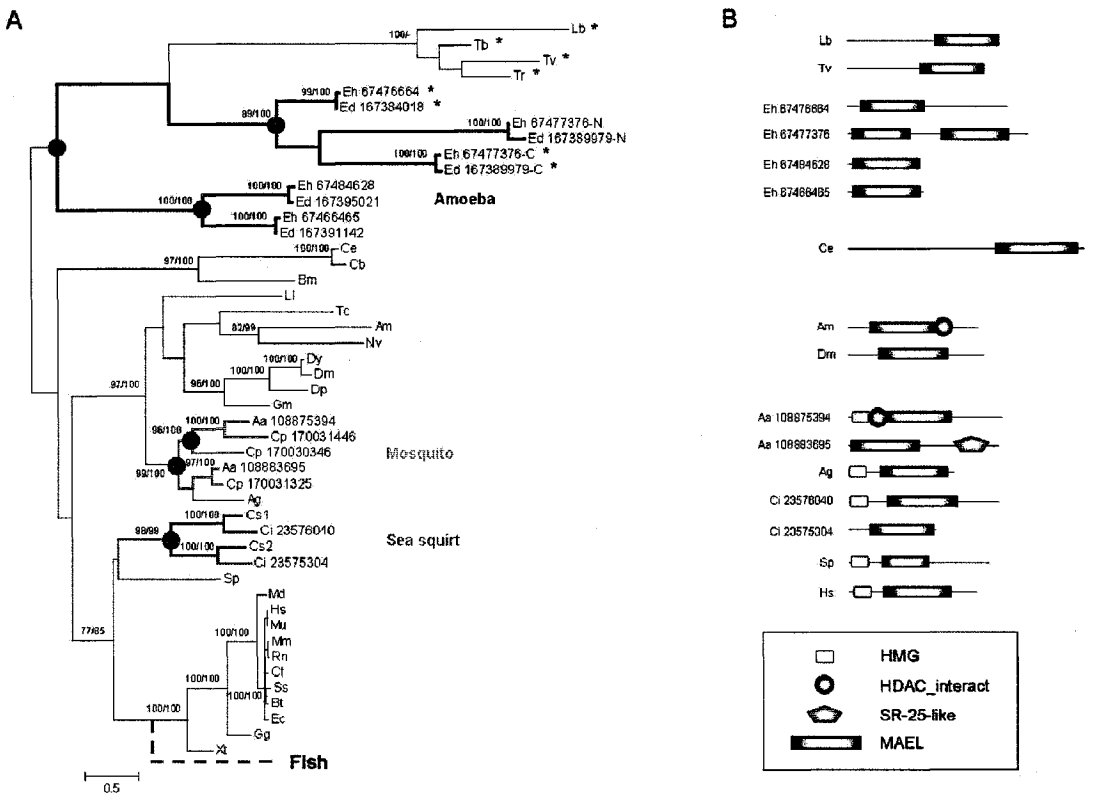

A
Lb *
Tb *
Tv *
Tr *
Eh 67476664 *
Ed 167384018 *
Eh 67477376-N
Ed 167389979-N
Eh 67477376-C *
Ed 167389979-C *
Eh 67484628
Ed 167395021
Eh 67466465
Ed 167391142
Amoeba
Ce
Cb
Bm
Li
Tc
Am
Nv
Dy
Dm
Dp
Gm
Aa 108875394
Cp 170031446
Cp 170030346
Aa 108883695
Cp 170031325
Ag
Mosquito
Cs1
Ci 23576040
Cs2
Ci 23575304
Sea squirt
Sp
Md
Hs
Mu
Mm
Rn
Cf
Ss
Bt
Ec
Gg
Xt
Fish
0.5
B
Lb
Tv
Eh 67476664
Eh 67477376
Eh 67484628
Eh 67466465
Ce
Am
Dm
Aa 108875394
Aa 108883695
Ag
Ci 23576040
Ci 23575304
Sp
Hs
HMG
HDAC_interact
SR-25-like
MAEL

Figure 19. Sequence and structure similarity between MAEL and DnaQ-H domains

(A) Sequence and the secondary structure alignment of MAEL and DnaQ-H domains. MAEL domains are represented by an abbreviation of the species name followed by Genbank ID, while DnaQ-H domains are represented by an abbreviation of the species name followed by PDB code and protein names. Five DnaQ-H domains with 3-D structures are included: *Thermotaga maritime* ε exonuclease (Tm ε, 2P1J:A), *Escherichia coli* ε exonuclease (Ec_ε, 1J53:A), *Haemophilus influenzae* oligoribonuclease (Hi_Orn, 1J9A:A), human Trex2 exonuclease (Hs_Trex2, 1Y97:A) and human 3'-5' exoribonuclease (Hs_Exo, 1W0H:A). The consensus in 80% of the sequences is shown below the alignment. The conserved DnaQ-H specific residues (DEDHD) are highlighted with the blue background; whereas the conserved MAEL-specific residues (EHHCHC) are highlighted with the red background. The MAEL-specific residues (EHH) also exist at counterpart positions in some DnaQ-H domains, and were highlighted with pink background. Detailed secondary structures for MAEL domains and DnaQ-H domains are obtained from secondary structure predictions and the 3-D structures, respectively; they are shown below the alignment (h in red, α helix; e in blue, β sheet). The structural sequence alignment of MAEL domains and DnaQ domains with PDB structures was established carefully by hand on the basis of CE-MC results, alignment in fold recognition, literature information, and predicted secondary structures. The numbers in bracket are indicative of the excluded residues from sequences. New species name abbreviations: Tm, *Thermotaga maritime*; Ac , *Alteromonas macleodii* 'Deep ecotype'; Ma , *marine gamma proteobacterium* HTCC2143. (B-E) NewCartoon diagrams for DnaQ-H domains (1J53:A and 1W0H:A) and the homology model of two MAEL domains (Eh 67476664 and chicken MAEL). The α helices are shown in pink, β sheets in yellow, and loops in white; Their spatial locations are labeled in 1J53:A. Strictly conserved DnaQ-H active site residues DEDHD of protist MAEL domain (Eh 67476664) are highlighted in licorice drawing with acidic residues (D and E) in red and basic His in blue. The MAEL-specific residues EHHCHC of chicken MAEL domain are highlighted with Glu in red, His in blue and Cys in orange.

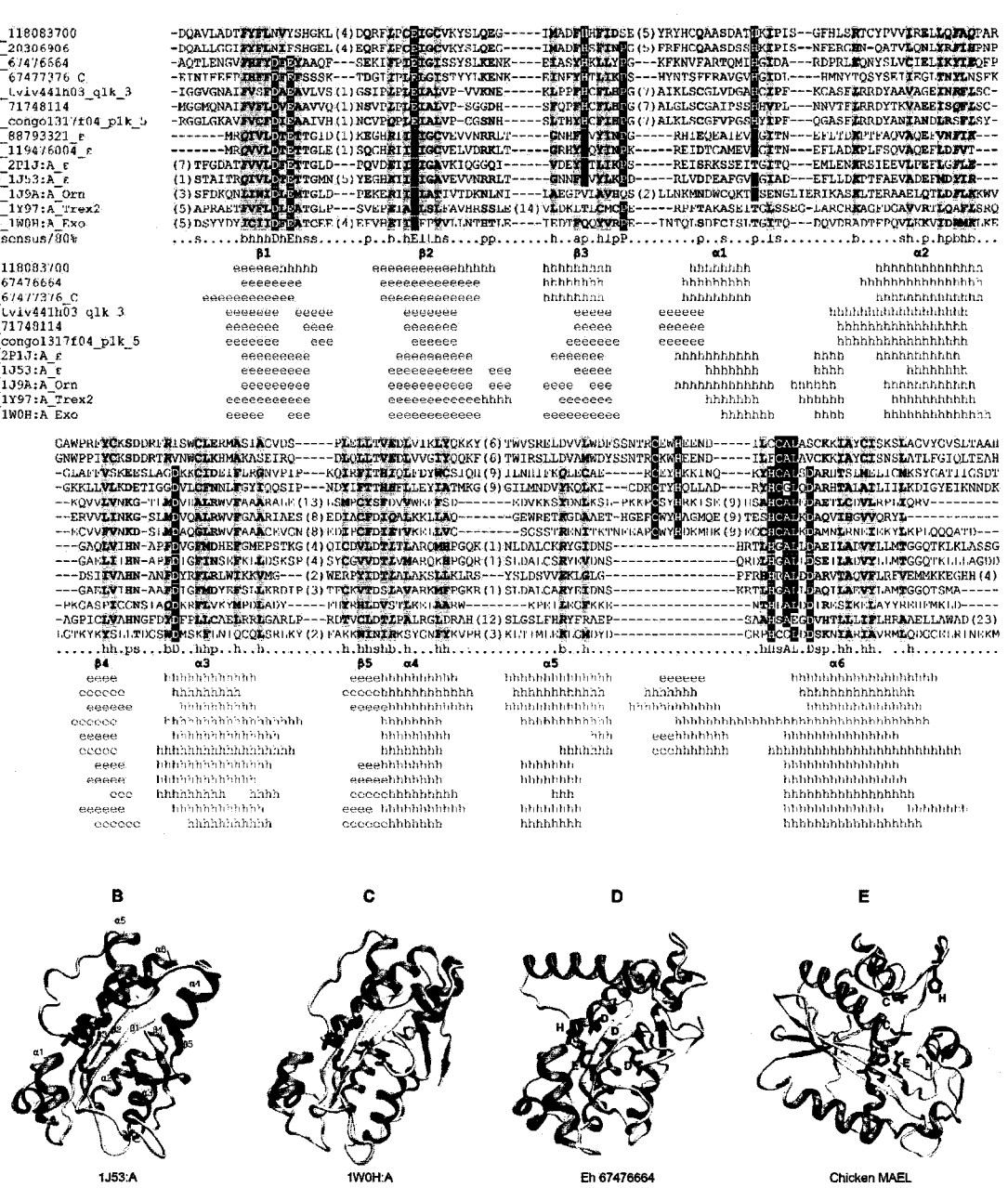

A
B
C
D
E
1J53:A
1W0H:A
Eh 67476664
Chicken MAEL

Figure 20. The conservation of MAEL-specific residues in *E. coli* DnaQ-H domain (1J53:A)

The cartoon drawing of protein structure is shown. The α helices are shown in pink, β sheets in yellow and loops in cyan. The DnaQ-H specific residues (DEDHD) are highlighted with acidic residues (D and E) in light red and basic His in light blue, whereas three MAEL-specific residues (EHH) are highlighted with acidic Glu in red and basic His in blue.

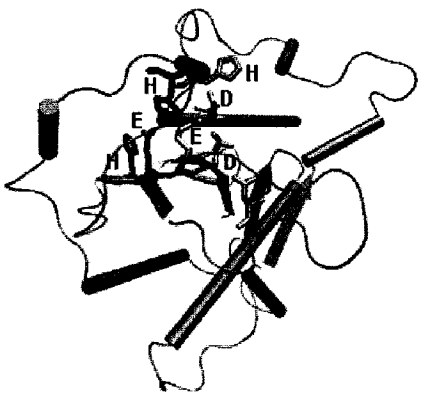


Figure 21. Evolutionary relationship between DnaQ-H and MAEL domains

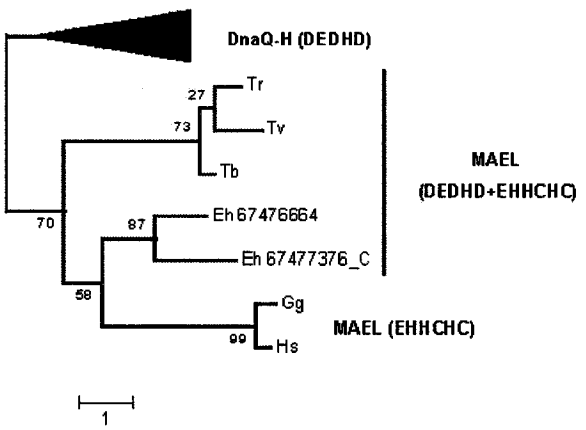
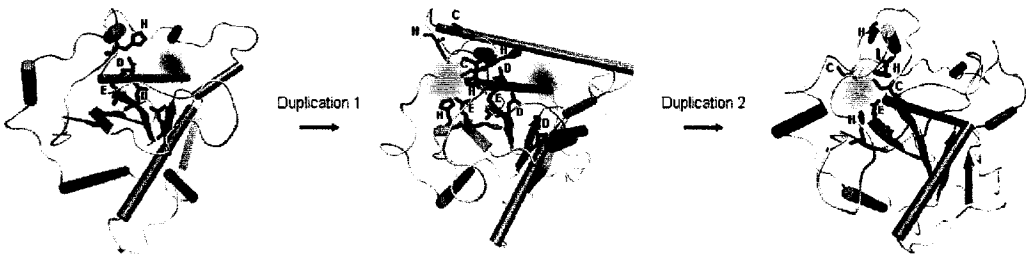


Figure 22. A proposed "active site switch" mode for MAEL domain function evolution
The cartoon drawing of protein structure is shown. The α helices are shown in pink, β sheets in yellow and loops in white. The DnaQ-H specific residues (DEDHD) are highlighted in blue, whereas MAEL specific residues (EHHCHC) are highlighted in red. Red cloud and green cloud indicate DnaQ-H active site and MAEL-specific active site, respectively.



CHAPTER 5: GENERAL CONCLUSION AND FUTURE DIRECTIONS

5.1 The E2-regulated genes in the fish brain

Blood E2 levels exhibit a seasonal profile during the reproductive cycle, and thus E2 regulation of target genes also varies temporally. Since there is an increase for both E2 blood level and brain aromatase activity around April and May, corresponding to the prespawning period, I hypothesized this season of the year is a critical period for physiological E2 action in regulating its target gene expression in the neuroendocrine brains (Goal 1, Figure 23). In the first study, I utilized fadrozole, a specific aromatase inhibitor, to inhibit the E2 production and evaluated the effect of this fadrozole-induced E2 decline on global gene expression change in the female goldfish brain. I have identified numerous E2-regulated genes among which some genes have shown to be estrogen-responsive in either fish or other species.

Moreover, 12 regulated genes are coding calcium binding proteins, indicating regulatory action of E2 on calcium signaling in the fish brain. The physiological significance of calcium signaling in the fish brain is still largely unknown. There is a need to identify the precise mechanism during E2 regulation on these genes. To clarify these points, classical electrophysiological studies on calcium signaling in the fish brain and the anatomical relationship between these calcium binding proteins, nER and aromatase should be investigated in the future. In this way, the goldfish can serve as an ideal model for glial to neuronal signaling through estrogen. It will be interesting to also study the distribution of mER relative to the genes identified here. There are many examples where there is estrogen regulation of gene expression but little evidence for the presence of the classical nERs. As one example, a previous study has shown that in the monkey forebrain, a lack of co-localization of ERα and parvalbumin, a E2-regulated calcium binding protein (Perez et al., 2004). The recent discovery of the mER (reviewed in (Zhang and Trudeau, 2006) and a multitude of gene targets that could potentially be regulated through the membrane receptor pathway begs the question of the relative importance of the nER and mER in the the vertebrate brain.

5.2 The putative crosstalk between E2 and GABA in neuroendocrine brain

Interestingly, by comparing the result from fadrozole treatment and another result from baclofen treatment, I observed that a large set of genes are commonly regulated by both E2 and GABA. There is a possible crosstalk between E2 and GABA in regulating gene expression. I further verified this possibility by showing that the expression of activin βA is subjected to regulation from both E2 and GABA. I also observed that the common regulation of gene sets in goldfish brain can be extended to other neurotransmitters, for example dopamine (J. Popesku, personal communication). What is the putative mechanisms underlying the regulation on these common genes? This is a matter of speculation. I hypothesize that membrane receptor-initiated signaling pathway crosstalk may contribute to this (Figure 23). The regulatory model may be similar to the one we proposed in Figure 3 (Chapter 1). Both E2 and GABA can bind to their own membrane receptors which are co-localized on the membrane of the same cells. Thereafter, the same GPCR-initiated signaling pathway can be activated, which further generates similar or reverse effect on gene expression according to diverse hormones. A possible strategy to study this question is to identify the involvement of second signaling pathways using specific inhibitors for blocking these pathways.

5.3 Seasonal gene expression patterns associated with hormone profiles

I identified seasonal gene expression patterns across the goldfish reproductive cycle (Goal 2, Figure 23). The differential expression of these genes may account for the establishment of seasonal hormone profiles and reflect the seasonal actions of these hormones. Indeed, I have observed that aromatase B expression in goldfish brain exhibits a similar profile as blood E2 level. Moreover, a large set of genes share a similar gene expression pattern which has a higher expression around May compared to August and December. Their expression change appears to be regulated by photoperiod, a known dominant factor in regulating fish seasonality. Gene ontology analysis shows that most of them are functionally related to neuroendocrine processes. Thus the increased expression of these neuroendocrine regulated genes in May may be crucial for the ensuing spawning period. Therefore, I hypothesize that the gene expression patterns may account for dynamic neuroendocrine regulaton on fish seasonal reproductive development.

To my knowledge, this study is the first one to define the global neuroendocrine gene expression patterns throughout a whole breeding cycle. The identified differential genes provide a basis for understanding the molecular mechanisms determining the fish reproductive seasonality. However, how to "dissect out" the effects of diverse sex hormones and other neurotransmitters is still a challenge. I have attempted to compare the E2-regulated genes and these seasonally-regulated genes. However, I found that no single regulatory mode could be retrieved (analysis not shown). For example, the seasonal expression of aromatase B shows a similar pattern as the profile of blood E2 level, indicating the direct regulation by E2. In contrast, although calmodulin expression was repressed by E2, it still followed the general HLL pattern described in chapter 3. This likely reflects a complex regulatory mode by diverse hormones and neurotransmitters. It will be important to identify the genes regulated by other hormones and neurotransmitters, which Dr. Trudeau's lab is now attempting to do for dopamine, serotonin and GABA.

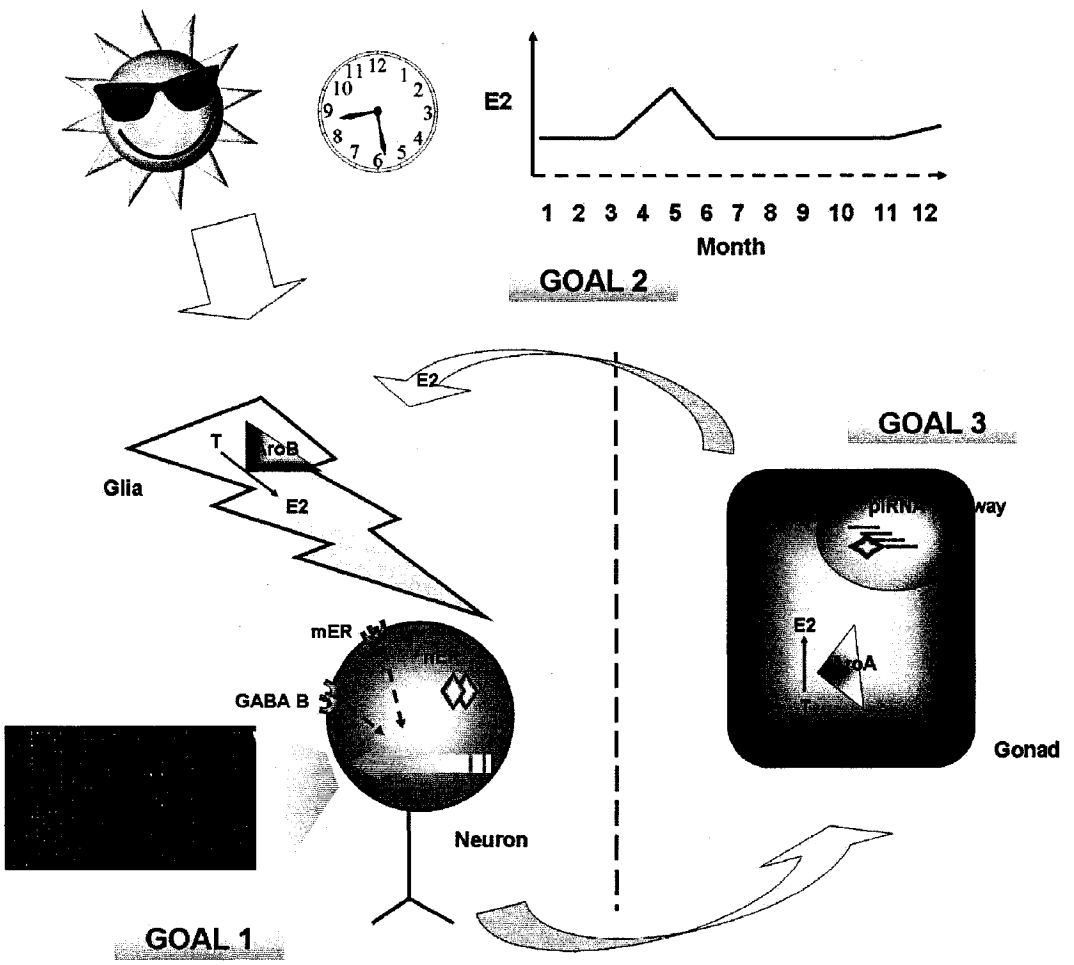
5.4 The piRNA pathway and hormone regulation of gonadal development

After fadrozole treatment of female goldfish (chapter 2), there was an overt change in ovarian morphology. This prompted me to explore the mechanisms underlying this observation (Goal 3, Figure 23). I focused my efforts on the MAEL protein, a piRNA pathway specific protein. As shown in section 4.2, and initially somewhat disappointingly, MAEL is not found in fish, and therefore MAEL is not depicted in the summary figure, but rather I speculate that a piRNA pathway could be involved. I hypothesized that the germline specific piRNA pathway may be one target of estrogen or androgen, and utilized the developing frog model rather than a fish model. Indeed, I observed that in *Xenopus tropicalis*, both fadrozole and finasteride treatments can increase the expression of MAEL. Both fadrozole and finasteride can change the ratios of E2 relative to DHT, which may disrupt normal germ cell development, or perhaps induce sex reversal or the intersex phenotype. Therefore, the increased expression of MAEL during this process suggests that the piRNA pathway may be involved in estrogen regulation of gonadal development. Since MAEL was not identified in any of the available fish genomes, whether E2 can regulate other genes in the piRNA pathway in fish is unanswered and will be an interesting line of investigation in the future. Moreover, MAEL expression change is known to affect piRNA production and

the expression level of transposon transcripts in cells, thus the effect of fadrozole-induced E2 decline on their abundance should also investigated in the frog model.

Figure 23. Cartoon showing the overall research goals in this thesis

Please refer to the text in the subsections of chapter 5 for details.



APPENDIX A: CROSSTALK BETWEEN ESTROGEN AND GABA SIGNALING REVEALED BY A COMMON GENE EXPRESSION SIGNATURE

Introduction

Neuroendocrine function is closely regulated by diverse hormones typified by estrogen (E2) and gamma-aminobutyric acid (GABA). Through its membrane and nuclear receptors, E2 affects signaling pathway activation and gene expression (Zhang and Trudeau, 2006). In contrast, GABA action is achieved by binding to its two types of membrane receptors, ionotropic ligand-gated ion channel (GABA-A and C) and metabotropic G protein-coupled receptors (GABA-B), which activate ion channel and cellular signaling pathway respectively (Popesku et al., 2008). In our previous study, E2 associated gene expression profile was characterized by mimicking E2 withdrawal using an aromatase inhibitor fadrozole (Chapter 2). In another study, GABA-B receptor signaling pathway-specific gene expression profiles were identified in goldfish Hyp after exposure to baclofen, a GABA-B receptor agonist. Interestingly, we observed that a set of genes are commonly identified in these two separate studies, but with inverted expression change. Activin βA is one example; expression of activin βA was decreased after fadrozole exposure whereas it was elevated after baclofen treatment. This observation suggests that there may be crosstalk between E2 and GABA signaling to regulate gene expression. Here we used *meta* analysis to re-evaluate the common genes of E2 and GABA, and illustrated their crosstalk in regulating expression of activin βA using independent samples and real-time PCR.

Materials and methods

1. Microarray data sets

Microarray data from fadrozole exposure in female goldfish Tel were retrieved from my previous study (Chapter 2). The baclofen-induced gene expression data was produced by Dr. Christopher Martyniuk in our lab (Martyniuk et al., 2007a; Popesku et al., 2008). In his

study, in early September adult female goldfish were injected with baclofen (10 ug/g of body weight). Six hours later, hypothalami (Hyp) and telecephelon (Tel) were collected for total RNA isolation. The Hyp RNA from control and treated fish were hybridized on microarrays. For both datasets, intensity-dependent Lowess method was used for within normalizations.

2. Meta analysis of microarray data

We used *meta* analysis strategy to further to identify the candidate genes, whose expression are commonly changed following both Fadrozole and Baclofen treatments. This analysis is based on a general hypothesis that a common set of differential genes exist for both studies. Since the genes of interests have opposite expression change after fadrozole and baclofen treatments. We reversed the expression direction prior to analysis. Both the effect size model (Choi et al., 2003) and rank product (Hong et al., 2006) methods were used.

For the effect size model, a z score of the null average effect size was obtained for each gene. The genes with /*z* score/>1.6 were chosen. This *z* score cutoff is corresponding to an estimated false discovery rate (FDR) of 10%. For rank product method, the genes with pfp (FDR) lower than 5% were chosen. The heat map of gene expression change was generated by MeV.

3. Fadrozole-Baclofen treatment and real-time RT-PCR assay

Adult female goldfish were exposed to fadrozole (50 μg/L) for one month. Thereafter, both control and fadrozole-treated fish were injected with baclofen (10 μg/g of body weight, injection volume of 1 μl/g) dissolved in 6% saline solution and sampled six hours later. Therefore, there were four groups: control fish, fadrozole, baclofen and fadrozole-baclofen treated fish. Two or three hypothalami and telencephali were pooled for a total number of 8-10 samples in each group.

The detailed procedures for RNA isolation, cDNA synthesis and real-time RT-PCR assay refer to Chapter 2. EF1α was used as an internal reference gene to normalize activin βA expression between samples. The final normalized data from biological replicates are presented as means +SEM of fold changes relative to control group, which was set at 1. For statistical analysis, significant changes in gene expression between control group and three

treated groups were determined by using one way ANOVA and Holm-Sidak post-hoc test in SigmaStat3.5 (SPSS Inc.).

Results

1. Common gene expression signature by both estrogen and GABA

Two popular methods of meta analysis were used to combine the results of different studies into a single, more powerful result. Since we wanted to identify all possible gene candidates for both the fadrozole and baclofen groups, we extracted the common genes that were retrieved by two individual methods. This resulted in about 100 gene candidates. Heat map of these genes were shown in Figure 1. Our result indicates that E2 and GABA may cooperate to exert their actions in brain.

2. Real-time PCR verification for activin βA

To verify our hypothesis, we exposed female goldfish with both fadrozole and baclofen. The expression of activin βA, in both Tel and Hyp was examined by using real-time PCR. In our previous study (Chapter 2), activin βA in both Tel and Hyp was down-regulated by fadrozole treatment. However, in Martyniuk's data, activin βA was up-regulated by baclofen treatment (Martyniuk et al., 2007a).

In the current study, we observed that the similar gene expression change for activin βA in Tel after either fadrozole or baclofen treatments (Figure 2A). However, we further observed that the fadrozole-baclofen treatment showed a similar activin βA mRNA level as control. In other words, estrogen facilitates the increased expression of activin βA by baclofen. Therefore, this result illustrated that activin βA is subjected to transcriptional regulation by both E2 and GABA signaling.

We did not observe a similar regulatory pattern for activin βA expression in Hyp. Even individual treatment with either fadrozole or baclofen in the current study could not significantly change expression of activin βA. This may be due to the different stages of the fish used in those treatments.

Figure 1. Heat map of gene expression for common genes which are regulated by both E2 and GABA

Down expression of the gene was shown in blue whereas up expression was shown in yellow.

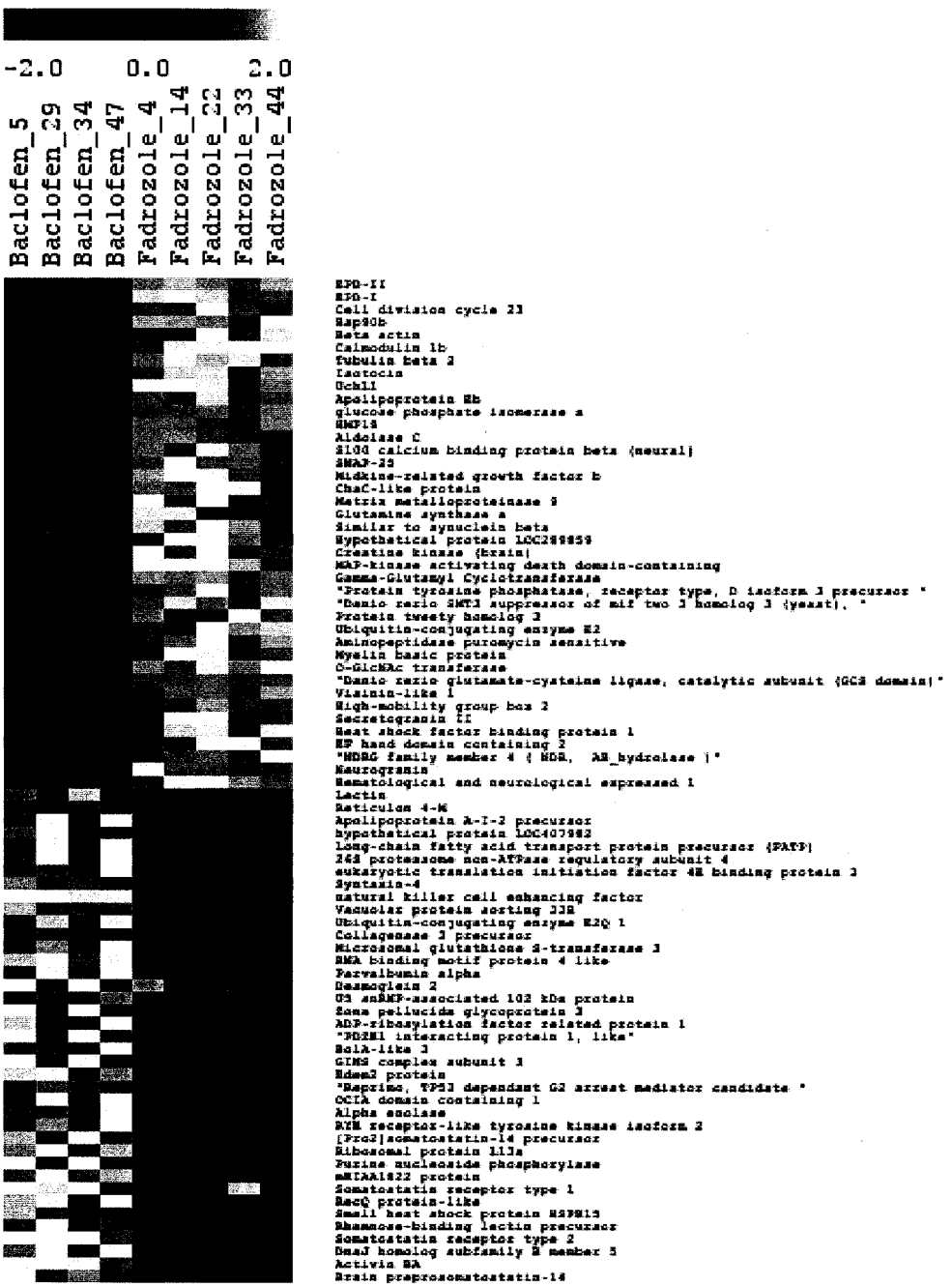
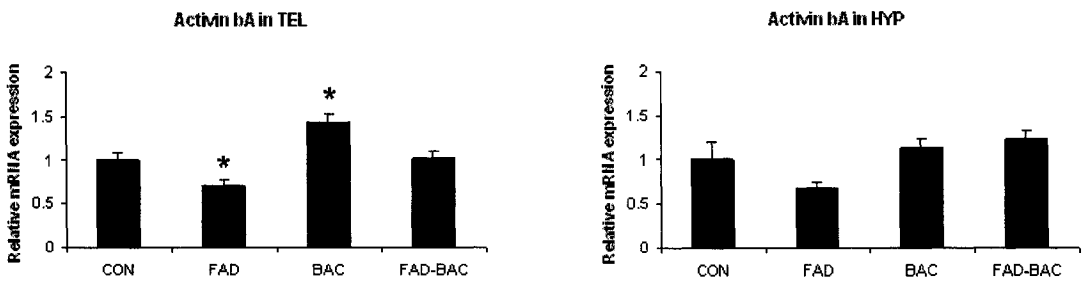


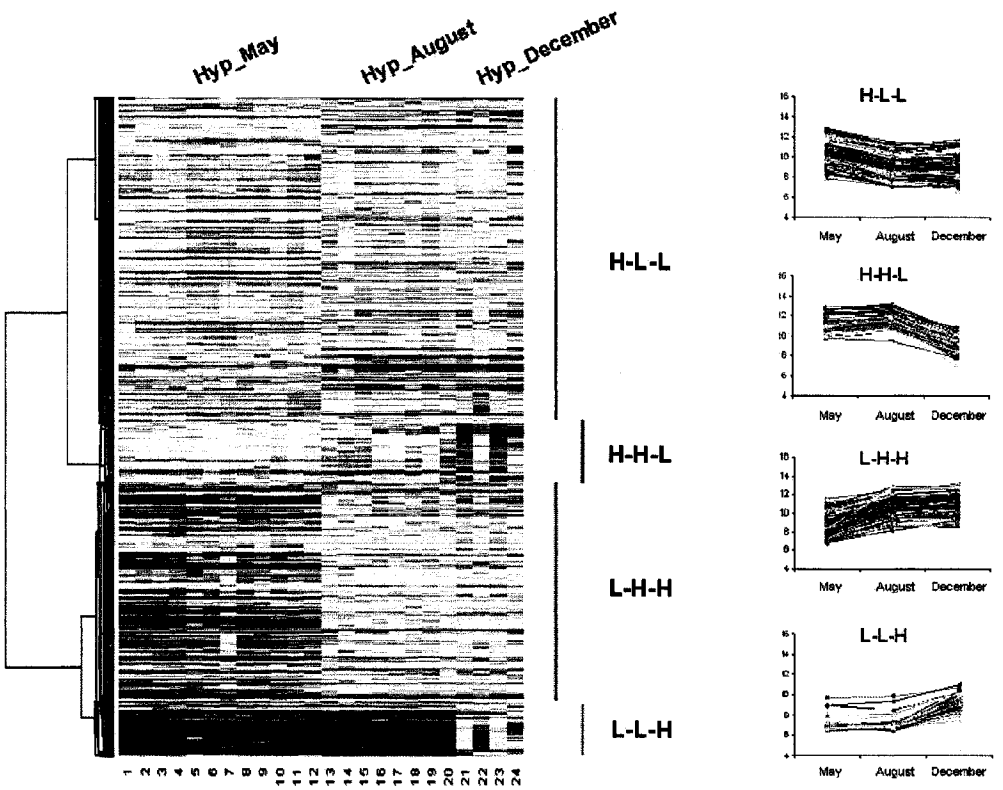
Figure 2. Relative gene expression change of activin βA in both Tel and Hyp after fadrozole and baclofen treatments

Asterisks (*) indicate significant differences between control and treatment groups ($p<0.05$ by one way ANOVA and Holm-Sidak post-hoc test)



APPENDIX B: HIERARCHICAL CLUSTERING OF EXPRESSION PROFILES OF SIGNIFICANTLY DIFFERENTIALLY EXPRESSED GENES BETWEEN THREE REPRODUCTIVE SEASONAL TIME POINTS (MAY, AUGUST, AND DECEMBER)

Signal represents log ratio intensity of the gene expression. Red indicates relatively high expression and blue indicates relatively low expression. This figure is from Xiong's thesis (2008), page No. 70.



APPENDIX C: MANUSCRIPTS PUBLISHED DURING MY PHD STUDY OR CURRENTLY IN PRESS OR SUBMITTED

Zhang D, Popesku JT, Martyniuk CJ, Xiong H, Duarte P, Yao L, Xia X, Trudeau VL. (2009) Profiling neuroendocrine gene expression changes following fadrozole-induced estrogen decline in the female goldfish. (Submitted)

Zhang D*, Xiong H*, Mennigen JA, Popesku JT, Marlatt VL, Martyniuk CJ, Crump K, Xia X, Cossins AR, Trudeau VL. (2009) Defining neuroendocrine gene expression patterns associated with reproductive seasonality in fish. (*Equally contributed, submitted)

Zhao E, **Zhang D**, Basak A, Trudeau VL. (2009) New insights into granin-derived peptides: evolution and endocrine roles. *General and Comparative Endocrinology, accepted*

Zhang D, Trudeau VL. (2008) Estrogen signaling mechanisms. Book chapter in "*Signal transduction: pathways, mechanisms and diseases*", which is edited by Dr. Sitaramayya Ari and will be published by Springer. *In press*

Zhang D, Xiong H, Shan J, Xia X, Trudeau VL. (2008) Functional insight into Maelstrom in the germline piRNA pathway: a unique domain homologous to the DnaQ-H 3'-5' exonuclease, its lineage-specific expansion/loss and evolutionarily active site switch. *Biology Direct* 2008, 3:48.

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