ROLE OF CYCLOOXYGENASES IN ZEBRAFISH OOGENESIS AND THEIR TRANSCRIPTIONAL REGULATION BY PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA

by

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DEDICATION

I dedicate this thesis to my father Dr. Prem Kumar Khare, a scientist, a teacher and my inspiration.

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Vani Khare **February 2013**

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LIST OF SYMBOLS

Nl	Nanoliter
µl	Microliter
Ml	milliliter
Ng	nanogram
µg	microgram
Mg	milligram
µM	micromolar
mM	Gmail.com
A	alpha
B	beta
Γ	gamma
Ω	Omega
Δ	Delta

LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
mRNA	messenger RNA
PG	Prostaglandin
AA	Arachidonic Acid
PUFA	Poly Unsaturated Fatty Acid
LH	Luteinizing Hormone
FSH	Follicle Stimulating Hormone
EMV	Early and mid vitellogenic
MV	Mid vitellogenic
LV	Late vitellogenic/Full grown
M	Mature
GVBD	Germinal Vesicle Break Down
MO	Morpholino
mmMO	Mismatch Morpholino
DB	Danieau Buffer
PCR	Polymerase Chain Reaction
EMSA	Electrophoretic mobility shift assay
PAGE	PolyAcrylamide Gel Electrophoresis
TBE	Tris Borate EDTA
TE	Tris EDTA
PBS	Phosphate Buffer Saline
EDTA	EthyleneDiamineTetraAceticAcid

TRIS	Tris(hydroxymethyl)aminomethane
TEMED	Tetramethylethylenediamine
APS	Ammonium persulphate
SDS	Sodium dodecyl sulphate
kDa	Kilodalton
kb	Kilobase
bp	Basepair
PVDF	Polyvinylidene fluoride
LB	Luria Bertani
dH2O	Distilled water
COX	Cyclooxygenase
PPAR	Peroxisome Proliferator Activated Receptor

PERANAN SIKLOOKSIGENASES DALAM OOGENESIS DAN PERATURAN TRANSKRIPSI MEREKA OLEH RESEPTOR AKTIVASI PEMBIAKAN PEROKSISOM GAMMA

ABSTRAK

Prostaglandin adalah perlu untuk kejayaan perkembangan dan kefungsian sistem pembiakan betina veterbrata. Sintesis prostaglandin bergantung kepada aktiviti pemangkin dua isobentuk siklooksigenase (COX-1 dan COX-2). Pelbagai penemuan telah menunjukkan bahawa kedua-dua isobentuk COX berperanan penting dalam penghasilan prostaglandin, dan juga semasa pertumbuhan, kematangan serta ovulasi oosit. Namun masih terdapat jurang maklumat tentang peranan COX-1 jika dibandingkan dengan COX-2 terutamanya di dalam pembiakan. Walaupun COX-2 telah dikenalpasti sebagai penanda yang kukuh semasa ovulasi berlaku, pada masa yang sama fungsinya dalam perkembangan dan kematangan oosit masih perlu diterokai. Oleh itu, untuk membezakan peranan COX-1 dan COX-2 semasa kematangan dan ovulasi oosit ikan zebrafish, ekspresi mRNA kedua-dua isobentuk ini mula-mula dikaji dalam peringkat-peringkat oosit tersebut. Peningkatan ekspresi mRNA COX-1 folikel zebrafish diperhatikan berlaku semasa oosit tumbuh dan matang. Walau bagaimanpun, ekspresi COX-2 telah terencat secara drastik dalam oosit berkenaan. Lebih-lebih lagi, kepentingan fungsi COX-1 dalam kematangan oosit telah dikenalpasti melalui eksperimen penindasan penghantaran-pengantara morfolino telah mendedahkan bahawa terdapat penurunan peratus folikel vitelogenik yang melalui kematangan spontan *in vitro*. Tambahan lagi, siasatan tentang regulasi COX-1 dan COX-2 oleh reseptor aktivasi pembiakan peroksisom gamma (PPARγ)

yang merupakan peregulasi COX dalam pelbagai aspek kesuburan betina juga dijalankan. Analisis PCR-masa nyata menunjukkan penurunan PPARγ yang signifikan dalam folikel zebrafish yang sedang berkembang dan matang. Apabila dibandingkan dengan COX-1, PPARγ menunjukkan corak mRNA terbalik yang mana ianya menyokong fakta bahawa regulasi transkripsi pengantara PPARγ menghasilkan perencatan gen sasaran. Tiada kolerasi dilihat antara COX-2 dan corak expresi PPARγ. Untuk mengesahkan kebarangkalian bahawa terdapat regulasi transkripsi langsung pengantara PPARγ untuk COX-1 dan COX-2, fragmen promoter 3kb kedua-dua gen diimbas menggunakan perisian Matinspector untuk kehadiran tapak pelekatan PPARγ (PPRE). PPRE telah ditemui di kawasan -2573/-1952 bp promoter COX-1. PPRE tidak ditemui pada COX-2, yang mana ini memansuhkan anggapan bahawa gen ini diregulasi secara langsung oleh PPARγ. Keputusan juga menunjukkan bahawa PPARγ telah menekan COX-1 melalui PPRE yang terletak di kawasan -2573/-1952 bp menggunakan sel-sel HepG2 yang telah di transfeksi dengan pengantar luciferase yang terdiri daripada 2.7 kb promoter COX-1 ikan zebrafish. Esei anjakan mobiliti elektroforetik menunjukkan pelekatan khusus PPARγ kepada kawasan PPRE. Keseluruhannya, kajian ini telah menyerlahkan fungsi COX-1 dalam kematangan oosit dan potensi regulasi oleh PPARγ.

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ABSTRACT

Prostaglandins are required for the successful development and functioning of the female reproductive system in vertebrates. The synthesis of prostaglandin relies on the catalytic activities of two cyclooxygenase isoforms (COX-1 and COX-2). Various reports emphasize that both COX isoforms are essential for the production of prostaglandin during various stages of oogenesis, such as oocyte growth, maturation and ovulation. In comparison to COX-2, there is a paucity of knowledge on the role of COX-1, especially in oogenesis. At the same time, while COX-2 is a well established marker in ovulation, its function in growth and maturation of oocyte requires further exploration. This study, thereby, distinguishes the role of COX-1 and COX-2 during zebrafish oocyte growth and maturation, firstly by examining the mRNA expression of both the isoforms in these oocyte stages. An increase in zebrafish follicle COX-1 mRNA expression was observed as the oocytes develop and mature. However, the expression of COX-2 was drastically inhibited in these oocytes. Further, functional importance of COX-1 in oocyte maturation was verified in a morpholino delivery-mediated knockdown experiment, which revealed a decrease in the percentage of zebrafish vitellogenic follicles undergoing *in vitro* spontaneous maturation. Additionally, regulation of COX-1 and COX-2 by a known COX regulator, peroxisome-proliferator-activated receptor gamma (PPARγ), which

also regulates various aspects of female fertility, was investigated. Real time-PCR analysis showed a significant reduction of PPARγ in developing and mature zebrafish follicles. When compared with COX-1, PPARγ exhibited inverse mRNA pattern in oocytes, which was in agreement with the fact that PPARγ mediated transcriptional regulation results into the inhibition of its target gene. No such correlation was seen in between COX-2 and PPARγ expression pattern. To confirm the possibility of the PPARγ mediated direct transcriptional regulation of COX-1 and COX-2, 3kb promoter fragments of both the genes were scanned using Matinspector software for the presence of PPARγ binding site (PPRE). The PPRE was found to be located within the -2573/-1952 bp region of COX-1 promoter. PPRE was not found on COX-2 promoter, which eliminated the possibility of this gene being directly regulated by PPARγ. Using HepG2 cells transfected with luciferase reporter constructs of 2.7 kb zebrafish COX-1 promoter, it was demonstrated that PPARγ transcriptionally repressed COX-1 through PPRE which was located at -2573/-1952 bp region. Furthermore, electrophoretic mobility shift assay revealed the specific binding of PPARγ to this PPRE region. Overall, this study highlighted the function of COX-1 in oocyte maturation and its potential regulation by PPARγ

CHAPTER 1

INTRODUCTION

1.1 Background

The occurrence of Cyclooxygenases (COX) pathway is essential for the normal functioning of vertebrate physiology. It affects various aspects of gastrointestinal, cardiovascular, muscular, renal, immunological and reproductive systems. Inhibition or overexpression in normal physiological conditions, or even normal expression of COX in abnormal physiological conditions, results in progression of diseases such as cancer, Alzheimer's and heart disorders.COX act as the rate limiting enzymes in production of prostaglandin. COX enzymatically derives prostaglandin from essential fatty acids (EFAs), specifically, arachidonic acid (AA) in almost all the nucleated cells of human body. Two isoforms of COX (COX-1 and COX-2) produce prostaglandin in the vicinity of the target cells by cyclizing and oxygenating AA in autocrine and paracrine manner.

In this study, the role and regulation of COX is examined during oogenesis. Oogenesis is the process of development of the egg. It is one of the key events that determine the success of the reproductive process. Key features of reproduction such as establishment and continuation of pregnancy, embryo endurance and foetus development rely heavily on the health and quality of oocytes, acquired at the growth and maturation phase of oogenesis. A disrupted oogenesis not only results in

reproductive failure, but can also cause diseases in adults. As mentioned above oocyte growth and maturation are two critical events necessary to equip the oocytes with developmental competence. The oocytes go through the process of meiotic arrest at the time of their development from germ cell. Inside the ovary, they grow and attain vitality and vigor. Finally, with the advent of luteinizing hormone surge, they resume meiosis. The process of meiotic resumption is called maturation, a critical process necessary for the oocyte to attain haploid status and get ovulated as egg (ovulation). Even with numerous on-going studies, there is much to be discovered in the maturation process.

Experiments performed on various animal models like *Drosophila*, mouse and zebrafish show that COX derived prostaglandins are indeed required for the successful oogenesis. The role of COX in ovulation is well-documented in humans and other vertebrates. Of the two isoforms, COX-2 is critically important for this process. The induction of COX-2 is believed to be the marker of ovulation. But at the same time, the role of COX-2 in oocyte maturation needs further exploration. It has been reported that COX-1 homologue of *Drosophila* is critical for oocyte maturation (Tootle & Spradling, 2008). However it has not been explored yet, if COX-1 is indispensible for maturation in higher animals as well. Recently, COX-1 expression was reported during human oogenesis along with COX-2. Taken together, it still needs to be determined whether only one or both isoforms of COX are required for the production of prostaglandin during growth and maturation of oocytes. At the same time it is also interesting to know about the regulation of COX during oogenesis.

When it comes to understanding the regulation of COX, its relationship with Peroxisome Proliferator Activated Receptor Gamma (PPARγ) cannot be overlooked.

PPARγ is a nuclear receptor which acts as a transcription factor. COX-2 isoform in human shows the presence of PPARγ binding site on its promoter and on frequent occasions, regulatory inhibition of COX-2 by PPARγ is demonstrated in humans. Similarly, reciprocal expression pattern between COX-1 and PPARγ is seen in diseases like cancer and Alzheimer's. However, a direct regulation of COX-1 by PPARγ still needs to be proven. Interestingly, the fact that PPARγ is known to regulate most of the genes involved with oogenesis and female fertility suggests the possible control of this gene on prostaglandin production during oocyte development via COX regulation.

The aim of this study is to demonstrate the functional role of COX isoforms in oocyte development and maturation. To facilitate the complete picture of COX regulation during oogenesis, this study also aims to subsequently determine the regulation of COX by PPARγ. Zebrafish has been used as the model organism in this study to achieve the aim mentioned above. Zebrafish is a well established model system which comes with a completely sequenced genome and the ease with which reverse genetics can be performed. Being a vertebrate, zebrafish physiological pathways and functions are highly similar to mammals and it does not come with the complications of mouse model or limitations of *Drosophila* model. The ability of zebrafish oocytes to mature *in vitro* is also taken into consideration when it was selected as the model used in this study. Zebrafish produces prostaglandins by COX enzymatic actions via AA pathway similar to human.

This study focuses on providing a clearer understanding of COX regulation during oocyte development (vitellogenesis in zebrafish) and maturation. Nevertheless by doing so, this study also opens up a possibility to use this understanding in acquiring high quality, healthy oocytes, in order to meet the

demands of breakthrough medical techniques like *in vitro* fertilization. Connection between PPARγ and COX can also lead towards the development of novel therapeutic approaches in diseases like cancer and Alzheimer's

1.2 Objective of the study

Target of present study is to establish the role of COX isoforms and their regulation by PPARγ during oogenesis, especially maturation. To achieve this target following objectives were determined.

- To ascertain the expression pattern of COX-1 and COX-2 isoforms in developing oocytes.
- To determine the effect of functional absence of COX on oocyte maturation.
- To elucidate the expression pattern of PPARγ in developing oocytes and its comparison with COX isoform expression.
- To establish the interaction between COX and PPARγ.
- To elucidate the regulation of COX promoter by PPARγ.

CHAPTER 2

Literature Review

2.1 Cyclooxygenase

Cyclooxygenase genes are present in all the vertebrates examined. However their

existence is not known in unicellular organisms, insects and plants. In mammalian species, two different COX isozymes, encoded by separate genes have been identified; COX-1 and COX-2. Both COX proteins have 60% conserved amino acid sequence. They have identical three dimensional structure and they catalyze the same reaction (Kujubu *et al.,* 1991; O'Banion *et al.,* 1991; W. L. Xie *et al.,* 1991)

Arachidonic Acids (AA) are produced by the enzymatic action of COX. AA is a polyunsaturated fatty acid present in the cell membranes. Basically it is a 20-carbon tetraenoic fatty acid (C20:4 ω6) that acts as a precursor for the synthesis of PGs (Bergstroem *et al.,* 1964; Van D *et al.,* 1964). The process of conversion of AA to PGs involves two critical steps. In the first step, AA undergoes cyclization and oxygenation reactions catalyzed by COX enzyme in order to produce prostaglandin G2 (PGG2) which contains endoperoxyside. The second step involves the reduction of a hydroperoxyl present in PGG2 to a hydroxyl to form prostaglandin H2 (PGH2). This step is also catalyzed by COX, with the aid of a separate peroxidase site present on the enzyme. PGH2 is then utilized by numerous isomerases and oxidoreductases to produce various prostaglandins.

2.1.1 Historical background of Cyclooxygenase

COX was first purified from sheep and bovine seminal vesicle (Hemler & Lands, 1976; Minghetti *et al*., 1988). The purified COX enzyme demonstrated cyclooxygenase and peroxides activity on separate sites. It was categorized as integral microsomal membrane protein, based on the requirement of detergents like Tween-20 to solubilize it. The size of enzyme was approximately 67 kDa. COX was found to be inhibited by the action of popular nonsteroidal anti-inflammatory drugs (NSAIDs) (Vane & Williams, 1972). This discovery played a big role in determining the mode of actions of these popular drugs.

In the early 1970s, COX was commonly known as prostaglandin synthetase. However due to the non-requirement of ATP in the COX mediated reaction it was changed to synthase. Currently this enzyme is popularly referred as cyclooxygenase (COX), prostaglandin G/H synthase (PGHS) or prostaglandin endoperoxide synthase (E.C.1.14.99.1). COX (now known as COX-1) was cloned and characterized by De Witt and Smith from sheep vesicular gland. They isolated 2.7 kilobase complementary DNA (cDNA) coding for 600 amino acids (DeWitt *et al*., 1990).

2.1.2 Discovery of two Cyclooxygenase isozymes

Findings like inhibition of COX by acetaminophen in dog brain but not in rabbit spleen (Flower & Vane, 1972), and presence of two catalytically distinct COX activities in acetone powder extracts of sheep vesicular glands (Smith & Lands, 1972) led many researchers to speculate the presence of more than one COX enzyme.

At the same time, researchers also observed the variation in the time course of prostaglandin synthesis. In platelet-derived-growth factor-treated Swiss 3T3 cells,

prostaglandin synthesis was reported twice, once in a matter of few minutes and then after few hours (Habenicht *et al*., 1985) indicating two distinct enzyme mediated activities. However the biggest limitation in the acknowledgement of the presence of another COX isoform was the unavailability of specific antibody and nucleic acid probes. Using the antibody and probes derived from the COX (COX-1) present in the seminal vesicle, only a marginal increase in COX was seen; while prostaglandin synthesis was increased many fold.

In the late 80s and early 90s the possibility of existence of two cyclooxygenases became evident. Rosen and colleagues reported the presence of an inducible 4.0-kb mRNA together with a 2.8-kb mRNA in their northern blot probed by ovine seminal vesicle COX cDNA (Rosen *et al.*,1989).

Finally, Simmons discovered the presence of another form of COX induced by Rous sarcoma virus in chicken embryo fibroblast. Analysis of the resultant protein sequence showed 59% amino acid similarity with sheep COX (COX-1) (Simmons *et al.*,1989). In the same year, Herschman cloned and characterized the COX-2 cDNA from Swiss 3T3 cells induced by 12-O-Tetradecanoylphorbol to produce rapid prostaglandin (Kujubu *et al*., 1991; Varnum *et al.,* 1989). However they called this gene TIS10.

A partial predicted sequence of COX-2 from mouse cDNA was subsequently reported. Using this cDNA as probe, human homologue was identified and named as COX-2 (O'Banion *et al*.1991), finally establishing the existence of two cyclooxygenase enzymes, COX-1 and COX-2.

2.1.3 Cyclooxygenase-1 (COX-1)

As mentioned in section 2.1.1, COX-1 enzyme was first purified from ovine and bovine vesicular glands. Since then, COX-1 cDNA of many species have been cloned successfully. COX-1 is a single copy gene mapped on chromosome number 9 in human and chromosome number 1 in mouse. The length of COX-1 gene is >22 kb with the coding region of 1797 bp. In northern blot analysis, COX-1 cDNA hybridizes with 2.8 kb mRNA species (DeWitt *et al.*, 1990; Diaz *et al.*, 1992; Yokoyama *et al.*, 1988; Yokoyama & Tanabe, 1989). Vertebrate COX-1 has 11 exons and 10 introns. 3' untranslated region of this gene contains only one polyadenylation site in all the species sequenced (Figure 2.1). 5' flanking region of COX-1 harbors several transcription binding sites like two Sp1 motifs, two AP-2 sites, an NF-IL6 motif, and a GATA sequence. COX-1 lacks TATA box (L. H. Wang *et al.*, 1993; W. Xie *et al.*, 1993).

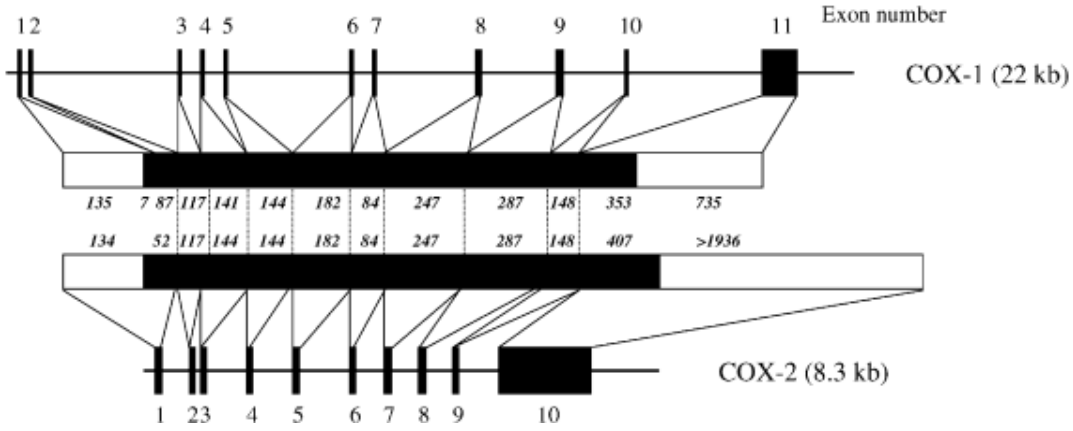


Figure 2.1 Gene structures of Human COX-1 and COX-2. COX-1 has 11 exons and COX-2 has 10 exons. Numbers in italics indicate nucleotides present in each exon. (Adapted from Tanabe and Tohnai 2002).

In mouse, human, rat and sheep COX-1 shares 90% homology of DNA. COX-1 protein is found in the lumen of the nuclear envelops and endoplasmic reticulum. It is characterized as membrane glycoprotein. On the SDS-PAGE it separates as a single band, with a molecular weight of 72kDa (Inoue *et al.*, 1994; Otto *et al.*, 1993). Long signal peptides of varying length in different species of COX-1 are reported. Removal of these signal peptides, produce mature 576 amino acid long COX-1. Like DNA, 90% similarity is reported in the amino acid sequences of mouse, human and sheep COX-1 as well.

2.1.4 Cyclooxygenase-2 (COX-2)

Like COX-1, COX-2 is a single copy gene present on chromosome number 1 of human and mouse (Jones *et al.*, 1993; Kosaka *et al*., 1994; W. Xie *et al*., 1993). The length of this gene is 8-9 kb with 10 exons, 9 introns and a coding region of 1812 bp (Figure 2.1). Its cDNA probe hybridizes around 4-4.5 kb mRNA. The 3' untranslated region of COX-2 is remarkably different from COX-1 due to the presence of 23 Shaw-Kamen motifs (ATTTA) which are related to enhanced mRNA degradation contributing to the RNA instability of this isoform (Shaw & Kamen, 1986). The 3'untranslated region of COX-2 also contains multiple polyadynelation sites, a feature which is different from COX-1. The 5' flanking region of this gene has several potential transcription regulatory elements, including TATA box, an NF-IL6 motif, two AP-2 sites, three Sp1 sites, two NF- κB sites, a CRE motif and an E-box. cDNA of COX-2 of mouse, human and rat show 90% homology (Kosaka *et al*., 1994; Kraemer *et al.*, 1992). All the exon-intron junctions are strikingly conserved in COX-1 and COX-2. However both the genes still have few differences, COX-2 does

not possess the first intron of COX-1 and the other introns of COX-2 are smaller in length than COX-1.

COX-2 protein contains 603 to 604 amino acids. It separates as two bands on SDS-PAGE at 72 and 74 kDa (Inoue *et al*., 1994; Otto *et al*., 1993). COX-2 protein shares 70 to 90% homology across the vertebrate species and shows 60 to 65% similarity with COX-1 protein.

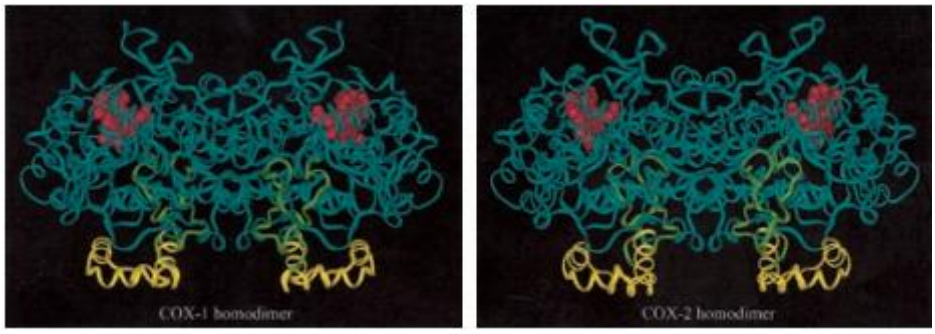
Among the differences between the two COX proteins, the most remarkable is the presence of a large hydrophobic signal peptide at the N-terminus in COX-1 which is replaced by a cleaved smaller signal peptide in COX-2. Similarly at the C-terminus, COX-2 has 18 amino acid insert which is absent in COX-1. This difference of amino acid at the C-terminus contributes towards the specificity of antibodies for both the isoforms.

As stated previously, both COX isozymes have similar enzymatic action and their protein structures are identical; however differences do exist. In section 2.1.5, the protein structure of COX has been described in detail. Differences between the structures of both the isoforms are also mentioned in the same section.

2.1.5 Structure of Cyclooxygenase protein

Both COX isozymes are found in the lumen of nuclear envelop and endoplasmic reticulum. They comprise of four domains. The three dimensional structures of both COX isoforms are almost super imposable (Garavito *et al.,* 2002; Picot *et al.,* 1994). Figure 2.2 B represents the four domains of COX.

A)



B)

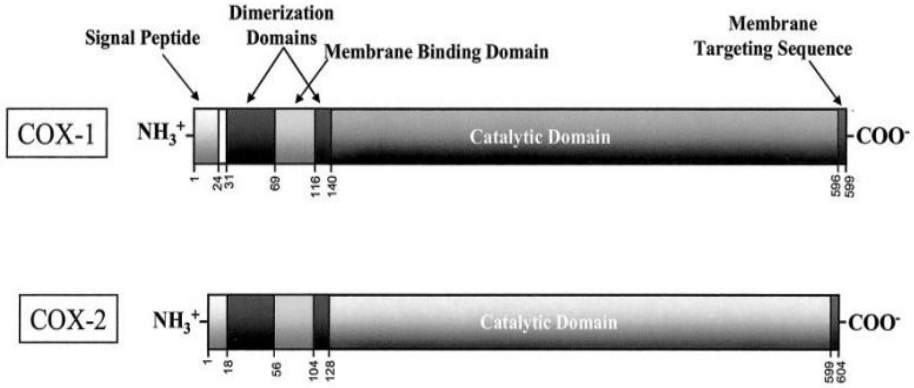


Figure 2.2 Domain structure of COX-1 and COX-2. A) Crystallographic structure of homodimers of ovine COX-1 and murine COX-2 showing membrane binding domain (yellow), dimerization domain (green) catalytic domain (blue) and bound heme (red). B) Diagrammatic representation of COX-1 and COX-2 domains. Numbers represent amino acids comprising each domain. (Modified and adapted from Simmons *et al*., 2004).

Amino Terminal Signal Peptide Domain

Amino terminal signal peptides facilitate the delivery of nascent COX in the lumen of nuclear envelop and endoplasmic reticulum. Signal peptides are hydrophobic and their numbers vary in both protein. The COX-1 signal peptide

consists of 22 to 26 amino acids and a large hydrophobic core with lucines and isolucines. On the other hand, the COX-2 signal peptide is made up of 17 amino acids and is less hydrophobic as compared to COX-1. Also, the signal peptide of COX-1 is followed by 8 amino acids which are missing in COX-2.

Dimerization Domain

COX-1 and COX-2 both exist in the form of dimer (Figure 2.2 A). Each monomer of COX is held together in a dimerization domain by hydrophobic interaction, involving hydrogen bond and salt bridges. There are no reports of heterodimerization between COX-1 and COX-2. This domain consists of 50 amino acids. It is held together by three hydrogen bonds to form a structure resembling the epidermal growth factor. Dimerization domain is linked to the globular catalytic domain by fourth hydrogen bond.

Membrane Binding Domain

As the name suggests, this domain binds cyclooxygenase to the microsomal membrane. The domain is encoded by 50 amino acids, forming a series of four amphipathic helices. These helices create a hydrophobic surface which penetrates the hydrophobic core of the lipid bilayer towards lumen. These helices facilitate the floating of the COX dimer on the surface of lumen. Most of the protein protrudes out in the luminal space. This domain also forms the mouth of the narrow hydrophobic channel of the cyclooxygenase active site.

Catalytic Domain

This domain is made up of 480 amino acid representing 80% of the total COX protein. It is globular with two intervening lobes. It has two individual enzymatically active sites. In the peroxidase active site the intersurface of the intervening lobes create a shallow cleft which acts as the site of peroxidase activity.

Heme binding occurs at this site by iron-histidine bond. Heme binds on this site in such a way that its large portion is exposed in the cleft for its interaction with PGG2 and other lipid peroxides. The cyclooxygenase active site is composed by four amphepathic helices of membrane binding domain mentioned above. It is a long, narrow, dead-end channel of hydrophobic nature. It is 8Å wide and spans around 25Å in globular catalytic domain. The channel narrows down further, Arginine 120 (numbering of amino acids is deduced from sheep COX-1) protrudes in the channel and creates a hydrogen bond network with Glutamate 524 and Tyrosine 355. In COX-1, Arginine 120 is required for the binding of carboxylate containing NSAIDs and other substrates. In COX-2 the presence of Arginine 120 is not required for the binding of substrates and NSAID to happen. The catalytic pocket of the channel consists of Tyrosine 385 which abstracts hydrogen bond from pro-S side of carbon 13 of Arachidonic Acid to form the Arachidonyl radical. The Arachidonyl radical then undergoes cyclization/oxygenation. The hydrophobic pocket of channel contains Serine 530 which together with Valine 349 controls the stereochemistry of the oxygen attack happening on 15 carbon of AA to produce PGG2.

2.1.6 Evolution of Cyclooxygenase

COX-1 and COX-2 are characterized from various vertebrate species, including

bony and cartilaginous fish, birds and mammals. COX is also present in invertebrate organisms. COX of coral and sea squirts have been identified indicating the presence of the Arachidonic Acid pathway in early invertebrate speciation. The presence of isoforms, COX-1 and COX-2 occur to be the result of gene duplication which took place either during early vertebrate speciation or before that (Jarving *et al.,* 2004;

Valmsen *et al*., 2001). Dendrogram in Figure 2.3 shows the phylogenetic relationship between COX of different vertebrate species and their invertebrate ancestry.

COX has not been reported in unicellular organism, insects and plants. However in monocotyledons, dicotyledonous plants, *C. elegans* (*Caenorhabditis elegans*) and bacteria, enzymes known as pathogen-inducible oxygenases (PIOXs) are involved with the oxygenation of polyunsaturated fatty acids. Interestingly, PIOXs share 30% identity with COX (Hornsten *et al*., 1999; Sanz *et al.*,1998).

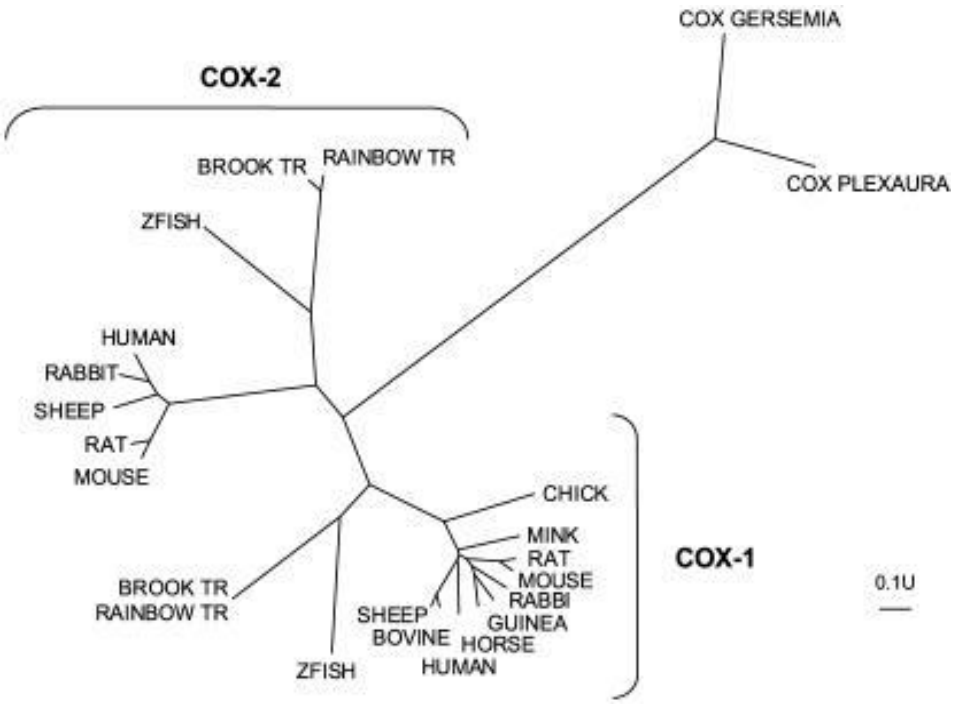


Figure 2.3 Dendrogram representing phylogenetic relationships between vertebrate COX-1 and COX-2 enzymes and invertebrate (Coral) COX enzymes. Genetic distances were calculated using neighbour joining methodology. (Adapted from FitzGerald *et al*.2002)

2.1.7 Mode of Action of Cyclooxygenase enzyme

Cyclooxygenase reaction mechanism has been reviewed in great detail (Chandrasekharan & Simmons, 2004; Simmons *et al.,* 2004; Van der Donk *et al.,* 2002). COX facilitates two activities while converting AA to PGs, a cyclooxygenase activity and a peroxidase activity. The cyclooxgenase activity of COX oxygenates AA to a cyclopentane hydroperoxy endoperoxide known as prostaglandin G2 (PGG2). PGG2 is then reduced to the corresponding alcohol prostaglandin H2 by peroxidase activity of COX (Figure 2.4 A and B). The cyclooxygenase catalysis begins with the activation of the COX enzyme. This process depends on the peroxidase activity of enzyme and thus involves the peroxidase site of catalytic domain. Two electrons are reduced from peroxide substrate causing the oxidation of ferric heme to the oxo-ferryl porphyrin radical cation. An electron is transferred from Tyr-385 of the COX protein to heme resulting in the generation of a tyrosyl radical in the cyclooxygenase active site. This radical abstracts the Pro-S hydrogen from carbon-13 (C-13) of AA, to convert it into Arachidonyl radical. The first molecule of oxygen is then added at C-11 and the second oxygen is added at C-15 to yield PGG2. After the initiation of cyclooxygenase activity, peroxidase reduces the 15 hydroperoxy of PGG2 to form PGH2 (Figure 2.4 C). Due to the regeneration of tyrosyl radical in each catalytic cycle, continues peroxidase activation of cyclooxygenase activity is not required. The catalytic time span of COX is very short (1-2 minutes V max *in vitro*) and the enzyme is auto-inactivated.

PGH2 is the root prostaglandin which is later converted to the prostaglandin isomers like prostacyclin and thromboxane by isomerization and oxidation or reduction.

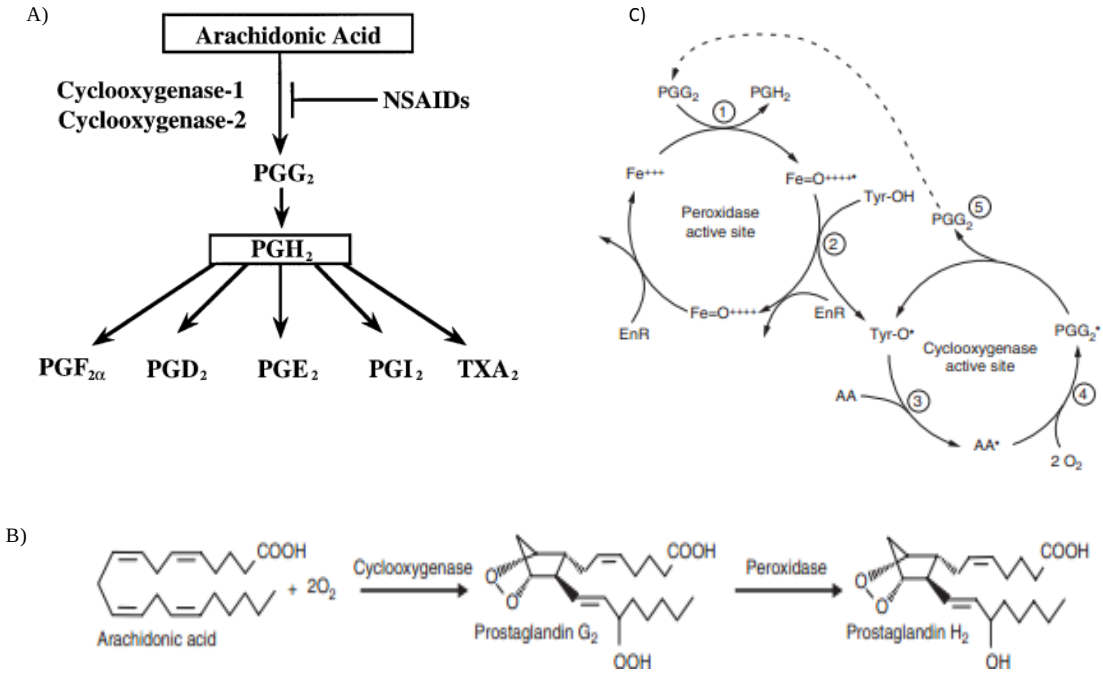


Figure 2.4 Mechanism of action of cyclooxygenases. A) Arachidonic acid pathway flowchart. B) Cyclooxygenase activity of COX enzymes converts AA to PGG2 and peroxidase activity causes conversion of PGG2 to PGH2. C) Cyclooxygenase and peroxidase activity (Adapted and modified from Dubois *et al.*, 1998. Chandrashekharan and Simmons 2004).

2.1.8 Functional Expression of Cyclooxygenase

COX-1 mRNA and protein are expressed constitutively in most of the tissues and cells. It is believed to be a housekeeping gene (Crofford LJ. 1997), attributed to its ubiquitous expression, presence of GC rich 5' flanking region and lack of TATA or CAAT box. COX-1 expression is higher than normal in differentiated cells. Vascular endothelia (DeWitt *et al.*, 1983), platelets (Funk *et al.*, 1991), renal collecting tubule epithelia (Huslig *et al.*, 1979), and monocytes (Lee *et al.*, 1992) are reported to have elevated expression of COX-1.

In normal conditions COX-2 is barely detectable in the tissues (Feng *et al*., 1993). However expression of COX-2 increases significantly in response to proinflammatory factors like IL-1 (Jones *et al*., 1993), TNF α, INFγ, LPS and TPA; hormones: follicle-stimulating hormone (FSH), luteinizing hormone (LH) and estrogen; growth factors: EGF, PDGF and FGF; oncogenes: v-Src and v-Ras (Tanabe & Tohnai, 2002). Even though COX-2 expression is induced, it's constitutive expression is also reported from mouse prostate, brain and rat kidney (Harris *et al*., 1994). High levels of COX-2 have been detected by RT –PCR in human prostate and lungs. Intermediate and low levels of COX-2 are found in human uterus, small intestine, mammary gland, stomach, thymus, liver, kidney, testis pancreas, and brain (O'Neill & Ford-Hutchinson, 1993).

Similarly, even though COX-1 is known to be constitutive in expression, many reports confirm its induction during the differentiation of the cell lines. Induction of COX-1 has been documented even without differentiation. Treatment of TPA causes the maturation of human megakaryoblastic cells to megakaryocyte-like cells, at the same time increasing the expression of COX-1 mRNA and protein 5-20 folds (Ueda *et al.,* 1997). Sheer stress also causes the increase in COX-1 level in HUVEC (Human Umbilical Vein Endothelial Cell) which lasted for 12 hours (Okahara *et al.*, 1998). COX-1 is also induced in during lipopolysaccharide (LPS)-mediated inflammatory response. TGFβ, VEGF, tobacco carcinogen and retinoic acid are some of the known COX-1 inducers which are extensively studied and reviewed (Goppelt-Struebe, 1995). Based on the findings mentioned above, it can be stated that the constitutive ness and induction of COX-1 and COX-2 is process and tissue specific. Their expression cannot be categorized as either constitutive or induced.

2.1.9 Physiological and pathophysiological functions of Cyclooxygenase

Both COX isoforms are encoded by different genes, however the difference doesn't end here. COX-1 knock-in at COX-2 locus of mouse shows that COX-1 does not completely compensate the PGI2 deficiency resulting in the phenotypes with renal and reproductive defects (Yu *et al*., 2007). This study clearly demonstrates that both the isoforms are not interchangeable at the protein level. It is evident from the studies that both of the isoforms have different expression patterns and they play different roles physiologically. The physiological and pathophysiological functions of COX have been reviewed elsewhere (Morita, 2002; Simmons *et al*., 2004).

COX-1 is expressed in vascular endothelial cells and smooth muscle cells. COX-1 derived prostacyclin is critical for blood flow, blood pressure and anti-aggregation of platelets. In platelets only COX-1 is present, and thromboxane A2 generated from COX-1 is required for thrombosis (Matijevic-Aleksic *et al.,* 1995). During megakaryogenesis both COX-1 and COX-2 are detected (Morita *et al*., 1995). When increased COX-2 expression is reported in pathological condition, it is known to promote the hypotension (Leach *et al.,*1998).

COX-1 is important for the normal function of kidney. It is involved in glomerulogenesis, and regulation of renal blood flow. In adult humans, COX-1 signals are detected in collecting ducts, the loops of Henle, interstitial cells, endothelial cells, smooth muscle cells and pre- or post-glomerular vessels. Fetal kidney also shows the expression of COX-1 in podocytes and collecting duct cells (Komhoff *et al.,*1997).

COX-2 is expressed in renal vasculature, medullary interstitial cells, and the

macula densa. COX-2 is also detected in the podocytes at advanced stages of renal development suggesting the requirement of COX-2 in renal perfusion and glomerular hemodynamics (Nantel *et al*., 1999).

COX-2 is involved in the functioning of the brain, it plays a key role in neuronal development and adaptation. COX-2 is activated during the advanced stage of brain development and brain modeling. Its activation corresponds to the environmental influences on developing brain (Kaufmann *et al.,* 1997). COX-2 remains involved with the neuronal responses in adult life. Dramatic increase in the level of COX-2 has been reported after seizures. COX-2 has also been associated with neuronal degeneration (Tocco *et al*., 1997).

Much less is known about the involvement of COX-1 in brain functions. Many findings suggest that COX-1 has been detected brain related disease and brain injuries. It has been proposed that COX-1 may contribute to CNS pathology as it has been detected in various parts of injured and Alzheimer-affected human brains (Yermakova *et al.,* 1999).

COX-1 is known to play major role in the maintenance of the glandular architecture. It is also involved with the crypt cell regeneration. COX-1 expression is inhibited during the exposure of intestinal epithelial cell to radiations that also cause the death of these cells (Cohn *et al.,* 1997). COX-2 expression is induced in response to infection or bacterial invasion in epithelial cells. It mediates the flushing of bacteria from the intestine (Eckmann *et al*., 1997).

Amniotic fluid is discovered as one of the earliest sites where PGs were found. Similarly contraction of the uterine myometrium was one of the first known biological function attributed to PGs. Thus it is evident that COX play major role in female reproductive physiology. Both the isoforms are responsible for the

development and successful function of female reproductive system. As this study has been conducted in the same area, the topic is described in detail in section 2.1.10.

Both the isoforms are involved with the process of inflammation. However their functions are different. COX-1 is involved in the resolution of inflammation and COX-2 is required for the progress of inflammation.COX-1 expression in glomeruli during the repair from experimentally induced mesangioproliferative glomerulonephritis and delaying of the ulcer healing process in the presence of COX-1 selective inhibitors, indicates that COX-1 is involved in healing (Hartner *et al.,* 2000).

Dramatic increases in COX-2 mRNA level in inflamed tissues and its inhibition by NSAIDs which are also known to reduce inflammation exhibit that COX-2 is involved in the progression of inflammation (Hempel *et al.,* 1994; Samad *et al*., 2001).

Cyclooxygenases are involved in the development of malignant tumors. COX-1 expression is detected in vascular endothelial cells. COX-1 is involved in angiogenesis, growth of tumors, endometrial growth, wound healing and inflammation. Inhibition of COX-1 using antisense oligo suppresses tube formation induced by colon cancer (Tsujii *et al*., 1998). While there is little knowledge about COX-1 in tumor formation, many findings show the heavy involvement of COX-2 in tumorigenesis. Tumor cells escape the apoptosis and enter the matrix when COX-2 is over expressed (Sheng *et al.,* 1998; Tsujii & DuBois, 1995).

2.1.10 Role of cyclooxygenases in female reproduction

Success of reproduction largely depends on the successful development and function of the female reproductive system. A well-developed healthy egg is the

prerequisite for successful fertilization and embryonic development. The process of development of egg is called oogenesis which involves development of oocytes, their maturation (resumption of meiosis) and ovulation (rupture of follicle and release of egg). The step by step process of oogenesis is described in section 2.3 and 1.2.4.3. Oogenesis is a complicated process involving intervention of various enzymes, growth factors and hormones. A lot of signaling occurs between oocytes and follicles (somatic cells surrounding oocytes) in order to deliver the mature egg. Developing oocytes are arrested in Meiotic prophase-1. Resumption of meiosis occurs during oocyte maturation. The process of oocyte maturation which is described in section 2.3.2 in detail is a developmental milestone for oocytes, as the release or ovulation of egg depends on maturational success. However due to the lack of complete knowledge of involvement and regulation of hormones, enzymes and growth factors which come into play, extensive exploration of this process is still required.

Cyclooxygenase produced prostaglandin are known to influence various aspects of female reproduction. Many studies have established the involvement of prostaglandin and COX in oogenesis. Prostaglandins have the reputation of being the modulator of oogenesis. In other words, they do not induce oogenesis but their presence or absence transforms oogenesis in a positive or negative way. Prostaglandins are known to mimic the actions of luteinizing hormone (LH). They are required for follicle maturation and rupture, oocyte maturation, ovulation (Armstrong, 1981; Armstrong *et al.*,1974; Lau *et al.,* 1974) and formation and regression of corpus luteum (Howard & Britt, 1990). PGs are also required for the menstrual shedding of the endometrium of primates (Eldering *et al.,* 1993). Most of these actions are reported to be obliterated in the presence of cyclooxygenase

inhibitors including germinal vesicle breakdown which happens in the oocytes when they undergo maturation (Takahashi *et al.*, 2006).

Researchers have shown the requirement of both the COX isoforms to ensure the success of female reproductive process. However, compared to COX-1, COX-2 has been studied extensively in the process of oogenesis. COX-2 null mice demonstrate infertility, abnormal ovulation, implantation and fertilization (Lim *et al*., 1997). It has also been shown that COX-2 derived PGE_2 is critical for oocyte maturation (Takahashi *et al*., 2006). Also COX-2 selective inhibitor is known to inhibit the Mitogen Activated Protein Kinase (MAPK) activation. The COX-1 null mice were completely fertile although their parturition was impaired (Langenbach *et al*., 1995). Recent studies like enhanced COX-1 derived PGE_2 level in monkey ovarian surface epithelium (Cabrera *et al.*, 2006), increase in the level of COX-1 in salmon and brook trout during ovulatory stage (Daikoku *et al*., 2005; Roberts *et al.*, 2000), continuous increase in the amount of COX-1 as zebrafish oocytes reach towards maturation (Grosser *et al.*, 2002; Lister & Van Der Kraak, 2008; Lister & Van Der Kraak, 2009), indicate the definite but poorly understood role of this isoform in oogenesis. The involvement of COX-1 in the major events of oogenesis has gained strong support in recent times, ever since its expression was reported in human ovary. Like COX-2, COX-1 was also detected in cumulus cell and was shown to be regulated by follicle stimulating hormone (FSH) in the same way as COX-2 (Adriaenssens *et al* 2010.), thus indicating the possibility of synergy between COX-1 and COX-2 in oogenesis.

It can be deduced from the facts mentioned above that these isoforms are involved in oocyte development and maturation, but the degree of their involvement and regulation remains unclear.

2.1.11 Regulation of Cyclooxygenase

Cyclooxygenase regulation is a complicated process as it differs in different cell types as well as in the same cells type of different species. Many stimuli via various signaling pathways activate both, the COX-1 and COX-2, genes. The type of stimulus, time of stimulus and cell type determines which isoform of COX will contribute to a given signaling pathway at that point in time. These conditions also determine the association of the transcription factor with COX promoter. The basic understanding of COX-2 regulation has been established now but same is not true for COX-1. There still is paucity of knowledge about the regulation of COX-1.

Several potential transcriptional regulatory element binding sites have been identified in the 5' flanking region of human COX-2 including a peroxisome proliferator response element (PPRE), two cyclic AMP response elements (CRE), a sterol response element (SRE), two nuclear factor kappa B (NF-κB) sites, an SP1 site, a CAAT enhancer binding protein (C/EBP, or nuclear factor for interleukin-6 expression (NF-IL6) motif, two AP-2 sites, an E-box, and a TATA box (Kang *et al.*, 2007) . Interspecies differences have also been found in the promoter region of COX-2 in terms of presence, absence and arrangement of these binding sites in human, mouse, rat, cow and horse. Involvement of COX-2 in various cell types and in various signaling pathways has been studied extensively. Still, the fact that regulation of COX depends on various stimuli at different time points and in different signaling pathways makes it difficult to establish the complete understanding of its regulation.

Classically, it has been believed that COX-1 exhibits housekeeping features while COX-2 is inducible. The COX-1 promoter has many transcriptional start sites, its GC rich and it lacks canonical TATA or CAAT box attributing to its

housekeeping job (Figure 2.5). Transcription factor SP1 is known to be involved with the constitutive COX-1 expression in human umbilical vein endothelial cells (HUVEC). Mutation in one or both the sites reduces the COX-1 expression by 29% (Tanabe & Tohnai, 2002). In many cases, COX-1 expression is induced contrary to the belief that its expression is constitutive . The promoter of COX-1 also consists of many putative binding sites for different transcription activators. Inducing agents for COX-1 are mentioned in section 2.1.8. However the potential of COX-1 promoter has not been studied and other than a few reports, the regulation of COX-1 remains unknown.

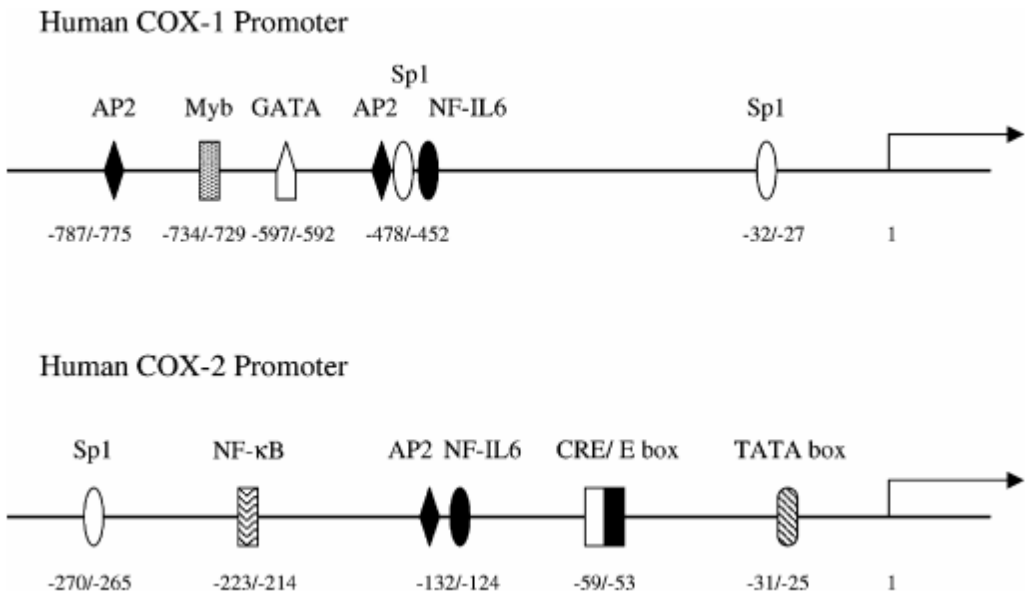


Figure 2.5 Schematic presentation of regulatory elements in Human COX-1 and COX-2 promoter. Arrow indicates transcription start site. (Adapted from Tanabe and Tohnai 2002).

Together with induction, transcription factors are also involved in cyclooxygenase suppression. Peroxisome proliferator activated receptor gamma

(PPARγ) and glucocorticoids are major COX suppressers (Inoue *et al.,* 2000; Newton *et al.,* 1998; Scheinman *et al.,* 1995; Subbaramaiah *et al.,* 2001). Glucocorticoids are known to suppress the action of both COX-1 and COX-2. PPARγ is known to be the master regulator of female fertility. Like prostaglandin, PPARγ is also expressed in granulosa cells of ovary and is regulated by LH. Thus the possibility of PPARγ mediated regulation of COX in oogenesis cannot be ruled out.

However, to date, PPARγ mediated suppression is only studied in COX-2. The presence of PPRE (binding site for PPARγ) is reported on human COX-2 promoter but direct suppression of COX-2 expression by PPARγ is not shown distinctly and PPARγ- mediated suppression of COX-1 has not been explored but the likelihood of this event cannot be overlooked

2.2 Peroxisome proliferator activated receptor gamma (PPARγ)

Peroxisome proliferator activated receptor gamma (PPARγ**)** is a ligand inducible transcription factor which belongs to the nuclear hormone receptor (NHR) super family. Nuclear receptors are transcription factors with multiple domains, which regulate the expression of their target gene by binding to a specific DNA sequence present in the promoter of that gene. NHR super family contains receptors for thyroid hormone, steroid hormones and vitamin D, together with PPAR. PPAR has three isoforms, PPARα, PPARβ/δ and PPARγ**.** Each isoform is encoded by different genes present on different chromosome (Desvergne & Wahli, 1999; Hihi *et al.*, 2002). The expression patterns of these isoforms are distinct from each other. To date PPARs have been identified from a variety of species including human, rodents,

fish and insects (Escher & Wahli, 2000; Ibabe *et al.*, 2002; Meng *et al.*, 2005). PPARγ and PPARα isoforms are studied in great detail compared to PPARβ/δ.

2.2.1 Structure, function and tissue distribution of PPAR isoforms

Structurally, PPAR isoforms resemble each other and their domain structures are similar to any other steroid receptors. PPAR consists of four domains A/B, C, D and E/F (Desvergne & Wahli, 1999; Diradourian *et al.,* 2005; Lazennec *et al.*,2000).

A/B domain is proximal to N terminal and is the most poorly conserved domain. It acts as a ligand independent transcription activator. This domain either provides site for protein phosphorylation or it directly interacts with other regulatory proteins and receptor domains. Due to their ligand independent transcription activation function they are also designated as AF-1.

C domain is the central DNA Binding Domain (DBD). This domain is highly conserved and contains 2 zinc finger binding sites which recognize the PPRE (peroxisome proliferated response element) present at the 5' flanking region of the targeted gene. PPRE sites are discussed in detail in section 2.2.4.

D domain, or the hinge region, plays a role in receptor dimerization and cofactors interaction. E/F domain is also known as Ligand Binding Domain (LBD). Hydrophobic molecules bind to LBD to facilitate the binding of co-regulators. As LBD exhibits ligand dependent transcriptional activation function, it is also referred to as AF-2. Structural and functional domains of PPARγ are shown in Figure 2.6.

PPARα is highly expressed in liver, heart, small intestine, kidney and brown adipose tissues. It is known to play a major role in fatty acid metabolism and it is also involved in inflammation. PPARβ/δ is ubiquitously expressed and is basically involved in development, embryo implantation, wound healing, proliferation of

epidermal cell and lipid metabolism. PPARγ is expressed in adipose tissues mainly; however it is also expressed in colon, retina, immune systems, ovary and testis. It is involved in adipocyte differentiation, glucose and lipid homeostasis, cell cycle, carcinogenesis and inflammation (Desvergne & Wahli, 1999; Kersten *et al.*, 2000).

Of the three isoforms, PPARγ is mostly involved with various aspects of female fertility. PPARγ is detected in the ovaries of the mouse (Cui *et al*., 2002), rat (Komar *et al.*, 2001), pig (Schoppee *et al.*, 2002), sheep (Froment *et al*., 2003), cow (Lohrke *et al*., 1998), human (Lambe & Tugwood, 1996) and zebrafish (Ibabe *et al*., 2002). Strong expression of PPAR γ is detected in granulosa cells of various species. PPAR γ is also expressed in different developmental stages of follicles and is down regulated when LH surge occurs (Froment *et al*., 2003; Komar *et al*., 2001; Komar & Curry, 2002). Direct and indirect involvement of PPAR γ in oocyte maturation has been speculated; however there are no direct reports to prove such involvement.

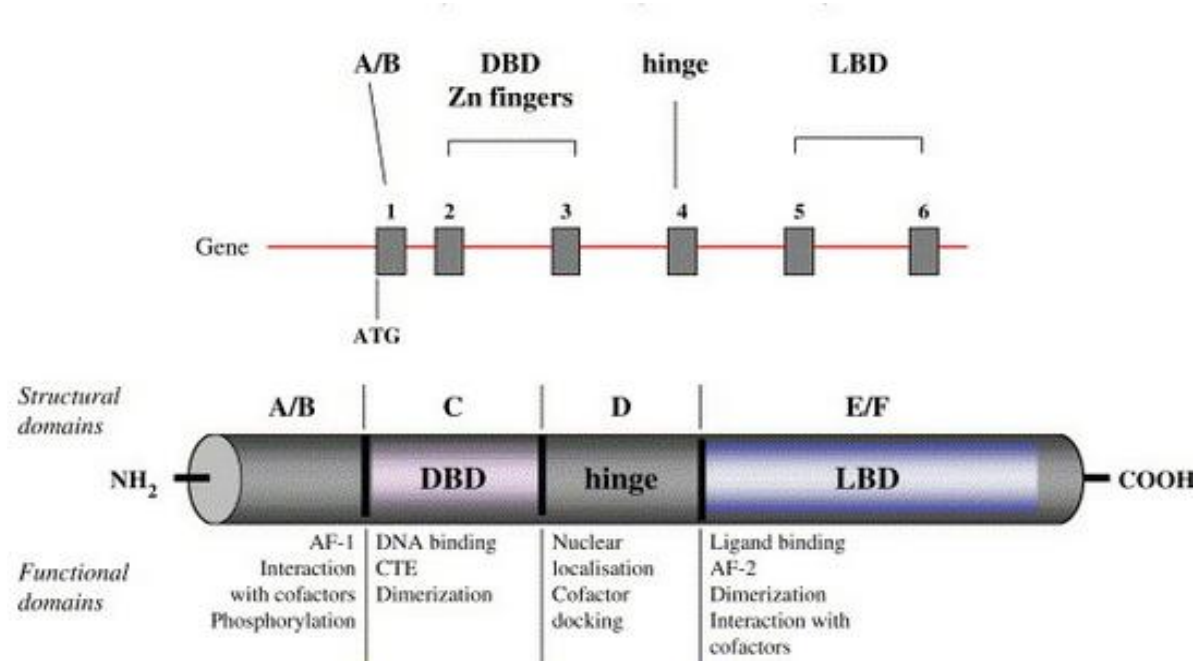


Figure 2.6 Structural and functional domains of PPARγ. Dark boxes represent the exonic region of four domains. (Adapted and modified from Evain-Brion *et al*., 2007)

2.2.2 Activation of PPAR

PPAR are basically involved in the transcriptional activation of their target gene. They were first identified as the receptors that are activated in the presence of broad spectrum of chemicals known as peroxisome proliferators. These chemicals are known to be involved in the function of peroxisome proliferation in rodents (Issemann & Green, 1990). To date, diverse range of natural and synthetic PPAR ligands have been identified including polyunsaturated fatty acids, eicosanoids, antidiabetic drugs and hipolypidemic agents (Forman *et al.*, 1997; Kliewer *et al*., 1997; Krey *et al*., 1997). PPAR binds to the targeted gene on the special sequence present on their promoter known as PPRE. Classically, PPRE are the direct repeat element of DR-1 types separated by one base pair. However the PPRE sequences are

not completely conserved; they show sequence diversity in different genes as well as different species. PPRE are discussed in detail in section 2.2.4.

PPAR forms heterodimer with retinoid X receptor on PPRE of target gene (Keller *et al.*, 1993; Kliewer *et al.*, 1992). Presence of the ligands for PPAR and RXR results in the conformational changes in LBD domain (E domain) which causes the release and recruitment of corepressors and coactivator proteins. As shown in Figure 2.7, this process is followed by the assembly of protein complex and subsequent transcriptional activation of the targeted gene (Xu *et al.*, 1999). Most of the PPAR targeted genes have roles in lipid and glucose metabolism and most of the PPAR ligands are the substrate and /or the product of the enzymes encoded by genes regulated by PPAR.

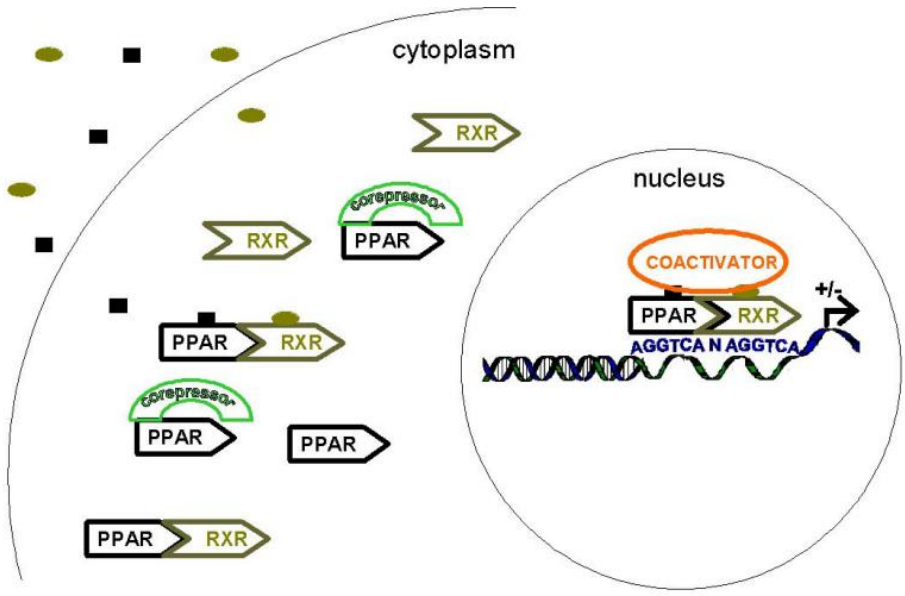


Figure 2.7 Mechanism of PPARγ action. PPARγ heterodimerizes with RXR in the presence and absence of ligands, causing conformational changes in LBD. The conformational changes result in dissociation of corepressor and binding of coactivators. Activated PPARγ protein complex binds to DR1 site present on the promoter of target gene. (Adapted from Komar 2005).

2.2.3 PPAR ligands and cofactors

Specificity of some degree can be seen in the ligands of PPAR isoforms. Thiazolidinediones like troglitazone, ciglitazone, pioglitazone, rosiglitazone and NSAID which inhibit COX actions are specifically PPAR γ activating ligands (Escher & Wahli, 2000). Prostaglandins can activate all PPAR members but some specific prostaglandins preferentially activate PPAR γ and PPARδ (Forman *et al*., 1995). Fibrates and polyunsaturated fatty acids prefer to activate PPARα. Their higher concentrations also activate PPARγ (Desvergne & Wahli, 1999).

PPAR cofactors can be corepressors or coactivators. Conformational changes in the LBD that occur due to the ligand binding cause the dissociation of corepressor from the receptor and binding of the coactivators resulting the activation of repressed receptor. Nuclear receptor corepressor (NCoR) and silencing mediator for retinoid- and thyroid-hormone receptors (SMRT) are some commonly known receptor repressors (Zhu *et al*., 2000).

Steroid receptor coactivator-1 (SRC-1) and CREB binding protein/p300 (CBP), RIP140, ARA70, members of the DRIP/TRAP family, PPAR interacting protein PPAR γ coactivator-1 and PPAR binding protein (PBP) are PPAR specific coactivators which mostly bind to PPAR in ligand dependent manner (Berger & Moller, 2002). However some of them also bind to PPAR in ligand independent manner.

2.2.4 Peroxisome proliferator response element

PPAR-RXR heterodimer binds to the DNA sequence present on the promoter of the target gene. This DNA sequence is known as Peroxisome Proliferator activated Receptor Element or PPRE. In a classical case, PPRE is a direct repeat of

sequence AGGTCA separated by a random base (AGGTCA N AGGTCA). The first of the PPRE is taken by PPAR and second half by RXR. The 5' flanking region adjacent to PPRE has been reported to play some role with the PPAR binding to DNA (Chandra *et al*., 2008).

Analysis of different genes in the same and different species shows that PPRE sequence is not completely conserved. A lot of variations from the above-mentioned wild type sequence have been seen. In mouse, human and chimpanzee, PPRE sequences for Catalase gene differ from each other (Okuno *et al*.).Sequence of PPRE in human carnitine palmitoyltransferase (CPT) is only partially conserved (Barrero *et al.,* 2003). Meta analysis of large number of PPRE obtained from genome wide screening of PPAR targeted genes shows a lot of variation from the normal consensus. Occurrence of bases and their positions on PPRE can result into strong, medium or weak binding of PPAR protein. For example, presence of G continuously on first, second and third position shows strong PPARγ binding (Heinaniemi *et al.,* 2007).

2.2.5 Role of PPARγ in oogenesis

As mentioned above, PPARγ acts by regulating its target genes, involved in various pathways. Many important pathways are known to be active at the time of oogenesis and it has been reported that PPARγ regulates the rate limiting enzymes involved in these pathways. PPARγ can suppress or enhance the expression of same gene based on the availability of ligands, cofactors, cellular environment and requirement. It has been seen that PPARγ mostly regulates genes involved in the critical oogenetic events and causes inhibition of their expression.

PPARγ is presumed to be the master regulator of female fertility; disruption of PPARγ in female mice resulted in sterile and sub-fertile females with severe inconsistency in their normal ovarian function (Cui *et al*., 2002). As mentioned in section 2.2.1, its expression is detected in the ovary of various species. PPARγ expression is high in granulosa and theca cells (Schoppee *et al*., 2002). It is also detected from the oocytes of zebrafish (Ibabe *et al*., 2002), cattle (Mohan *et al*., 2002) and xenopus (Dreyer & Ellinger-Ziegelbauer, 1996). In mammals, like sheep and rats, PPARγ expression is restricted to the granulosa cells of developing follicles. Other isoforms of PPAR expressed in ovary have consistent expression throughout, contrary to the expression of PPARγ that downregulates with the LH surge. It has been reported that PPARγ is endogenously active in the ovary because the promoter reporter construct the expression of which is driven by PPRE was transfected in granulosa cell of sheep and rat, showed enhanced activity in the presence and absence of exogenous agonists (Froment *et al*., 2003). Thus, it can be deduced that PPARγ is functional in granulosa cells and the ligands required for its activation are also endogenously present in granulosa cells. Presence of complete PPARγ machinery granulosa cells and its downregulation with the arrival of LH surge establishes that PPARγ regulates oocyte maturation.

PPARγ is also involved in the regulation of steroidogenesis. The ovaries with disrupted PPARγ show reduction in the level of progesterone. It regulates the remodeling of ovary in all the aspects right from the development of a primordial follicle to the ovulated egg. PPARγ is also involved in cell cycle regulation and ovarian tumor development (Komar, 2005).

2.2.6 PPARγ mediated regulation of genes involved in oogenesis

As pointed out in section 2.2.5, there is a significant involvement of PPARγ in regulation of ovarian functions. As a well known transcriptional regulator, PPARγ is known to control the transcription of the genes critically involved with oogenesis. Interestingly, the preferred mode of control that PPARγ exhibits on the ovarian genes is downregulation. For example, PPARγ inhibits the gonadotropin induced progesterone production and level of aromatase in granulosa cells (Yanase *et al*., 2001). An inverse relationship between the mRNA of PPARγ and the mRNA of P450 the rate limiting enzyme in the production of progesterone in rat garnulosa cell also goes in favor of this argument (Keller *et al.,* 1995; Komar & Curry, 2003). PPARγ mediated direct transcriptional downregulation of promoter of proteases like MMP-3 and MMP-9 has been demonstrated by ensuring the presence of PPRE site on them, suggesting the involvement of PPARγ in tissue remodeling(Eberhardt *et al*., 2002; Yee *et al.,* 1997). Similarly, PPARγ also binds with Estrogen Response Elements (EREs) and regulate the ability of estradiol to produce the cellular responses which influence ovarian functions (Keller *et al*., 1995; Nunez *et al*., 1997).

Few more examples of the genes involed with ovarian functions and exhibiting the downregulational control of PPAR γ are stated further. Genes involved in the blood vessels formation in ovary, like vascular endothelial growth factors and their receptors are also reported to be inhibited by PPARγ. Likewise endothelin-1 (ET-1) and nitrous oxide synthase (NOS) genes, which contribute in ovarian vasculature formation as well as in ovarian cyclicity, ovulation, oocyte maturation and follicular development are also reported to be inhibited by the intervention of PPARγ (Jablonka-Shariff & Olson, 1998; Jablonka-Shariff *et al.,* 1999; Matsumi *et al*., 1998; Nakamura *et al*., 1999). Figure 2.8 demonstrates how

PPARγ controls various aspects of female fertility by regulating the genes involved in ovarian functions.

2.2.7 PPARγ-mediated regulation of Cyclooxygenase

Interestingly, it has been frequently reported by researchers that AA which is a substrate of COX (Kliewer *et al*., 1997) and PGs which are product of COX act as the activators of PPARγ (Negishi & Katoh, 2002) . Even the selective and non selective COX inhibitors are reported to activate PPARγ in higher concentration (Eibl *et al.,* 2004; Funahashi *et al*., 2007). In various forms of cancers like colon cancer, pancreatic cancer, ovarian cancer etc, increased expression of COX and reduced expression of PPARγ is evident (H. J. Kim *et al*., 2007). While most of the researchers are focused on the COX-2 and PPARγ expression patterns, upregulation of COX-1 in ovarian cancer indicates that possibility of COX-1 regulation by PPARγ cannot be denied as well. (Daikoku *et al*., 2006). In the gastric mucosa of children infected with *Helicobacter pylori* COX-1 and COX-2 expression was upregulated and PPARγ expression was downregulated (Haruna *et al*., 2008).

Findings like induction of COX-2 promoter getting inhibited by PPARγ agonists like rosiglitazone, clearly points out towards direct or indirect possibility of regulation of COX by PPARγ (Funahashi *et al*., 2007). The Cox-2 promoter is reported to contain an upstream PPRE in human at the position -3721/-3707 (Meade *et al.,* 1999; Pontsler *et al.,* 2002). The reciprocal and negative regulation between COX-2 and PPARγ has been demonstrated in human cervical cancer cells (Daikoku *et al*., 2006). However a clear approach showing that PPARγ transcriptionally downregulates COX is in normal physiological processes is still not available.

Prostaglandins are the modulator of oogenesis, influencing development, maturation, ovulation and oocyte growth and PPARγ is the key regulator of female fertility (Minge *et al.,* 2008). The speculations, that PPARγ can mediate COX regulation during the events of female reproduction are obvious because both prostaglandin and PPARγ are detected in granulosa cells (Froment *et al*., 2003) and low expression of PPARγ and increase in amount of PGs is seen with the occurrence of the LH surge (Banerjee & Komar, 2006; Duffy & Stouffer, 2001). As mentioned before, prostaglandins are also PPARγ ligands which can endogenously activate PPARγ. Their presence together in granulosa cells of developing and mature follicles implies the possibility of PPARγ mediated regulation of the production of prostaglandins by regulating the rate limiting enzyme COX. A cyclic relationship between the presence and absence of prostaglandins, activation and/or inhibition of PPARs and feedback to the prostaglandin synthesizing enzyme COX is evident.

PPARγ mediated regulation of COX is not a mere presumption, presence of PPRE has been reported long back on human COX-2 promoter (Meade *et al*., 1999), indicating direct control of PPARγ on COX-2 gene. An inverse relationship between COX-2 and PPARγ mRNA expression has been seen in placenta (Dunn-Albanese *et al.,* 2004). In rat granulosa cells, reduction of PPARγ and increment in COX-2 has been reported in the follicles nearing ovulation (Komar *et al*., 2001; Sirois *et al.,* 1992).

LH surge causes the maturation and ovulation of oocytes. While maturation is the process where oocytes resume the meiotic division and develop into egg, ovulation is the process of release of this egg from ovarian follicles. Connections have already been established between PPARγ and COX-2 in ovulation (J. Kim *et al*., 2008). Nonetheless, oocyte maturation is a lesser-explored field in this context.

With the studies being conducted recently, even the role of COX-1 cannot be denied in oogenesis. As we have seen earlier, PPARγ-mediated regulation is dependent of cell type, cofactors, ligand, environment and process; so the possibility of other COX isozymes (COX-1) being regulated by PPARγ cannot be overlooked (Figure 2.8).

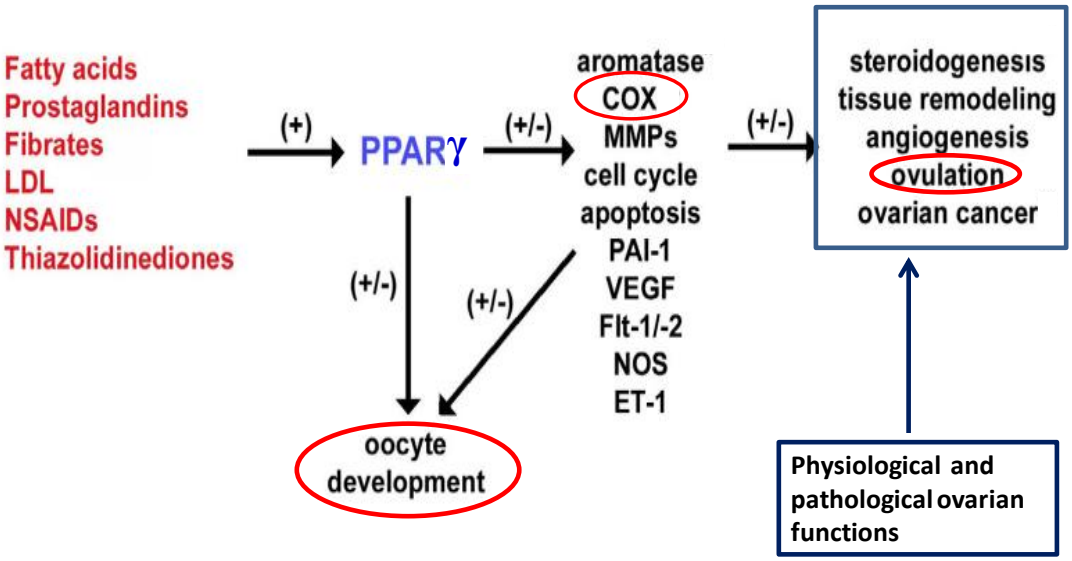


Figure 2.8 Impact of PPARγ on ovarian function. PPARγ controls all the aspects of female fertility by inhibiting or enhancing the expression of genes (including COX) involved in ovarian functions. Possible interaction of COX, PPARγ and ovarian function is demonstrated by encircling them. (Adapted and modified from Komar 2005).

2.3 Vertebrate oocyte development (Oogenesis)

The fact that oocytes have the capacity to transform from a single cell into a totipotent linage, which produces the early embryo, makes oogenesis a critical process for reproductive success (Rodrigues *et al.*, 2008). All vertebrate eggs are developed from the primordial germ cells (PGCs). PGCs develop from the zygote

and migrate to the genital ridge. The bipotential genital ridge either develops into ovary or testis. Eventually when the ovary is formed from the undifferentiated gonad it allows the migrated PGCs to differentiate into primary oocytes (Edson *et al.*, 2009). These primary oocytes get surrounded by the single layer of flattened epithelial cells and become primordial follicle. The first step of meiosis occurs in the primordial follicle, after which its DNA replicates, followed by the immediate meiotic arrest at diplotene step of prophase 1. With the advent of the LH surge, the oocytes again resume meiosis and transform themselves to metaphase 1. This process is known as meiotic maturation. At the end of this, ovulation takes place. The mammalian female reproductive cycle is represented in Figure 2.9.

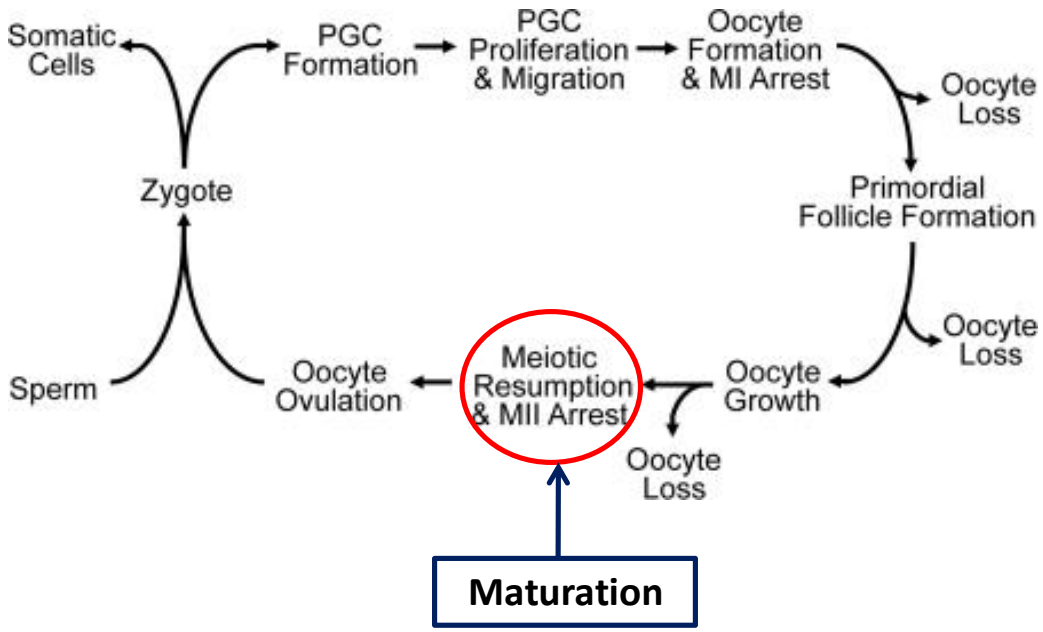


Figure 2.9 Reproductive cycle of a mammalian Female. Sperm and egg fuse to form a diploid zygote which divides to form somatic and primordial germ cells (PGC). PGCs migrate and form oocytes. Oocytes undergo first meiotic division (M1) but get arrested at prophase 1. They grow and develop eventually and resume

meiosis after which the haploid egg (ovulation) is formed. (Adapted from Matzuk *et al*., 2009).

2.3.1 Meiotic arrest (Oocyte growth and vitellogenesis)

As described in section 2.3.1 primordial follicles enter into meiosis and get arrested in the diplotene stage of prophase 1 (Figure 2.9). In fish and amphibians, during this arrest, oocytes undergo massive growth and vitellogenesis where these oocytes assemble nutritional reserve required for the development of embryo after fertilization. At the same time these oocytes also accumulate maternal RNA and differentiate their cellular and noncellular envelope. Oocytes complete their vitellogenesis before undergoing meiosis resumption (Lubzens *et al.*, 2010). In many vertebrates including humans, oocyte development is suspended after birth for months or years, and gets resumed when the female reaches puberty. The oocytes undergo further development and they grow up to a thousand-fold in size (Wolpert, 2002).

2.3.2 Meiotic resumption (Oocyte maturation)

Oocytes of vertebrates are arrested in the meiotic prophase-1 and complete the meiosis in response to the intracellular signaling that occurs during the process called maturation. In other words, maturation is the elevation of oocytes arrested at the diplotine stage of prophase-1 to the metaphase-2. This resumption of meiosis is essential for the normal ovulation and the fertilization (Jamnongjit & Hammes, 2005). The term ‘meiotic maturation’ was coined by Wilson, describing it as the cascade of physiological changes that occur once the meiosis is resumed in the oocytes until zygote formation (Wilson, 1925). Germinal vesicle break down,

chromosome condensation, spindle assembly and the formation of first polar body are the milestones achieved by oocytes during maturation (Senthilkumaran, 2011).

As the oocytes progress towards maturation it requires the change in the balance created by factors which promote meiotic arrest and meiotic resumption (Jamnongjit & Hammes, 2005). Thus it is evident that maturation is a tightly regulated process which involves complicated intracellular signaling.

2.4 Zebrafish as a model organism for studying the role of cyclooxygenase in oocyte development and maturation.

Zebrafish comes with a huge bioinformatical database and a completely sequenced genome. Zebrafish has fully functional arachidonic acid pathway. Both the COX isoforms and all the PPAR isoforms are present in zebrafish and expressed in the ovary. The fact that all the stages of developing oocytes are available in large number in zebrafish ovary makes it a suitable model for developmental biologists. The ease with which the zebrafish oocytes can be grown and made to undergo maturation *in vitro*, together with their effortless micro-manipulation for applications such as gene knockdown and over-expression, provide the fascinating opportunity to understand role of important genes involved in ovarian physiology.

2.4.1 Cyclooxygenase in zebrafish

Both the isoforms of Cyclooxygenase (COX-1 and COX-2) have been characterized in zebrafish. In zebrafish, COX is involved with formation of prostaglandin in the same way as in other species (Grosser *et al*., 2002). Zebrafish COX-1 (zCOX-1) is located on chromosome number 5 with RXRG and NOTCH1B. Its position and synteny with the other two genes mentioned above is similar to

human COX-1 present at chromosome 9. zCOX-1 contains 2094 bp of cDNA which includes 1794 bp of ORF, 126 bp of 5' UTR and 124 bp of 3' UTR. The cDNA of zCOX-1 is shorter than that of human, as in human 3' UTR extends to 1.5 bp. zCOX-1 translates into 597 amino acid protein.

Zebrafish COX-2 (zCOX-2) is located on chromosome 2 in close vicinity with CPLA-2. The synteny and position of zCOX-2 is similar to human COX-2 present on chromosome 1. zCOX-2, cDNA consists of 2150 bp including 1806 bp of ORF, 68 bp of 5'UTR and 276 bp of 3' UTR. 3' UTR has AU rich elements, a typical characteristic of COX-2. Human COX-2 cDNA has almost similar length of 2539bp. zCOX-2 translates into a 601 amino acid protein.

Like COX isoforms in other species, the distinct N-terminal and C-terminal features are conserved. zCOX-1 has 12 amino acid N-terminal motif which is absent in COX-2, and COX-2 has 18 amino acid C-terminal motif absent in COX-1. Each isoform of zebrafish COX shares 67% identity with other species. More specifically 72% homology was present in epidermal growth factor domain and catalytic domain. Figure 2.10 demonstrates the alignment of human and zebrafish COX proteins, showing the conserved and differing amino acids. Almost all the residues including glycosylation site and potential heme coordinating histidines required for catalysis are conserved in zCOXs. One surprising difference that is seen in zCOX-1 from human COX-1 is the presence of Val and Arg in position 523 and 513; These amino acids are present in the same position in human COX-2 creating an access in the form of side pocket to the arachidonate channel for the selective COX-2 inhibitor. How this orientation of COX-1 affects its function is unknown.

zCOX-1 expression is found to be high in ovary, gut and gills followed by

muscles, heart, testis and liver. zCOX-1 is not expressed in brain. zCOX-2 is expressed in gut, testis , heart, muscle and brain. Very low expression was seen in ovary and it was completely undetectable in liver. The dominant product of arachidonic acid catalysis by COX in zebrafish is PGE_2.

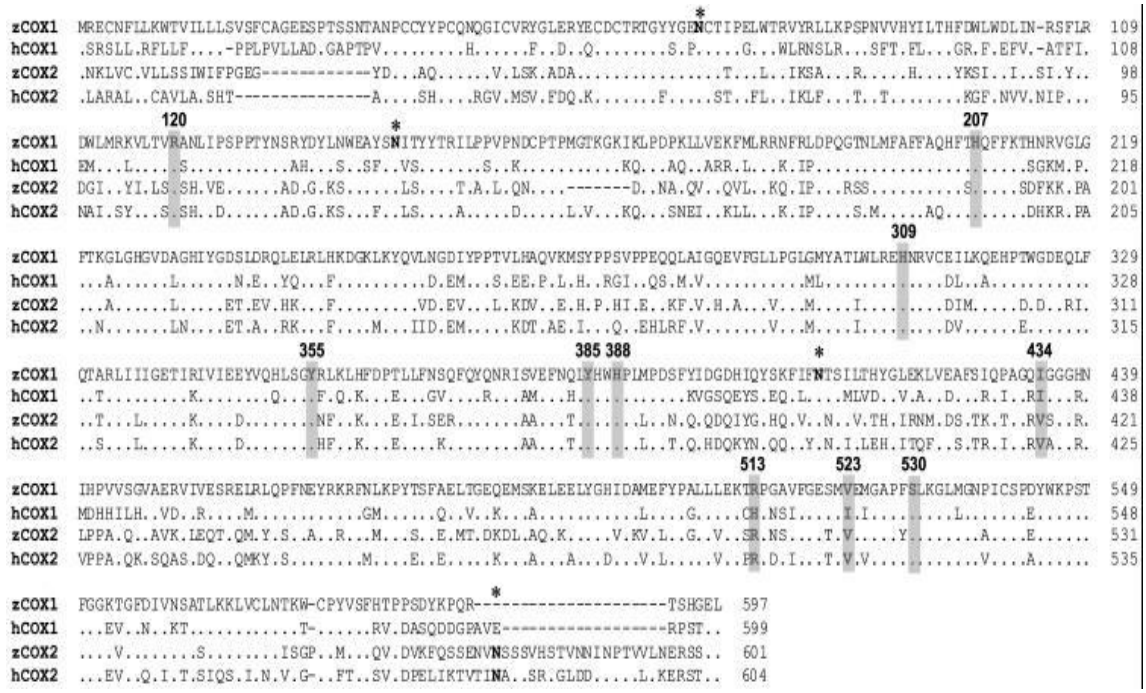


Figure 2.10 Amino acid alignment of zebrafish COX-1 and COX-2 with its human orthologs. Identical regions are indicated by dots. Gaps are represented by dashes. Highlighted boxes represent residues important for enzyme catalysis. N-glycosylation sites are marked by * (Adapted from Grosser *et al*., 2002).

2.4.2 Zebrafish PPARγ

In zebrafish, study of PPAR isoforms is rather new. 100bp DBD of all the PPARwas cloned in 1997 but there are no morphological or expression studies done till 2004. In 2004, Cajaraville *et al*., for the first time did the expression profiling of PPAR in male, female, adult, juvenile, and larvae (Ibabe *et al.,* 2005). All the isoforms of PPAR are encoded by separate genes. PPARγ is found on chromosome

number 11 (Gene ID: 557037). Recently, studies revolving around obesity, diabetes, muscle and neural development are looking into the involvement of PPARγ using zebrafish as the model organism. PPARγ shows different expression patterns in juvenile, adult and larvae indicating its involvement in the regulation of various developmental pathways. PPARγ is expressed in hepatocytes, adipocytes, pancreatic cells, skin, kidney proximal tubule, glomeruli, intestine, speramatogonia, ovary and skeletal muscles.

Immunostaining of PPARγ in oocytes shows strong expression in early staged oocytes and weak expression in late-stage oocytes.

2.4.3 Zebrafish oogenesis

Development, differentiation and maturation of zebrafish oocyte is similar to higher vertebrates and is regulated by the hormones of hypothalamus-pituitary-gonadal axis. Similar to other species, Follicle Stimulating Hormone (FSH) promotes the growth and vitellogenesis of oocytes by inducing estradiols. LH stimulates the production of 17α,20-β-dihydroxy-4-pregnen-3-one (17,20β DHP) a fish specific maturation inducing hormone (MIH) which induces the maturation promoting factor which promotes the oocyte maturation (E. Clelland & Peng, 2009).

In zebrafish, ovary development is initiated ten days after hatching. In three months the fish become sexually mature with an ovary containing large amount of follicles of all the developmental stages. In proper conditions, spawning can be achieved easily (Ge, 2005). Development of oocytes in zebrafish is asyncronic, which means all the stages of development can be seen in the ovary at any given point of time. During oogenesis, oocytes transform themselves from primary ovarian follicles to egg. Primordial Germ Cells (PGC) migrate towards genetic ridge and

transform into oogonia. While meiosis starts, they develop into primary oocytes. Once the meiosis is arrested in diplotene stage, oocytes grow in size, followed by vitellogenesis and development of inner and outer envelope. After the required growth and vitellogenesis is achieved, meiosis is resumed. Resumption of meiosis marks the advent of maturation in oocytes. Oocyte maturation accompanies Germinal Vesicle Break Down (GVBD), causing oocytes to become transparent. Completion of first meiotic division results in formation of cells with two different sizes. The small cell with a polar body ends up getting degenerated and the large cell left is known as secondary oocyte. Process of maturation concludes at this point. The secondary oocyte comes out of its follicular casing and goes to the abdominal cavity. This step is called ovulation. Occurrence of second meiotic division produces haploid female gamete or ovum. During fertilization, the haploid ovum nucleus fuses with the haploid nucleus of sperm.

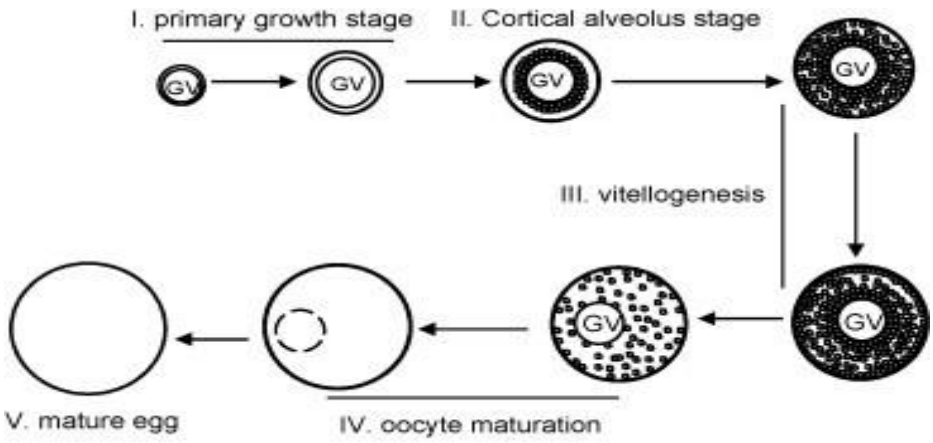


Figure 2.11 Schematic drawing of five developmental stages of zebrafish oocytes. based on the classification (Selman *et al*., 1993). (Adapted from Clelland and Peng 2009).

2.4.4 Developmental stages of Zebrafish oocytes

Zebrafish ovary is a bilobed structure consisting of a thin epithelium, oogonia and follicles. Follicles contain oocytes surrounded by somatic cells and interstitial tissues (stroma). Follicles of different developmental stages are randomly arranged in ovigerous lamellae, the chambers made up of connective tissues projecting longitudinally from the ovarian wall into the ovary. The ovary is connected to the ovarian lumen, where the oocytes are ovulated. A small oviduct connects ovarian lumen to a genital opening following the anus (Kelly Selman, 1993).

Zebrafish oocyte is surrounded by a layer of vitelline envelope, known as zona radiata which connects the oocyte to a single layer of granulosa cells which is further surrounded by a vascularized theca layer. The theca layer contains fibroblast and steroidgenic theca cells. Though structurally different, these granulosa and theca cell layers are found to be functionally homologous to mammals (Ge, 2005).

Oocytes pass through various developmental stages during oogenesis. Based on the size and structure of developing oocytes, Wallace and Selman have classified them into 5 stages (Kelly Selman, 1993). This classification is mostly followed in most of the zebrafish oogenetic studies, including this study. These stages are primary growth stage, cortical alveolus stage, vitellogenesis, maturation and mature egg (Figure 2.11, 2.12).

Figure 2.12 Zebrafish oocyte in different stages of development. Stage PV is pre vitellogenic and Stage EV is early vitellogenic. Smaller dark oocytes of stage MV are mid vitellogenic and stage LV late vitellogenic. Stage M represents mature oocytes. Pictures from PV to LV are provided by Ms Annette Jayaram and M is Adapted from (Kelly Selman, 1993).

Primary Growth Stage (PG) The follicle size at this stage, ranges from 7-140µm in diameter. During this phase of development, oocyte grows and undergoes the prophase of meiosis 1 and follicles are formed. At the end of this stage, meiotic division is arrested. Based on the fact whether the developing oocyte is resting in a nest or in follicle, this stage is subdivided into two more stages.

1. Pre follicle phase The oocytes at this stage measure 7-20 µm and lie in the nest. The oocyte nest is surrounded by a single layer of prefollicle cells which segregates it from oocyte stroma and other follicles. At this stage the oocyte nucleus is larger than the cytoplasm. Chromosomes start appearing in the form of thin thread; nucleoli also appear. Now these oocytes are in the leptotene stage of prophase. The oocytes grow and further condensation of chromosomes continues. Finally the oocytes form

a layer of definite prefollicle sheath around it. At this time, the oocytes go through the zygotene and pachyene phase. They are released from the nest but do not enter the diplotene stage yet.

2. Follicle Phase The follicles at this stage are transparent with a nucleus in the center, their size ranges from 20-140µm. A single layer of follicle cells surrounds the oocytes. Inside the nucleus, decondensation of chromosome starts and the oocyte enters the diplotene stage. Meiosis gets arrested now. From this point onwards, oocytes grow and develop until meiosis is resumed. By the end of this stage, the germinal vesicle enlarges. Oocyte becomes a definite follicle by getting surrounded with few cellular envelope layers. The first layer surrounding the oocyte follicle cells is a basement membrane. This whole complex opens up in a chamber of connective tissues known as theca which is covered by surface epithelium. The end of this stage can be marked by the appearance of the vitelline envelope.

Cortical alveolus stage At the beginning of this stage, cortical alveoli appears in the oocytes. The size of oocyte ranges from 0.14 to 0.34 mm in diameter. Cortical alveoli are also known as yolk vesicle. At this stage, the follicles appear translucent and foamy. But as the follicles grow in size, the yolk vesicle also grows and increases in number making the oocytes opaque. Now the germinal vesicle is not visible. At the end of this stage due to the rapid growth of cortical alveoli, almost all the ooplasm is covered. Germinal vesicle grows and becomes irregular in shape. Other cellular organelles also grow and differentiate. A tripartite vitelline envelop is formed with the three layers, known as zona externa (outer), zona radiata (middle) and zona interna. The follicle cells surrounding the oocytes also continue to grow and divide. Ovarian interstitial cells start to appear and increase in number, these cells are believed to be steroid secreting cells.

Vitellogenesis stage The follicle size ranges from 0.34 to 0.69 in diameter, at this stage. This is the major growth phase for the oocytes. At the end of this stage they acquire maturational competence. This stage is marked by the voluminous increase in the protein and yolk inside the oocyte. During vitellogenesis follicles become increasingly opaque and the germinal vesicle is completely murky. Female yolk precursor protein, vitellogenin, is hepatically derived and transformed into yolk protein and gets collected in yolk bodies. These yolk bodies start appearing at 0.34 mm sized follicles and increase in number as the follicles enlarge. The vitelline envelope starts thinning; theca cells enlarge and keep increasing in size. Germinal vesicle acquires a smooth outline and at the end of this stage, it migrates away from the center of the ooplasm. Yolk bodies come towards the center and the cortical alveoli move towards the periphery. The most important event that occurs at the end of this stage is that oocytes become competent and respond to the endogenous hormones. These oocytes now contain maternal mRNAs, proteins, lipids, carbohydrate, vitamins and hormones that are important for the proper development of the embryo. Based on the acquired maturational competence in response to the hormones and steroids, vitellogenic stage has been subdivided into two stages (Kohli *et al*., 2003).

1. **Early vitellogenic stage** These are oocytes of the size below 0.52 mm in size. At this stage, oocytes are not able to respond to the hormones which lead them towards maturation. Thus the oocytes are maturationally incompetent. When cultured, *in vitro* in leibovitz L-15 medium these oocytes fail to respond to the MIH and hCG (human gonadotropin hormone).

2. **Late vitellogenic stage** Oocyte are now sized above 0.52 mm, acquire

maturational competence and respond to the maturation promoting hormones. These oocytes have capacity to undergo maturation in response to fish- specific MIH and hCG when they are cultured *in vitro*. The oocytes of this size are being utilized by developmental biologists for hormonally induced *in vitro* maturation to understand the intricate signaling that happens while the oocyte matures.

Meiotic Maturation Zebrafish oocyte maturation is a complex event that involves a number of cellular changes. Meiosis resumes in maturational competent vitellogenic oocytes, in response to the LH surge. Germinal vesicle migrates towards the periphery and the nuclear envelop breaks down. First meiotic division occurs and chromosomes proceed towards the second meiotic division metaphase. The division is again arrested here and oocytes are converted to egg now. As the maturation proceeds, oocytes become translucent, at this stage oocytes can be matured *in vitro* in the medium alone without any assistance of external supply of hormones. Major events that take place while oocytes undergo maturation are,

1. Germinal vesicle migration (GVM)
2. Dissolution of germinal vesicle, which is also known as Germinal Vesicle Break Down (GVBD)
3. Ooplasm clearing
4. Development of osmoregulation in fresh water
5. Formation of future animal pole

Zebrafish maturation is very well studied. It is essentially a three step process, involving induction of gonadotropin (LH), maturation inducing hormone (MIH) and maturation promoting factor (MPF). LH acts on the follicles cells to induce the production of MIH which is 17alpha, 20beta-dihydroxy-4-pregnen-3-one (DHP) in

zebrafish. DHP in-turn stimulates the production MPF to trigger maturation (Nagahama & Yamashita 2008).

Zebrafish oocyte maturation can also be performed *in vitro*. If late vitellogenic oocytes, typically sized from 0.575 to 0.624 mm diameter are incubated in Leibovitz L-15 medium containing 1 μg/ml of DHP for 8-24 hours at room temperature, they undergo maturation. The *in vitro* occurrence of maturation is tracked by germinal vesicle breakdown event. Morphologically, the dense dark oocytes become transparent with the advent of maturation. At the same time maturation can also be induced in midvitellogenic oocytes (which are incompetent to undergo meiosis), by incubating them with Human Gonadotropin Hormone (HCG) in L-15 medium prior to the addition of DHP (Selman *et al.,* 1994). Detailed description of *in vitro* induced maturation is provided in chapter 4, section 4.1.

Due to the ease at which zebrafish maturation assay can be performed *in vitro*, it has emerged as a very powerful model for maturation related studies. Transcriptomic and proteiomic studies have been carried out in order to understand the molecular pathway of maturation (Knoll-Gellida *et al*., 2006). Zebrafish studies have contributed largely to understand the transcriptional, endocrine, and paracrine control of maturation. Multidimensional studies are in progress, to elucidate various pathways which promote maturation. One such pathway that involves the critical interaction of activins, inhibins, and follistatins with LH and MIH is being explored in detail (Wang & Ge 2003; Wu *et al*., 2000). Simultaneously the studies looking at the involvement of growth factors during maturation are also going on. Interesting findings, like TGF-beta-1 having inhibitory effect on maturation and BMP-15 preventing premature maturation increases the enthusiasm further to explore zebrafish maturation (Clelland *et al*., 2007; Kohli *et al.,* 2005). Researchers have also

connected the dots between Highly Unsaturated Fatty Acids (HUFA) biosynthetic pathway and zebrafish maturation. Specifically desaurase, an enzyme of HUFA pathway was found to be highly expressed in mature oocytes indicating the interaction of HUFA and its downstream products with the maturation process (Ishak *et al*., 2008). Having said that, it can also be perceived that COX pathway is involved in maturation and hence comes across as an area worth exploring.

Mature eggs (Ovulation) Translucent eggs of the size 0.73-0.75 mm are released from the surrounding follicle cells to be fertilized. The release of oocyte from the surrounding follicle cells to the ovarian or abdominal cavity is known as ovulation. The release of oocyte happens after the rupture of follicle wall. The rupture and release are the function of complex biophysical and biochemical events (Carnevali *et al*., 2010). Preovulatory LH surge is believed to trigger ovulation by increase in the production of prostaglanding. Marked in increase in COX-2 enzyme is reported in time dependent manner when follicles approach ovulation. This indicates like other species, in zebrafish COX-2 plays an important role during ovulation.

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

The focus of this study is to establish the role of cyclooxygenase enzyme isoforms COX-1 and COX-2 during oogenesis, specifically development and maturation of oocytes. To achieve this target zebrafish was used as model organism in this study because all developmental stages of oocytes are easily distinguishable and readily available in the ovaries of these fish. The core material utilized to peruse this work was zebrafish ovaries and oocytes collected from sexually mature female fish. Zebrafish attain sexual maturity at the age of 3 to 4 months. Collection of various stages of oocytes can be done easily with the help of forceps; under the microscope. Oocyte collection is described in detail in section 3.4.2.

3.2 Media and stock solutions

Culture media, stock solutions used for this study are listed in Table 3.1 and Table 3.2 together with their formulation.

3.3 Chemicals and consumables

The chemicals and other material used in this study are documented in Table 3.3.

Table 3.1 List of Culture Media

Medium	Composition
LB Broth	1.0% Tryptone, 0.5% yeast extract, 1% NaCl. pH 7.0
LB Agar	1.0% Tryptone, 0.5% yeast extract, 1% NaCl. pH 7.0, agar (15g/L), ampicillin (50mg/ml)
Dulbecco's modified eagle's medium (DMEM)	1X DMEM, 10% fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin
Leibovitz (L-15) medium	60% L-15 medium, 100u/ml penicillin, 100 µg/ml gentamycin

Table 3.2 List of Stock Solutions

Buffer	Composition	Volume
5 X TBE buffer	54.0 g tris base, 25.5 g boric acid, 0.5 M EDTA (20 ml)	1 L
10 X DNA gel loading buffer	500 µl 1X TBE, 500 µl glycerol, 2.5 µg bromophenol blue	1 ml
RNA loading buffer	50% Glycerol, 1mM EDTA, 0.4% bromophenol blue, 1mg/ml ethidium bromide	10 ml
RNA sample buffer	10.0 ml deionized formamide, 3.5ml 37% formaldehyde, 2.0ml of 5X MOPS	15 ml

10 X Phosphate-buffered saline (PBS)	80 g NaCl, 2 g KCL, 11.1 g anhydrous NA2HPO4, 2 g KH2PO4 in 1L sterile dH2O	1 L
10 X TBS	800 ml dd H2O, 12.11 g tris, 116.88 g NaCl pH 7.4.	1L
1 X TBST (Washing buffer)	50 ml 10 X TBS, 500 µl Tween 20, 400 ml ddH2O	500 ml
5X sample buffer	0. 3125 M Tris-Cl pH 6.8, 10%SDS, 25% 2-mercaptoethenol, 50% glycerol and 25 µg bromophenol blue	10 ml
10 X running buffer	800 ml ddH2O, 30 g tris, 144.4 g Glycene, 0.1% (w/v) SDS while making 1X running buffer	1L
Transfer buffer	100 ml 1 X running buffer, 200 ml 100% methanol, 700 ml ddH2O	1 L
50 X Danieau's buffer	33.9 mg NaCl, 0.52 mg KCl, 0.98 mg MgSO4, 1.4 mg Ca(NO3)2, 13 mg HEPES, 10 ml dH2O, pH 7.6	10 ml

Table 3.3 List of chemicals and consumables

Suppliers	Chemicals
Molecular Research Centre	TRI Reagent
Bioneer	Oligonucleotides
Vivantis	SDS, Glycine, Agarose, DNAladder
Promega	Renilla Luciferase Assay System,
	pGL-3 basic vector, pGEM®-T Easy Vector System, RQ1 RNase- Free DNase, TNT T7 Quick Couple transcription /translation system,TNTVR T7 PCR Quick master mix
Bio-Rad	Tris, TEMED, iScript™ MMLV Reverse Transcriptase, SYBR® Green
Qiagen	RQ1 RNase- Free DNase, QIAprep Spin Miniprep
Fermentas	PageRuler™ Prestained Protein Ladder, BglII restriction enzyme, HINDIII restriction enzyme
Merck	Methanol
USB Corporation	Ammonium persulphate
Fluka	D-mannitol
GeneTools LLC.	Antisense Morpholino
Invirogen	Lipofectin
Gibco	Dulbecco's modified eagle's medium (DMEM), Fetal bovine serum, L-glutamine, Penicillin, Streptomycin
Hyclone	Streptomycin
Addgene	pSVsport PPARγ vector
Pierce Biotechnology	NE-PER Nuclear and Cytoplasmic Extraction Kit, Biotin 3'-End DNA Labeling Kit, Light Shift Chemiluminescent EMSA Kit

Santacruz Biotechnology	PPARγ H-100 antibody
Sigma	COX-1 (PTGS-1) Antibody (Rabbit polyclonal), Leibovitz L-15 medium Chorionic gonadotropin human (hCG), 17α, 20β-dihydroxy-4-pregnen-3-one (DHP)
Abcam	Rabbit IgG-H&L (HRP)
Millipore	Immobilon-P PVDF membrane
GE Healthcare	ECL Western Blotting Detection Reagents
American Type Culture Collection	HePG2 cell lines
R&M Chemicals	Sodium chloride

3.4 Methods

3.4.1 Zebrafish maintenance

Wild type zebrafish (*Danio rerio*) were maintained in Aquaculture Research Complex (University Sains Malaysia, Penang) at 14 hours of light and 10 hours of dark period maintained using an automated timer. Windows were covered with dark sheets in order to block the light coming from outside during dark period. The fish were fed with the combination diet of blood worms and live brine shrimps, *Artemia* sp. twice daily until satiation. Excess food was removed from the fish tank in order to avoid water contamination. Male and female fish were maintained in separate tanks.

3.4.2 Oocyte collection

Sexually mature zebrafish ranging between four to eight months were collected randomly, few hours before the dark period usually around 1pm-3pm and the ovaries from 10-15 fish were dissected out after anesthetizing the fish with tricaine solution made from 0.2mg/ml tricaine methane sulphonate (MS222) in distilled water. Ovaries were recovered and placed in 60% Leibovitz-15 (L-15) medium. L-15 medium is used for CO_2 free cell culture systems, to maintain physiological pH balance. This medium supports the growth of various established cell lines in CO_2 free environment. L-15 medium is used in zebrafish labs as common practice to facilitate the growth of isolated follicles *in vitro*.

Follicles were separated manually with the help of fine tipped forceps. Follicles were staged based on classification given by Kelly Selman with some modification (k. Selman *et al.,* 1994; W. R. A. Selman *et al.,* 1993). Early and mid vitellogenic follicle (EMV 0.34-0.61mm) and late vitellogenic follicles that can also be termed as full grown but immature follicles (LV 0.62-0.70mm) were sampled separately. To collect mature follicles, female fish were kept together in the breeding tank separated by a plastic sheet in dark. Mature follicles, which were translucent due to *in vivo* germinal vesicle breakdown (M ~ 0.70 mm), were collected 30 minutes before the end of the dark period. EMV, LV and M follicle samples were used for RNA extraction (Section 4.2.1).

Mid vitellogenic (MV) zebrafish follicles ranging from 0.52 mm to 0.62 mm were collected for the knockdown study described in chapter 4 due to their known response to hormonal-assisted *in vitro* maturation (Selman *et al.* 1994).

3.4.3 Cell line culture and maintenance

HepG2 (Human liver hepatocellular carcinoma) cell lines were used in the experiments involving cell lines. HepG2 are adherent epithelial like cells, which grow in monolayer in small aggregates. This cell line was originally derived from 15 years old Caucasian male with differentiated hepatocellular carcinoma. These cells exhibit high degree of morphological and functional differentiation *in vitro* thus acting as a suitable model for the study of membrane protein, lipid and fatty acid metabolism.

Cells were grown in 1 X Dulbecco's modified eagle's medium (Gibco), supplemented with 10% fetal bovine serum (v/v), 2mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Hyclone). Cultures were maintained at 37°C with 5% CO_2 (v/v) supply.

Culture medium was replenished every 2 to 3 days and fresh medium. Cells were split 1:4 every third or fourth day. Before splitting, cells were washed by 1xPBS twice and passaged by adding prewarmed (37°C) 0.05% Trypsin-EDTA solution on the cell monolayer. Once the cells are dispersed, equal volume of complete growth medium was added to deactivate Trypsin and cells were distributed in fresh culture flasks replenished with the culture medium. Cells at 60% confluence on the day of experiment were used for transient transfection.

CHAPTER 4

EXPRESSION OF COX-1 AND COX-2 IN VITELLOGENIC AND MATURE ZEBRAFISH OOCYTES

4.1 Introduction

Successful maturation of oocytes is a prerequisite for successful ovulation and fertilization. Maturation involves the resumption of meiosis (Wilson, 1925) and thus requires complicated signaling process (Jamnongjit & Hammes, 2005). It has been well established that cyclooxygenase derived prostaglandins are required for developmental and maturational success of the oocytes (Armstrong, 1981; Armstrong *et al*., 1974; Lau *et al*., 1974). Prostaglandins are believed to be formed in the granulosa cells of the preovulatory follicles, by the enzymatic action of COX on AA. Their amount increases at the time of LH surge (while oocytes mature) and is highest in the oocytes nearing ovulation (Dennefors *et al*., 1983; Hanzen, 1984; Tsutsumi, 1993).

As cyclooxygenase is the rate limiting enzyme in the production of prostaglandin, it is important to understand the involvement of both the isoforms of cyclooxygenase in oogenesis. COX-2 is known to be induced few hours after the LH surge, specifically at the time while follicles approach ovulation in zebrafish (Lister & Van Der Kraak, 2009), mouse, bovine and rat (Sirois *et al*., 2004) indicating the requirement of COX-2 derived prostaglandin during ovulation. COX-2 derived

prostaglandins are also reported to be required for the oocyte maturation in mouse (Takahashi *et al*., 2006).

In comparison to COX-2, requirement of COX-1 in oogenesis is poorly understood, however emerging evidences strongly support involvement of COX-1 in oocyte development and maturation. For example COX-1 derived PGE2 was detected in monkey ovarian surface epithelium (Cabrera *et al*., 2006). Studies in both salmon and brook trout reveal increased COX-1 levels during ovulatory stage (Roberts *et al*., 2000). In medaka, both COX isoforms were shown to be necessary to sustain female reproductive performances. Continuous presence of COX-1 in human (Adriaenssens *et al*.) and adult zebrafish oocytes (Grosser *et al*., 2002; Lister & Van Der Kraak, 2008) strongly implies a plausible function for COX-1 during maturation or ovulation.

The study that marked COX-2 as critical factor for oogenetic success was based on the implications that COX-2 knockout mouse had disrupted ovarian functions like ovulation and fertilization (Langenbach *et al*., 1995; Lim *et al*.,1997). Interestingly, when COX-2 knockout was produced in the mouse under different genetic background (CD-1) the ovulatory phenotype was rescued. The rescue was contributed to the ability of COX-1 to replace COX-2 and perform the functions that are generally attributed to COX-2 (H. Wang *et al*., 2004) .

Although we have a clear understanding that COX are essential for success of ovarian functions, the extent of involvement of COX-1 during vitellogenesis and maturation is still unclear. Contrary to COX-1 which requires extensive investigation, COX-2 is marked essential for ovulation and its inhibition results in ovulatory disorders. In spite of having a significant effect on ovulation, a study

revealed that COX-2 specific inhibitor failed to inhibit zebrafish maturation and elevated COX-2 expression was not seen in vitellogenic and mature zebrafish follicles (Lister & Van Der Kraak, 2008). However, the results were regarded as non-conclusive due to the arguments about COX-2 inducibility, sample handling and lack of repeatability. Thus, in the wake of present scenario, analysis of mRNA expression of both COX isoforms, in order to elucidate their significance during vitellogenesis and maturation is required.

Zebrafish has a functional AA pathway, and both COX isoforms are expressed in this model system (Grosser *et al*., 2002). Zebrafish ovary is asynchronous, in other words it contains all the developmental stages of oocytes (E. Clelland & Peng, 2009). Also when it comes to understanding the functional role of a gene, the simplicity of this model system holds a great advantage. In order to distinguish the participation of COX isoforms during development and maturation of zebrafish oocytes, the mRNA expression pattern of COX-1 and COX-2 in EMV oocytes, LV oocytes and M oocytes was observed through semi quantitative real time PCR. It is one of the most sensitive ways to analyze and compare the mRNA expression, as the amplification is monitored in "real time" which means signal is detected in terms of fluorescence the moment amplification starts. Thus it also allows the accurate quantification of a target mRNA in biological samples at different times of development. The data obtained is in terms of Ct value (threshold cycle), which represents the number of cycle at which fluorescent signals are obtained from the amplification crosses the threshold (background). Ct values are inversely propositional to the amount of nucleic acid present in the sample. mRNA expression is calculated by using these Ct values (See section 4.2.4)

4.2 Method

4.2.1 Total RNA extraction

Total RNA was extracted from the oocytes of three developmental stages, early and mid-vitellogenic (EMV), Full grown (LV) and Mature (M) respectively. RNA extraction was done using TRI Reagent, following the manufacturer's specifications. Purity and concentration of RNA was determined by spectroscopy (Bio-Rad SmartSpec™Plus Spectrophotometer). RNA was subjected to gel electrophoresis (1% w/v agarose gel) to ensure the integrity. To eliminate the possibility of DNA contamination 2µg of total RNA was treated with RQ1 RNase Free DNase. Treated RNA was stored in -80˚C before using it for real-time PCR.

4.2.2 Primer Design

Gene specific primers were designed using the published mRNA sequences of zebrafish COX-1 (Genebank: BC116575), COX-2 (Genebank: BC063232.1) and β-actin (Genebank: AF057040). Primers were designed using Primer3 (version 0.4.0) online program (http://frodo.wi.mit.edu/primer3/). Primers present on the exon-exon junctions were selected. Strict parameters like 20-25bp length of primer, 40-60% of GC content, 55 to 65˚ C annealing temperature (Ta) and 100-250 bp product size were taken into account while designing the primers. Primers with the tendency of hairpin loop, homodimers and herterodimers formation were avoided. Sequences of the primers together with amplicon size are given in Table 4.1

Table 4.1 Primers used for real time PCR

Name	Sequence 5'-3'	Amplicon size (bp)
COX-1	Forward 5'GCTGAAGTGGACGGTGATTT 3' Reverse 5'GACAAGGGTAATAGCAACAAGG 3'	104
COX-2	Forward 5' CTGTCCGATGGTGTCCTTTC 3' Reverse 5' AACTCCGCTCGTTCAAAACA 3'	125
β-actin	Forward 5'CCGTGACATCAAGGAGAAGCT 3' Reverse 5'TCGTGGATACCGCAAGATTCC 3'	201

4.2.3 Amplification, Cloning and Analysis of mRNA fragment

Fragments of zebrafish COX-1 and COX-2 were amplified by PCR using the primers mentioned above. These fragments were ligated in pGEMT-easy vector and transformed in DH5α strain of *E.Coli* using heat shock method. Transformed bacterial cells were grown in LB medium till they reach exponential phase and spread on agar plate supplemented with LB medium. Recombinant colonies were selected via blue/white selection and inoculated on a seperate LB-agar plate. Fragment insert was confirmed in these selected colonies by PCR amplification of recombinant DNA using these colonies as template. Colonies containing the fragment matching the size were further propagated in LB medium overnight. Recombinant plasmids were extracted using QIAprep Spin Miniprep kit following the manufacturer's instructions. Extracted plasmids were sent for sequencing and retrieved sequences were verified using NCBI nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

4.2.4 Semi-quantitative real time PCR analysis

Semi-quantitave real time PCR was performed to check the expression of COX-1 and COX-2 in oocytes. RNA extracted from EMV, LV and M stages of oocytes was used as template for the PCR. DNAse treatment was first performed on the RNA, in order to get rid of DNA contamination. For DNase treatment, 2µg of sample RNA was mixed with 1µl of DNase (Promega, USA), 2µL 10X DNase buffer and sterilized MiliQ water to constitute the final reaction volume of 10µl. This mixtures was incubated at 37°C for 30 minutes, followed by 10 minutes incubation at 65°C to inactivate the enzyme.

One-step RT-PCR was conducted in iCycler iQ 4 Real Time PCR Detection System (Bio-Rad), using iScript^{TM} One-Step RT-PCR Kit (Bio-Rad, USA) along with DNAse treated RNA and COX-1, COX-2 and *β-actin* primers mentioned in table 4.1. Recipe of mastermix is given below:

RNA template	300ng
iScript reverse transcriptase	1 µl
2XSYBR Green RT-PCR reaction mix	15µl
Forward primer	0.2 µM
Reverse primer	0.2 µM
Nuclease-free water	Add up to 30 µL

At first, the RNA templates were reversed transcribed at 20°C for 20 minutes to generate the first cDNA strand, followed by heat inactivation of enzyme at 95°C

for 5 minutes. Similar PCR cycling conditions were used for COX-1, COX-2 and reference gene *β-actin*. Denaturation was performed at 90˚C for 20 seconds; annealing step was done for 30 seconds at specific melting temperature for COX-1 and COX- 2 and β-actin. Extension step was carried out for 72˚C for 30 seconds. After the last cycles of PCR reaction, the PCR products were subjected to melt curve analysis (81 repeats).

Samples were run in triplicates during each PCR run for the amplification of COX-1 and COX-2 along with reference gene β-actin. Data was obtained in the form of Ct (calculated threshold cycle) values, from which the gene expression was calculated using the iCycler iQ Real Time PCR Detection System software version 3.1 (Bio-Rad) provided by the manufacturer. The formula used to calculate the gene expression is given below.

Expression of COX = $2^{(Ct\ \beta\text{-actin} - Ct\ COX)}$

Expression (control) = $2^{(Ct\ \beta\text{-actin} - Ct\ COX)}$ lowest COX expression is used as control.

Expression (target) = $2^{(Ct\ \beta\text{-actin} - Ct\ COX)}$ for other two stages.

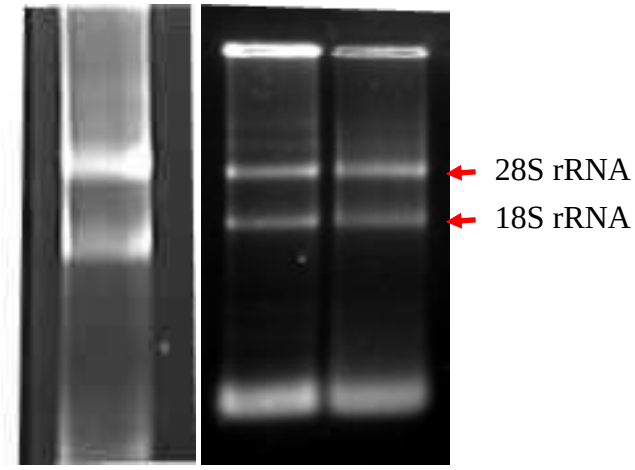
Expression level = Ratio expression (control/target)

The PCR was repeated with three biological replicates of the samples, in order to get statistically significant data. Results were analyzed by one-way analysis of variance (ANOVA) and Tukey's HSD (Honestly Significant Difference) post hoc test. The statistical analyses were performed using SPSS 10.0.1. The results are expressed as mean ± SEM for the number of experiments (n=3) performed. $P < 0.05$ was considered significant.

4.3 Results

4.3.1 RNA Isolation

Total RNA is used as the starting material for mRNA expression analysis, the success of downstream experiments depends on the high quality of total RNA. Total RNA isolated from the samples EMV, LV and M was separated as two bands on 1% (w/v) agarose gel. Presence of the intact, brighter 28S and a less bright 18S rRNA band ensured the integrity of total RNA extracted from the samples (Figure 4.1). Isolated RNA samples had the A260/A280 ratio and A260/230 ratio, ranging from1.8 to 2.0, indicating that the RNA samples used for downstream applications are free from protein, chaotropic salts or phenol impurities. Subsequent DNase treatment ensured that RNA is free from any DNA contamination



Lane 1 Early and Midvitellogenic oocytes (EMV)

Lane 2 Full grown but immature oocytes (LV)

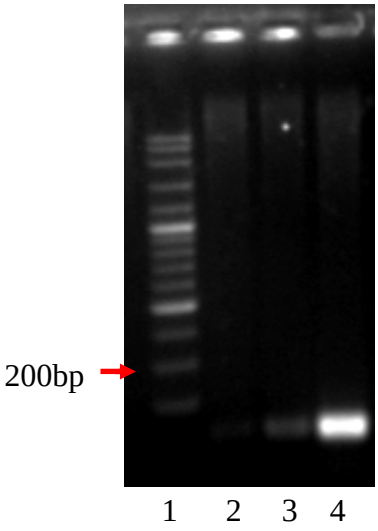
Lane 3 Mature oocyte

Figure 4.1 RNA extraction from zebrafish oocyte. Total RNA obtained from each oocyte stage resolved in the form of two bands on 1%w/v agarose gel.

4.3.2 Amplification and validation of zebrafish COX-1 and COX-2 fragments

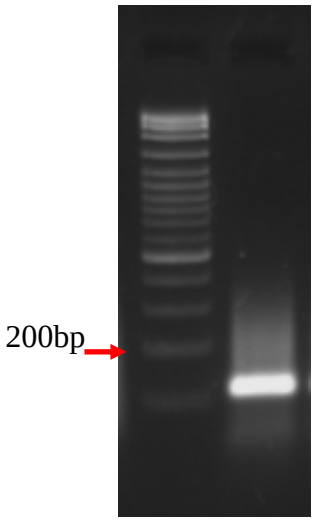
Validation of amplicons is required in order to be sure, beforehand, that the amplified PCR product is COX-1 and COX-2 indeed. Using the primers mentioned in Table 4.1 COX-1 and COX-2 were amplified using conventional PCR. PCR products were run on 1.5% w/v agarose gel. COX-1 and COX-2 were separated at 104bp (figure 4.2) and 125 bp (Figure 4.3) respectively in the form of single bright bands. There were no primer dimers seen on the gel. These amplicons were cloned in pGEMT easy vector and sequenced. Deduced sequences of both COX-1 and COX-2

were identical to zebrafish COX-1 and COX-2 published sequences mentioned in section 4.2.2 (Figure 4.4 and 4.5).



Lane 4 104 bp COX-1 fragment

Figure 4.2 Amplification of COX-1 COX-1 resolved on 1.5% agarose gel as sharp 104 bp band



Lane 2 125bp COX-2 fragment

Figure 4.3 **Amplification of COX-2** COX-2 resolved on 1.5% agarose gel as sharp 125 bp band

```
Amplicon   1 GCTGAAGTGGACGGTGATTTTGCTGCTGAGTGTGTCCTTTTGTGCTGGTGAAGAAAGCCC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
zCOX-1   147 GCTGAAGTGGACGGTGATTTTGCTGCTGAGTGTGTCCTTTTGTGCTGGTGAAGAAAGCCC  206

Amplicon  61 GACTTCATCAAACACTGCGAATCCTTGTTGCTATTACCCTTGTC  104
             |||||||||||||||||||||||||||||||||||||||||||
zCOX-1   207 GACTTCATCAAACACTGCGAATCCTTGTTGCTATTACCCTTGTC  250
```

Figure 4.4 **Sequence alignment of zebrafish COX-1** COX-1 sequence aligned with published zebrafish COX-1. Letters in bold represent primers used for PCR amplification

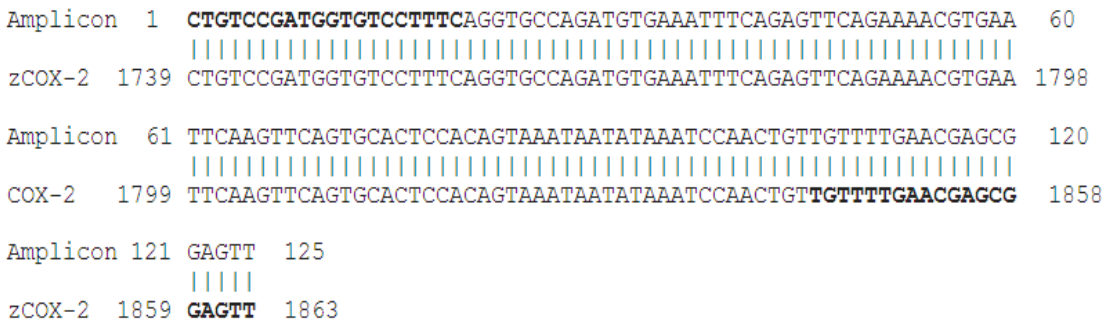


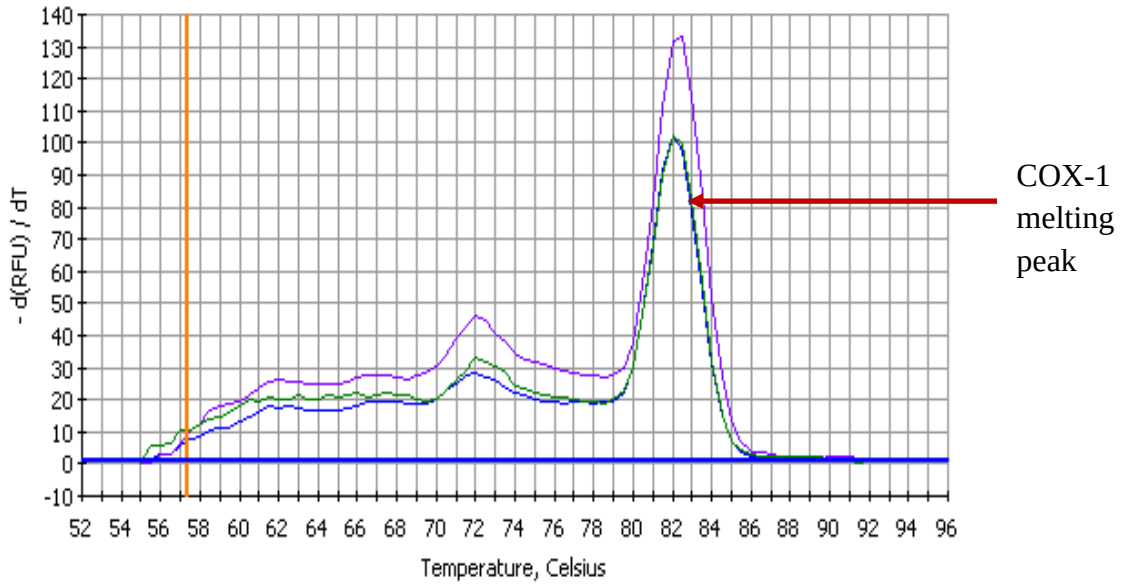
Figure 4.5 Sequence alignment of zebrafish COX-2 COX-2 sequence aligned with published zebrafish COX-2. Letters in bold represent primers used for PCR amplification

4.3.3 Expression analysis of COX-1 and COX-2 in vitellogenic and mature stage of oocytes

mRNA expression analysis is a reliable way of predicting the gene expression as it mostly correlates to the protein which is the functional expression of gene. To know the contribution of COX-1 and COX-2 in vitellogenesis, their mRNA was amplified in EMV, LV and M oocyte samples by real time PCR amplification. Data obtained at the end of the PCR was analyzed to get the expression of mRNA. The melt curve obtained at the end of PCR showed a single peak, demonstrating that only the desired PCR products are amplified, and the primers are very specific and free of dimer formation. (Figure 4.6 A, B). Data is collected in the form of Ct value from which mRNA expression of COX-1 and COX-2 was calculated as mentioned in section 4.2.4.

mRNA expression pattern of COX-1 showed a significant gradual increase from EMV to LV to M follicles COX-1 expression was increased at each oocyte stage analyzed, indicating the ongoing enhanced requirement of COX-1 at the time of vitellogenesis and maturation (Figure 4.7 A). Surprisingly COX-2 mRNA showed a significant gradual decrease of expression in EMV and LV follicles. The mRNA transcript of COX-2 was almost nil during maturation (Figure 4.7 B).

A



B

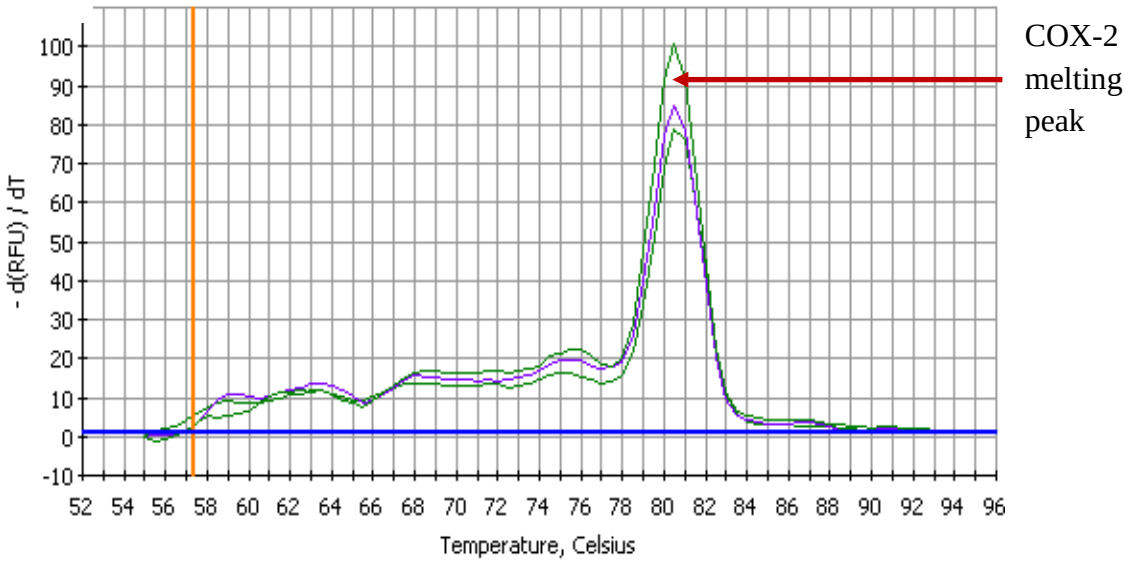
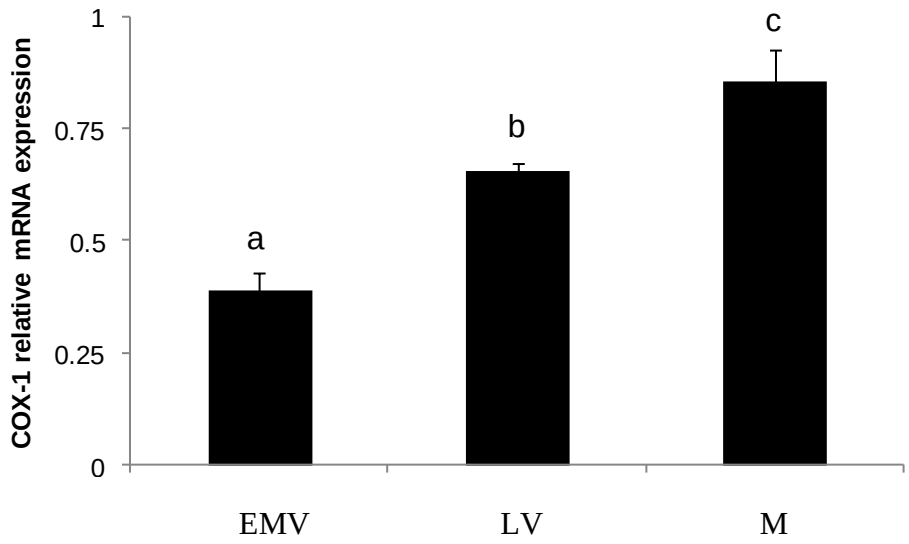


Figure 4.6 Melt curve analysis of COX-1 and COX-2 demonstrated single specific peak indicating the specificity of the primers and amplification.

A



B

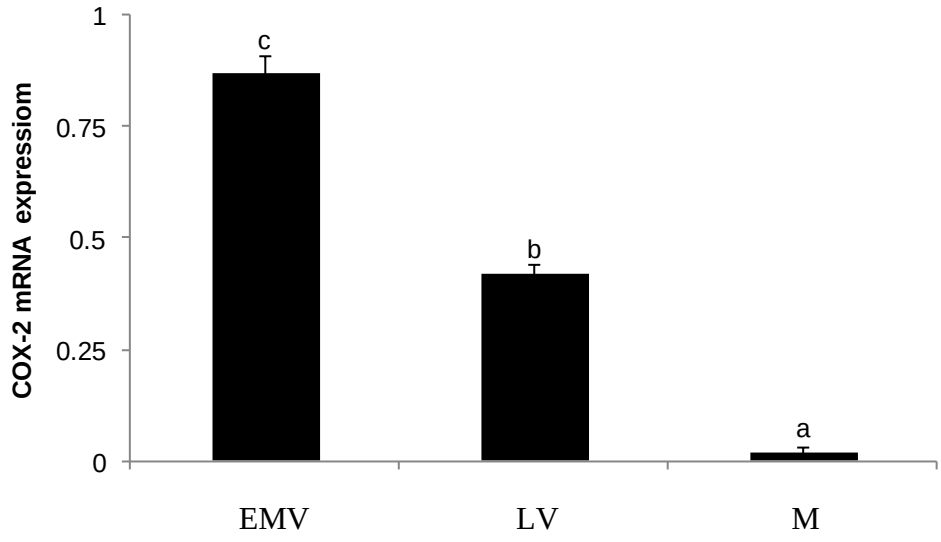


Figure 4.7 mRNA expression of COX-1 and COX-2 in early and mid vitellogenic (EMV), Full grown (LV) and matured (M) zebrafish follicles**.** Mean values with different letters are significantly different (P<0.05).

4.4 Discussion

Both COX-1 and COX-2 show a distinct expression pattern in vitellogenic and mature oocytes. Gradual increase in the expression of COX-1 and strong suppression of COX-2 from late vitellogenic to mature oocytes indicates that COX-1 can be critical during vitellogenesis and maturation.

In mice, it has been shown that COX derived PGE2 are critical for the induction of various pathways which leads to meiotic resumption, and cumulus cell expansion, the event that takes place during maturation (Takahashi *et al*., 2006). In bovine, it is exhibited that COX-1 expression increases in maturing oocytes and the expression of COX-2 remains unchanged (Nuttinck *et al*., 2008) which is also concurrent with increased expression of COX-1 in our study.

Low level of COX-2 expression in full grown and mature follicles of zebrafish indicates the non requirement of this isoform during vitellogenesis and maturation. COX-2 is also established as a marker for ovulation in various species. COX-2 is induced at the time of ovulation in zebrafish follicles (Lister & Van Der Kraak, 2009) suggesting the requirement of COX-2 in follicular rupture and ovulation. In other words, the possibility of COX-1 derived PGs modulating vitellogenic and maturational events and COX-2 derived PGs contributing towards ovulation in zebrafish cannot be overruled.

In teleosts, responsiveness of the oocytes towards maturation inducing hormone (MIH) is prerequisite for the resumption of meiosis and GVBD (Germinal Vesicle Break Down) . In zebrafish, the steroid 17α, 20β -DHP has been identified as a form of MIH capable of inducing maturation *in vitro* (Selman *et al*., 1994). COX derived prostaglandin are known to be necessary for the production of MIH in

various fish species. Elevated expression of COX-1 in this study suggests that COX-1 can be critical for the maturational events of zebrafish and its absence can hinder this process. To examine the effect of functional absence of COX-1 on oocyte maturation, COX-1 knockdown was performed in oocytes. This approach is described in next chapter in detail.

CHAPTER 5

EFFECT OF ANTISENSE MORHOLINO MEDIATED KNOCKDOWN OF COX-1 ON *IN VITRO* MATURATION OF ZEBRAFISH OOCYTES

5.1 Introduction

mRNA expression analysis of COX-1 and COX-2 revealed that contrary to COX-2, COX-1 is upregulated during vitellogenesis and maturation of oocytes. A significant increase in its expression from full grown to maturating oocytes indicates that COX-1 can be critical for the maturation of zebrafish oocytes. However, in order to demonstrate that COX-1 is functionally indispensible for this process, it is necessary to examine how absence of COX-1 affects the oocyte maturation *in vivo*. To achieve this target functional knockdown or knockout of COX-1 will be required.

The functional studies involve *in vivo* inhibition of protein function by either eliminating the protein (knockdown) or its corresponding DNA (knockout), thus directly establishing the function of a gene in a biological process. Using the knockout approach on mouse, it was demonstrated that knock out of COX-2 resulted in anovulation phenotype while knockout of COX-1 produced no phenotypic effects (Langenbach *et al*., 1995; Lim *et al*., 1997). However COX-2 knock out mouse of a different genetic background demonstrated that compensatory upregulation of COX-1 rescued the phenotypic defects caused by the loss of COX-2 (H. Wang *et al*., 2004). Hence in order to specifically understand the contribution of COX-1, a

simpler model system with a more streamlined *in vivo* inhibitory approach is required.

With the help of antisense technology, it is possible to specifically knockdown the protein expression of a gene on transcriptional or translational level. There are many powerful antisense agents available, but morpholino (MO) have gained popularity in vertebrate systems like zebrafish, xenopus, chick and mouse due to their strong and specific knockdown effects (Heasman, 2002).

Morpholinos are the radically redesigned nucleic acid analogs with modified sugar phosphate backbone. 5 ringed sugar and negatively charged phosphate intersubunit linkage of nucleic acid is replaced by 6 ringed morpholine ring and non ionic phosphorodiamidate linkage (Figure 5.1). These properties contribute towards morpholino stability, reduced toxicity and enhanced affinity to complementary mRNA in a biological system (Summerton, 2007).

Morpholinos work in two ways, either by blocking the translation of a protein or by modifying the splicing of pre mRNA. To inhibit the translation, morpholinos bind to the complementary mRNA through Watson and Crick pairing and causing the steric blocking of the start codon (Figure 5.2). Other type of morpholinos modify the splicing of pre mRNA by blocking the splice junctions and resulting into altered translation product (Karkare & Bhatnagar, 2006). To accomplish a successful knockdown experiment, it is imperative to also show that morpholino is able to inhibit the translation of targeted protein *in vitro* and *in vivo*. The former can be achieved by translating and knocking down the protein *in vitro*. If an antibody specific to the protein is available, morpholino mediated protein inhibition in an *in vivo* system can be shown with the help of a western blot. It is also necessary to have

a control morpholino, which is similar to the experimental morpholino but will not cause the inhibition of protein. This morpholino demonstrates that the presence of morpholino structure and the delivery method does not hinder normal biological function. This morpholino is called mismatch morpholino. A mismatch morpholino differs in 5 out of 25 nucleotides selected from its targeted mRNA sequence and as it is unable to bind the target site, it does not interfere with the translation process (Eisen & Smith, 2008).

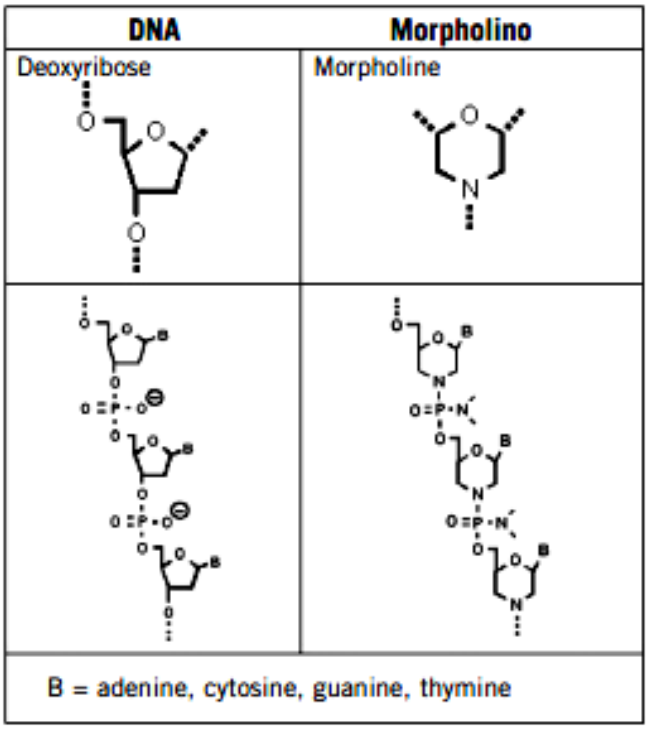


Figure 5.1 Structure of morpholino. Comparison of DNA and Morpholino structure. A five member morpholine ring can be distinguished from a six member sugar ring of DNA. Morpholino is non ionic compared to DNA which is negatively charged.

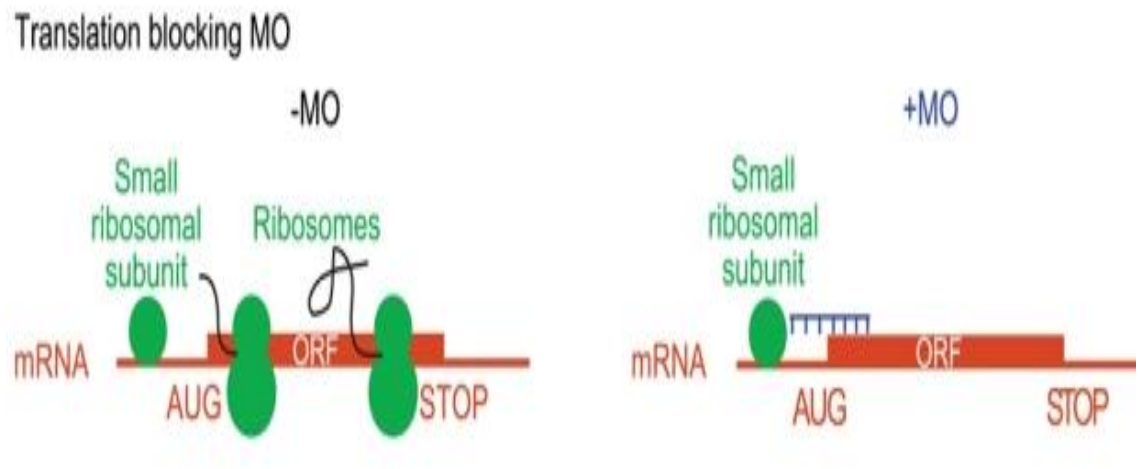


Figure 5.2 Mode of action of morpholino. By steric blocking of the start codon by morpholino does not allow the ribosomal units to assemble hence stopping the protein synthesis (B). (Adapted and modified from Summerton 2007).

Strong specificity of morpholino towards its target has been witnessed time and again as morpholino mediated knockdowns mimicked the known genetic mutants in various studies (Karlen & Rebagliati, 2001; Lele *et al*; 2001; Nasevicius & Ekker, 2000). In zebrafish, MOs are proved to produce specific and powerful knockdowns. Initially MOs were used to assign functions to the genes in zebrafish embryo development. However at present zebrafish embryo model system with the aid of MO is being used for drug resisitance, toxicity testing (Sipes, Padilla, and Knudsen 2011), hematopoieses (Ellett and Lieschke 2010), genetically modified platelets studies (Thijs, Deckmyn and Broos 2012), and for the study of diabetetic (Jörgens *et al*., 2012), cardicac (Miura and Yelon 2011), muscular diseases (Guyon *et al*., 2007) etc.

While not as abundant as embryonic studies, frequently oognetic studies have also been performed utilizing the MO antisense technology and *in vitro* oocyte

maturation assay. With the help of MO knockdown various genes were established as inhibitor or promoter of maturation and there potential role in the pathway that leads to maturation was determined. The control of Human G protein-coupled receptor 30 (GPR30) on meiotic arrest was realized when GPR 30 antisense moroholino blocked the inhibitory effect of estrogen on oocyte maturation (Pang, Dong and Thomas 2008). Knocking down of BMP-15 in oocytes resulted in maturation of premature oocytes, indicating its role in oocyte quality control (Peng, Clelland and Tan, 2009).

In the area of COX study, COX-1 MO knockdown has been performed on embryos establishing the requirement of COX-1 in embryogenesis (Grosser *et al*., 2002), vasculature formation and gastrulation (Cha, Kim, Solnica-Krezel, & Dubois, 2005). However functional requirement of COX-1 during oocyte maturation has not been demonstrated yet. Tracking the effect of functional absence of COX-1 protein in maturing oocyte is a straight forward approach to demonstrate that COX-1 acts as a rate limiting enzyme in AA pathway happening during maturation.

To elucidate the functionality of COX-1 during maturation, it is necessary to block the translation of COX-1 mRNA in oocytes by using translation blocking morpholino and observing if the maturation of oocytes is hindered which can be achieved in *in vitro* oocyte maturation assay.

Maturation can be induced *in vitro* in the zebrafish oocytes of certain size that are still meiotically incompetent. These oocytes are categorized as mid vitellogenic oocytes ranging from 0.52 mm to 0.62 mm (Pang & Ge, 2002). By maintaining oocytes in Leibovitz L-15 medium and incubating it with hCG (human gonadotropin hormone) maturational competence in the oocyte can be induced.

Later on administration of 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DHP) which acts as MIH in zebrafish induces the maturation. Success of these events requires enhanced supply of prostaglandin. Based on the results of real time PCR it can be stated that COX-1 is largely involved with oocyte maturation and the absence of COX-1 may affect the maturation process.

In order to confirm this hypothesis further morpholino mediated knockdown of COX-1 in MV oocyte followed by *in vitro* maturation assay was carried out.

5.2 Methods

5.2.1 Morpholino design, order and storage

Antisense translation blocking morpholino oligonucleotide covering the start codon of zebrafish COX-1 (5'-TCAGCAAAAAGTTACACTCTCTCAT-3') designated as COX-1 MO and its mismatched morpholino (5'-TCACCAAAAACTTAGACTGTGTCAT-3') designated as COX-1mm MO were designed and ordered from Gene Tool, LLC (Philomath, OR) at website http://www.gene-tools.com/. Few parameters that were taken into consideration while approving the morpholino design which are, length of morpholino around 25 bp, GC content around 50%, the sequence should snap between 5' cap and about 25 bases 3' of the AUG start codon. BLAST search was carried out to confirm that the morpholino sequence is not present anywhere in the zebrafish genome. Stock solution of 1mM concentration was prepared by dissolving the lyophilized

morpholinos in sterile distilled water. Morpholinos were stored at room temperature and diluted further for the injections.

5.2.2 Amplification of PCR template for *in vitro* transcription and translation

Primers were designed complementary to the open reading frame of COX-1. Start codon was included in the forward primer together with a T7 promoter and a Kozak sequence. Forward primer was designed according to this template: 5' (N)6–10-T7 Promoter-Spacer-Kozak-AUG-(N)17–22 3'. The N towards the 5' end of primer and the spacers can be any nucleotides. And the N after AUG represents the nucleotides complementary to zebrafish COX-1. T7 promoter sequence is TAATACGACTCACTATAGGG which is added to facilitate *in vitro* transcription. Kozak Sequence ACCACC was added after the spacer to initiate the translation process. Sequence 5'CAAAACTACA**TAATACGACTCACTATAGGG**AACA***ACCACC*ATG**AGAG AGTGTAACTTTTTGC 3' (T7 promoter indicated in bold, kozak sequence indicated in bold italics and started codon indicated in bold) was used as forward primer. At the 5' end of the reverse primer poly T sequence (reverse compliment of the poly A tail) and reverse compliment of stop codon (CTA) was added to stop the translation. Sequence 5'**TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT*CTA***CTGGTGTAGGTGATGT TGGAATAA 3' was used as reverse primer (Poly T sequence in bold and stop codon in bold italics). Using these forward and reverse primers, zebrafish COX-1 PCR fragment was amplified and used for *in vitro* transcription and translation.

5.2.3 *In vitro* transcription and translation for the expression of PCR template

TNTT7 Quick for PCR DNA kit (Promega) was used to achieve the transcription and translation of PCR templates. PCR fragment containing a T7 promoter was added to the TNTT7Quick for PCR master mix from the kit and incubated for 90 minutes at 30°C in the presence and absence of COX-1 MO and COX-1 mmMO. Synthesized proteins were subjected to western blotting for further analysis.

5.2.4 Protein extraction from the oocytes

Protein was extracted from the oocytes injected with COX-1 MO, COX-1 mmMO and Danieau's buffer respectively, after one hour of incubation in Leibovitz L-15 medium, using the protocol modified from ReadyPrep™ Sequential Extraction Kit (Bio-Rad). Concentration of protein was determined using RC DC protein assay (Bio-Rad). Various dilutions of BSA were used for the standard curve plotting. This plot was used as the reference to measure the protein concentrations. The proteins were subjected to western blot analysis.

5.2.5 SDS PAGE and Western blotting

SDS-PAGE gel was casted which consists of two gels. Upper 5% stacking gel (0.125M Tris-HCl pH6.8, 0.1% SDS, 5% acrylamide/bis, 0.2% TEMED, 0.1% APS) for the stacking of protein and lower 10% resolving gel (0.375M Tris-HCl pH8.8, 0.1% SDS, 10% acrylamide/bis, 0.1% TEMED, 0.1% APS) for the protein denaturation and separation according to their size. Presence of SDS causes the denaturing of the protein. All the protein samples were mixed in equal concentration with 1X sample buffer and loaded with 5µl of prestained protein ladder. Gel was run for 2 hours at 100 V. Proteins were transferred on the PVDF membrane (Millipore) by blotting the gel and membrane. Gel and membrane were aligned together

sandwiched between sponges and blotting papers in a cassette. The cassette was placed in the blotting apparatus which was run at 100 V for 2 hours at 4˚C in transfer buffer with continues stirring.

PVDF membrane with transferred proteins on it was blocked in 50 ml of 8% skimmed milk powder in TBST for 60 minutes. Blocking solution was discarded and membrane was washed five times in 1X TBST. Membrane was incubated with primary anti COX-1 antibody produced in rabbit (Sigma) in 1:1000 dilutions with 1xTBST. It was followed by the washes with 1xTBST (2 times, 5 minutes) and 5M $NaCl_2$ (1 time, 5 minutes) and then again with 1xTBST (2times, 5 minutes). Membrane was then incubated for two hours with anti-rabbit IgG-H&L (HRP) (Abcam) which was used as secondary antibody in 1:2500 dilutions. Washing step was repeated. ECL Western Blotting Detection Reagents (Amersham) was used to obtain chemiluminescence. Protein bands were visualized using ChemiDoc XRS+ System (Bio-Rad). Primary antibody step was eliminated while detecting the *in vitro* synthesized proteins because the proteins are biotin labeled and Streptavidin-Horseradish Peroxidase Conjugate was used as secondary antibody.

5.2.6 Morpholino microinjection in zebrafish oocytes

In order to examine the inhibition of COX-1 protein in oocytes, morpholinos were delivered in zebrafish oocytes by microinjection. Working solution of COX-1 MO and COX-1 mmMO was prepared by diluting the stock in 1 X Danieau's's buffer (DB). Final concentration of working solution was achieved by adding 0.05% phenol red dye, which helped to track the injection.

Injection needles were pulled from borosilicate glass capillaries using micropipette puller (Narishige). The tip of the needle was cut using sharp razor

under the microscope so that a fine opening is formed. Needle was filled with morpholino using microloader tips (Eppendorf). Needle was attached to the FemtoJet express injector (Eppendorf) which was connected to the InjectMan NI2 (Eppendorf) for the pressure supply and the manipulation of the injection. In most of the *in vitro* maturation studies, the volume of morpholino injected in the oocytes is approximately 1nl (E. S. Clelland *et al*., 2007; Zhu *et al*.,2003) which is also followed in this study. The volume of microinjection was adjusted with the help of InjectMan NI2 (Eppendorf) by optimizing the injection pressure (Pi) and injection timing (Ti). A constant compensation pressure was maintained throughout the injection in order to avoid the backflow in the capillary. Volume of each injection was calculated by mineral oil droplet method (Nüsslein-Volhard, 2002).

MV oocytes were used for morpholino microinjection, their separation process and timing are mentioned in section 3.4.2 of chapter 3. Oocytes were aligned on the stand specially prepared for oocyte microinjection in microinjection chamber. Oocytes were set in a desired direction after the removal of excess medium with the help of forceps. Needle filled with morpholino was positioned right above the oocytes and injections were performed with COX-1 MO, COX-1 mmMO and Danieau's buffer (DB). DB was used as negative control to demonstrate that oocytes were not harmed with injection process or the presence of buffer. After the injections oocytes were collected and kept in Leibovitz L-15 medium, only healthy looking oocytes were used for further applications.

5.2.7 *In vitro* maturation assay

Healthy looking injected oocytes (n=60) were used for the *in vitro* maturation assay (Selman *et al*., 1994). The oocytes were incubated in a six well plate with 2 ml of Leibovitz L-15 medium in which 20 IU/ml hCG was added. After 6 hours the medium was replaced with the fresh medium and 5ng/ml 17α, 20 β –DHP was added. Both the incubations were done at 28˚C. Oocytes were scored for %GVBD after 10 hours. Oocytes which turned transparent were counted and percentage was calculated. Experiment was repeated three times to confirm the results. Data regarding the percentage of maturation was analyzed by one way ANOVA and Tukey's HSD post hoc test.

5.3 Results

5.3.1 Determination of Efficacy of Morpholino

To examine the efficacy of morpholino, its *in vitro* and *in vivo* inhibition was observed by western blotting. The *in vitro* transcribed and translated protein using TNTT7 quick for PCR DNA, in the presence or absence of COX-1 MO and COX-1 mmMO were separated by SDS-PAGE and blotted. Low intensity protein bands (lane 3) obtained after western blotting, confirmed significantly inhibited protein expression of COX-1 protein, translated in the presence of COX-1 MO *in vitro*. Bright bands in lane 2 and lane 1 show that the expression of protein in case of COX-1 mmMO was equivalent to the expression of protein in the absence of any morpholinos (control) (Figure 5.3). Densitometric analysis of the protein bands obtained from western blotting demonstrates significant decreases in the COX-1

protein amount caused by the presence of COX-1 MO compared to Mismatch and Control.

Inside oocytes, COX-1 MO inhibited COX-1 protein production with in one hour. Sharp reduction in the protein band obtained from COX-1 MO injected oocytes can be seen in lane 3 compared to the bright bands obtained from COX-1 mmMO and Danieau's buffer injected oocytes in lane 2 and lane 1 (Figure 5.4).

Combined together, these results demonstrate that COX-1 MO knocked down the COX-1 protein with the same efficiency *in vitro* and *in vivo*. Strong suppression in the amount of COX-1 protein from the oocyte with in one hour demonstrates the strong inhibition caused by COX-1 MO. At the same time strong bands of protein obtained from COX-1 mmMO and Danieau's buffer injected oocytes show that presence of morpholino, buffer or injecting process did not have any effect on COX-1 protein expression.

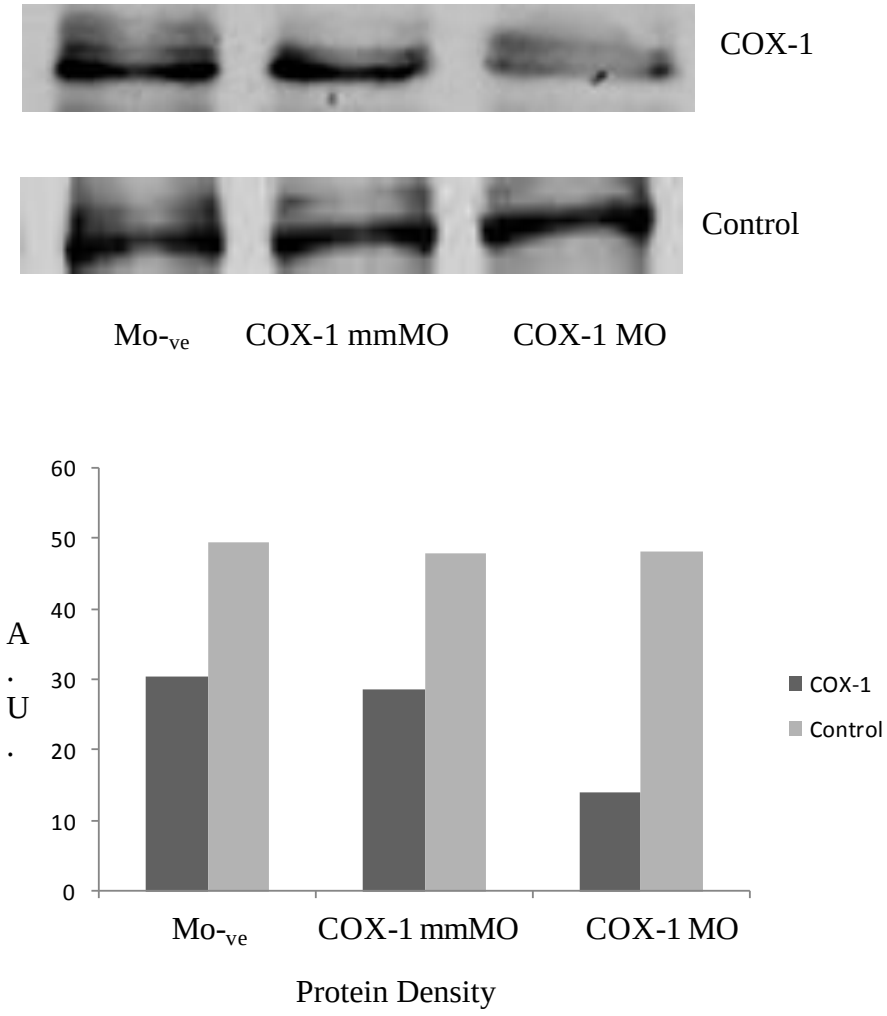


Figure 5.3 ***In vitro*** **measurement of COX-1 Morpholino efficacy**

Presence of COX-1 MO inhibits the *in vitro* translation of COX-1 protein which can be seen by decresed band intensity. In the presence of COX-mm MO and absence of MO (MO-$_{ve}$) in which no morpholinos were added (MO-$_{ve}$) translation is not affected. Protein density was measured and graphically represented in terms of arbitrary units (A.U.) to see the efficiency of inhibition.

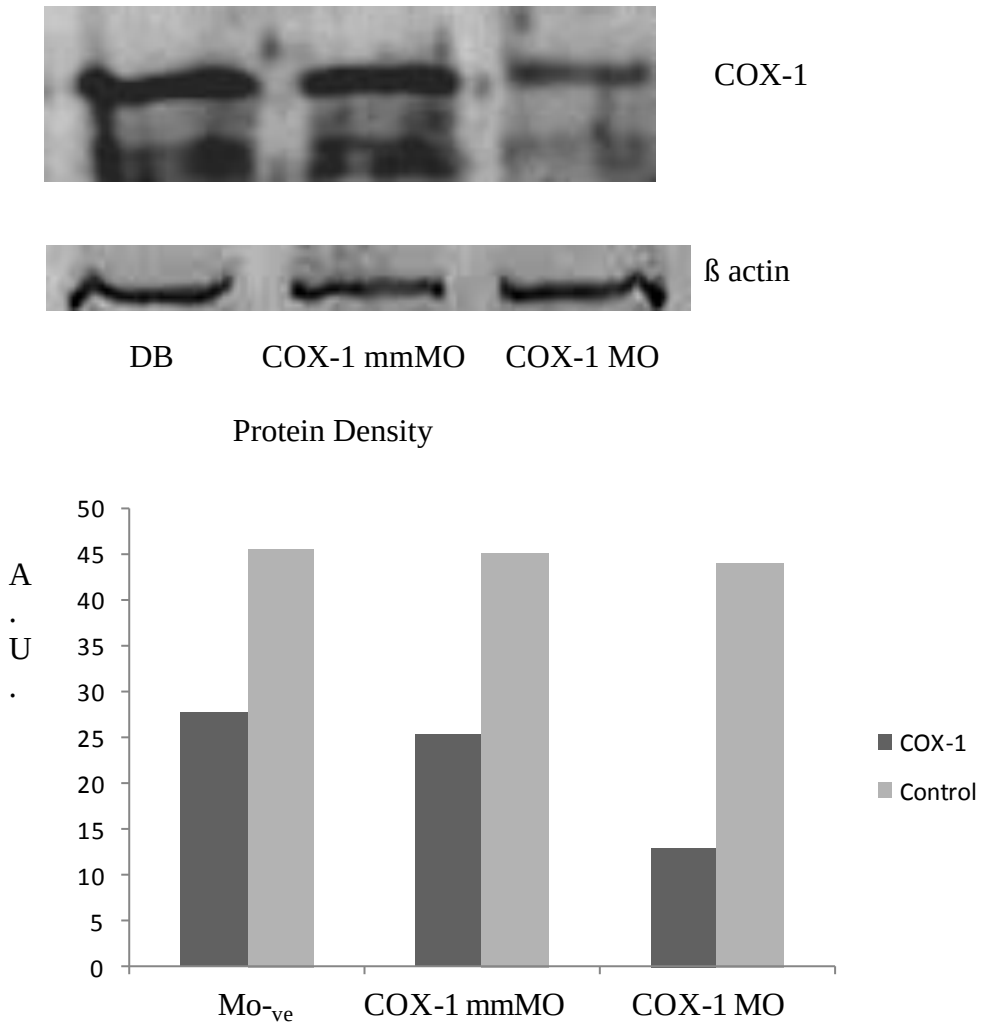


Figure 5.4 ***In vivo*** **measurement of COX-1 Morpholino efficacy** Presence of COX-1 MO inhibits the *in vivo* translation of COX-1 protein in oocytes. Protein level of oocyte microinjected with COX-1 MO goes down drastically compared to COX-1 mmMO and Danieau's buffer (DB) injected oocytes. ß actin was used as control. Protein density was measured and graphically represented in terms of arbitrary units (A.U.) to see the efficiency of inhibition.

5.3.2 Effect of COX-1 knockdown on *in vitro* maturation of zebrafish oocytes

In order to investigate the effect of absence of COX-1 on oocyte maturation, COX-1 protein synthesis was inhibited *in vivo* by microinjecting anti COX-1 morpholino (COX-1 MO) in MV oocytes. The strong inhibitory action of this morpholino was already displayed in section 5.3.1. Mismatch morpholino, COX-1 mmMO and Danieau's buffer were injected as control. Oocytes were then allowed to undergo *in vitro* maturation in the presence of hCG and DHP. Oocyte maturation accompanies GVBD which transforms dark MV oocytes into transparent mature oocyte. Transparent oocytes become the benchmark for maturation.

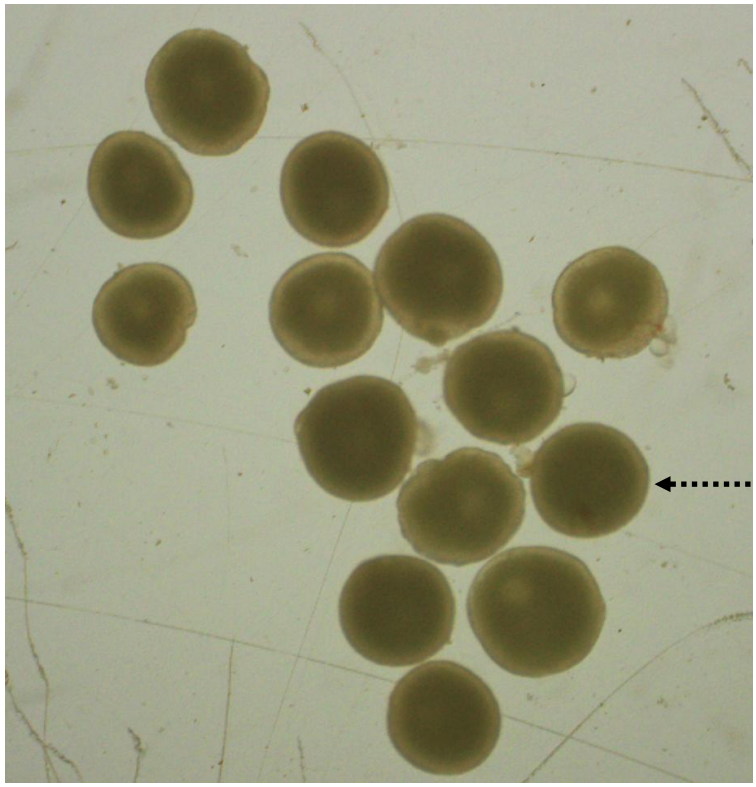
After 10 hours of incubation in DHP, it was observed that, most of the oocytes injected with COX-1 MO did not undergo GVBD. Morphologically, they did not turn transparent and remained same in appearance as before. In other words COX-1 MO injected oocytes did not mature and remained arrested in MV stage. (Figure 5.5 a). At the same time, oocytes injected with COX-1 mmMO matured successfully and became transparent due to GVBD. Similarly most of the DB injected oocytes also matured. (Figure 5.5 b and c.)

Percentage GVBD was calculated by counting the oocytes that went through GVBD after incubation of 10 hours in DHP. Figure 5.6 is the statistical representation of % GVBD that occurred in the oocytes injected with COX-1 MO, COX-1 mmMO and DB in three independent experiments.

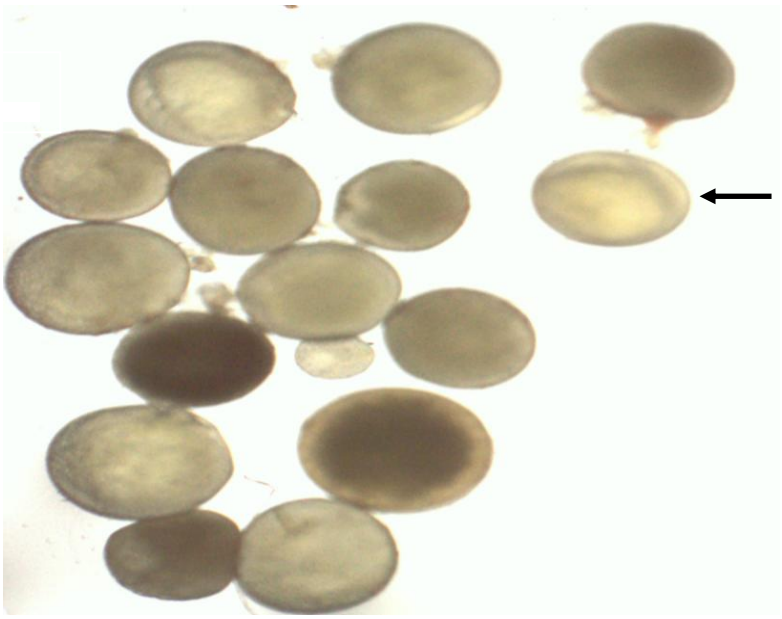
Morpholino mediated knockdown of COX-1 caused by COX-1 MO injection in MV oocytes, resulted in significant reduction in the percentage of maturation *in vitro*(10+/-1.2%). In comparison, more than 60% of the oocytes injected with COX-1 mmMO and DB matured *in vitro*.

It was demonstrated that knocking down the expression of COX-1, in oocytes, radically inhibits the maturation process. As the oocytes mature successfully in the presence of mismatch morpholino and Danieau's buffer, it demonstrates that the sole cause of maturation arrest is absence of COX-1 protein and not the injection process or presence of morpholino in the oocytes.

a



b



c

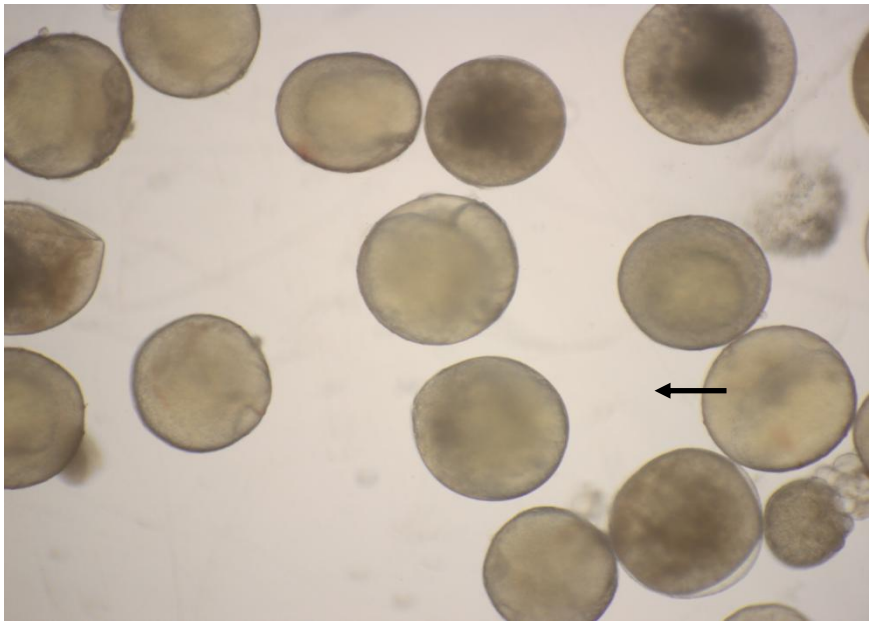


Figure 5.5 Effect of COX-1 knockdown on oocyte maturation. COX-1 MO treated oocytes failed to mature (a) Translucent matured oocytes acquired after COX-1 mmMO injection (b) Translucent mature oocytes obtained after DB injections (c). Mature oocytes are marked arrow and the oocytes arrested in MV stage are marked by dotted arrow.

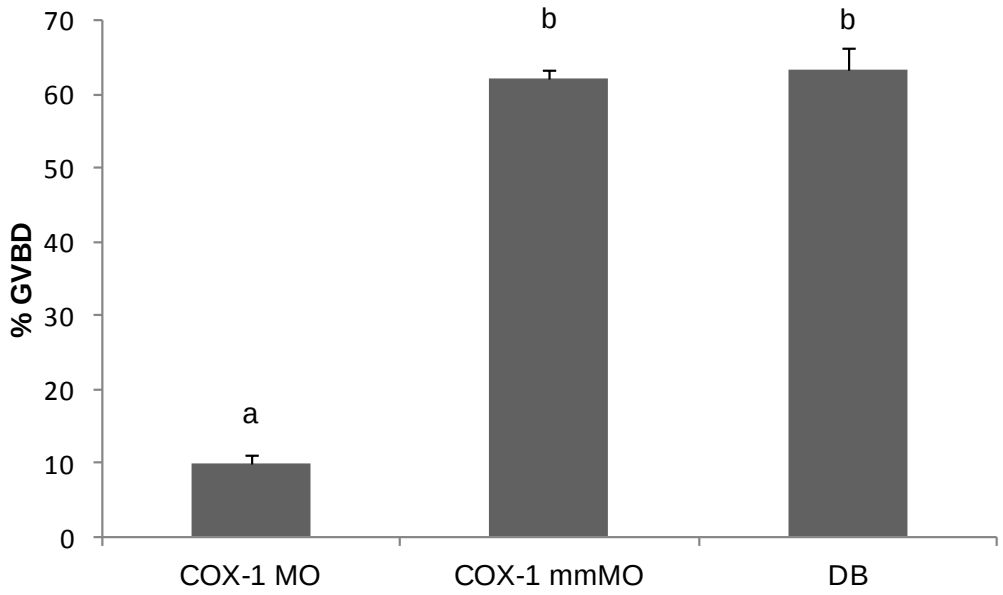


Figure 5.6 Graphical representation of COX-1 knockdown on oocyte maturation. COX-1 MO treated oocytes failed to mature (a) Translucent matured oocytes acquired after COX-1 mmMO injection (b) Translucent mature oocytes obtained after DB injections (c). Mature oocytes are marked arrow and the oocytes arrested in MV stage are marked by dotted arrow (A). Percentage (mean ± SEM) of COX-1 MO injected, COX-1 mmMO injected and DB injected MV follicles undergoing germinal vesicle breakdown (GVBD) in hCG and 17α, 20β-DHP induced *in vitro* maturation assay (B). Mean values with different letters are significantly different ($P<0.05$).

5.4 Discussion

In this chapter it was established that functional absence of COX-1 in zebrafish oocytes resulted in maturation failure. In chapter 4 it was observed that mRNA expression of COX-1 increases significantly during maturation. Taken together these findings indicate that the presence of COX-1 is critical for the maturational success of zebrafish oocytes. Based on the literature reviews, this is the first study which establishes the functional role of COX-1 in vertebrate oocyte maturation. Previously it has been demonstrated that COX non selective inhibitor indometacin (INDO) inhibits the zebrafish oocyte maturation. But inconsistent results were obtained with COX-1 and COX-2 selective inhibitors (Lister & Van Der Kraak, 2008). In medaka fish, exposure to COX-1 inhibitor reduced the amount of mature oocytes. Interestingly in invertebrate model system *Drosophila*, various COX-1 selective inhibitors disrupted the oocyte maturation and COX-2 selective inhibitor failed to do so (Tootle & Spradling, 2008). A *Drosophila* homologue of COX-1 known as Pxt was identified, which was highly expressed in late vitellogenic oocytes. Absence of Pxt resulted into the phenotypes with disrupted ovarian function. Interestingly, mouse COX-1 functionally rescued the Pxt phenotypes indicating that functional role of COX-1 in maturation might be evolutionary conserved (Tootle & Spradling, 2008).

The results of this study, taken together with the findings mentioned before strongly promote the role of COX-1 in vertebrate oocyte maturation. These results are also supported by the fact that non selective COX inhibitor indometacin (INDO), which is believed to be a stronger suppressor of COX-1 than COX-2 is reported to impede the GVBD of oocytes in mice (Downs & Longo, 1982, 1983) and European Sea bass (Sorbera, Asturiano, Carrillo, & Zanuy, 2001). It inhibits the early

development of follicles in rabbit (Spanel-Borowski, Sohn, & Schlegel, 1986) as well.

Previously the role of COX-1 was suggested to be that of a housekeeping gene (Crofford LJ. 1997). It was believed that it produces the basal level of PGs to maintain homeostasis. On the other hand COX-2, the inducible isoform of cyclooxygenase was believed to be involved with tumerogenesis, inflammation and processes like ovulation (Smith, DeWitt, & Garavito, 2000). As mentioned before in chapter 2, recently strong evidences which indicate the involvement of COX-1 in many degenerating conditions like ovarian and breast cancers, pain and neuro-inflammation are coming into picture (Niknami *et al*.). In zebrafish, it was already demonstrated that COX-1 is critically involved with various aspects of embryo development. COX-1 knockdown embryos resulted in the lethal developmental phenotypes. Surprisingly there was no effect of COX-2 knockdown on embryo development (Grosser *et al*., 2002). For example, COX-1 knockdown resulted in gastrulation arrest, impaired formation of vasculature and pronephric duct (Cha *et al*., 2005). In this chapter, it has been confirmed that COX-1 is also required for the successful maturation of zebrafish oocytes. Taken together these findings specify the role of COX-1 as an important developmental gene.

Subsequently it was also demonstrated that PGs produced during gastrulation period are solely sustained by COX-1 and COX-2 arises only at the end of the gastrulation (Cha, Solnica-Krezel, & DuBois, 2006). This indicates a switch in the isoforms from one embryonic event to another. A possibility of similar switching cannot be overruled in oogenesis. Oocytes could not escape the fate of maturation arrest caused by COX-1 knockdown indicating the possibility of COX-1 being the provider of PGs during vitellogenesis and maturation. Elsewhere it is shown that

COX-2 induction occurs when oocytes approach ovulation (Lister & Van Der Kraak, 2009). Taken together these findings indicate the possibility of COX isoform switching during oogenetic events as well. However this transitory role of COX isoform in oogenesis needs more exploration.

It is established in chapter 4 and 5 that COX-1 is required for folliculogenesis and maturation. As regulatory connection between COX-2 and PPARγ has been demonstrated in human on few occasions, possibility of COX-1 being regulated by PPARγ cannot be overruled as well, specially, because PPARγ is known to regulate various genes involved in oogenesis. In next chapter the possibility of PPARγ mediated transcriptional regulation of COX is explored.

CHAPTER 6

TRANSCRIPTIONAL REGULATION OF COX BY PPARγ DURING OOGENESIS

6.1 Introduction

Gene expression in eukaryotes is regulated at the level of replication, transcription and translation. Transcriptional regulation of a gene which is a primary level of expression control happens with the help of transcription factors. Transcription factors are regulatory DNA binding proteins which function to activate or inhibit the expression of genes by binding on the special sequences present on their promoter. In eukaryotes a combinatorial regulation of gene expression happens, involving transcription factors with various other proteins acting as ligands, corepressors or coactivators (Described in section 2.2.2 and 2.2.3 of chapter 2).

It has been described in Chapter 2, that PPARγ, a DNA binding protein, acts as transcription factor and controls most of the ovarian functions. It regulates the rate limiting enzymes of the pathways involved in oogenesis. PPARγ is known to control various reproductive events including follicular development, oocyte maturation and ovulation (Froment *et al*., 2006). PPARγ is expressed in the ovaries of various mammalian species. Genes targeted by PPARγ are expressed in inverse pattern of its own expression mostly. For example mRNA expression of PPARγ and Aromatase in granuslosa cells shows an inverse relationship (Yanase *et al*., 2001) . Similarly PPARγ and P450 the rate limiting enzyme for the production of progesterone, also show inverse mRNA expression pattern (Komar & Curry, 2003).

MMP-3 and MMP-9 genes, involved in tissue remodeling during oogenesis also have inverse relationship with mRNA expression of PPARγ (Yee *et al*., 1997).

It is known that both PPARγ and prostaglandins are found in the granulosa cells of the oocytes. These cells are the signaling hub for oocyte maturation. The process of maturation is triggered by LH surge. With the advent of LH surge prostaglandin production gets induced and PPARγ expression gets inhibited, indicating an inverse correlation between prostaglandin production and PPARγ expression (Froment *et al*., 2006; Komar *et al*., 2001; Komar & Curry, 2003).

It has already been established that COX-2, the rate limiting enzyme in prostaglandin production, exhibits inverse mRNA expression pattern with PPARγ in human placenta (Dunn-Albanese *et al*., 2004) and rat granulosa cells (Komar & Curry, 2002). However in chapter 4 it has been demonstrated that mRNA expression of COX-1 increases from full grown to mature oocytes while expression on COX-2 is low compared to COX-1 and it reduces from full grown to mature oocytes. In chapter 5 requirement of COX-1 during maturation has been demonstrated functionally. Based on these findings, it can be deduced that instead of COX-2, COX-1 acts as the rate limiting enzyme in prostaglandin production during vitellogenesis and maturation. Thus the possibility of COX-1 being regulated by PPARγ cannot be denied as well. While presence of PPRE has been reported on the human COX-2 promoter (Meade *et al*., 1999) there are no reports on human COX-1 promoter being explored for PPRE yet.

In this chapter, correlation in the expression pattern of COX-1, COX-2 and PPARγ in developing oocytes was studied. As well as, the possibility of direct transcriptional regulation of COX by PPARγ was also examined.

6.2 Methods

6.2.1 Semi-quantitative Real Time PCR analysis of PPARγ

Total RNA extraction, sequence cloning, validation and real time analysis of PPARγ was carried out as described in chapter 4 (from section 4.2.1 to section 4.2.4.) For real time PCR, Zebrafish PPARγ specific primers (5'-GTGGAAGGCGAGCAGATGAT-3' and 5'-GGACTGGTAGCTGTGGAAGAAG-3') were designed using published zebrafish PPARγ sequence (Genebank: U93477).

6.2.2 Bioinformatical analysis of COX-1 and COX-2 promoter

A 3kb promoter sequence, upstream of start codon from Zebrafish COX-1 and COX-2 gene was extracted with the help of Ensembl genome browser (www.ensembl.org). Promoter sequences were run through the MatInspector software in search of PPRE. This program is available online through the website http://www.genomatix.de. It is a reliable program and it utilizes the large library of matrix descriptions of transcription factor binding sites to locate their matches in DNA sequences.

6.2.3 Extraction of genomic DNA from zebrafish ovary

Genomic DNA was freshly extracted from zebrafish ovary using DNeasy Blood and Tissue kit (Qiagen). Manufactures' instructions were followed to extract the DNA. Integrity of genomic DNA was verified by running it on 0.7% (w/v) agarose gel. The concentration and purity of DNA was tested using spectroscopy.

6.2.4 Preparation of promoter reporter constructs of zebrafish COX-1 promoter

To examine the functional activity of zebrafish COX-1 promoter, promoter reporter constructs were prepared by cloning the PCR generated deletion fragments of 2.7kb zebrafish COX-1 promoter into pGL3 basic vector (Promega). pGL3 vector provides the platform for quantitative analysis of the factors that regulate gene expression such as promoters, enhancers, cis-acting elements and trans-acting elements. The vector contains modified coding region for firefly (*Photinus pyramids*) optimized to monitoring transcriptional activity in transfected eukaryotic cells. pGL3 vector lacks a eukaryotic promoter and enhancer sequence providing the maximum flexibility to clone a putative promoter (Figure 6.1).

Five 5' deletion fragments of different sizes covering 2.7 kb span of zebrafish COX-1 promoter were PCR amplified. These fragments were designated as A1 (-428/+134), A2 (-1067/+134), A3 (-1369/+134), A4 (-1952/+134) and A5 (-2573/+134). The forward primers used to amplify fragments A1, A2, A3 and A4 were A1 (-428/+134)F, A2 (-1067/+134)F, A3 (-1369/+134)F, A4 (-1952/+134)F, A5 (-2573/+134). A (+134) R was used as common reverse primer to amplify all the

fragments. To facilitate the cloning, restriction sites BglII and HindIII were added in the in the forward and reverse primers respectively. These primer sequences are mentioned in table 6.1. For PCR amplification, 34 cycles of denaturation at 94˚C for 60 seconds, annealing at primer specific temperature for 30 seconds, and extension 72˚C for 2 minutes was performed. The presence of PCR products was validated by gel electrophoresis. To create the promoter reporter constructs, these fragments were cloned in pGL3 basic vector and transformed in *Ecoli* JM109, screened and sent for sequencin

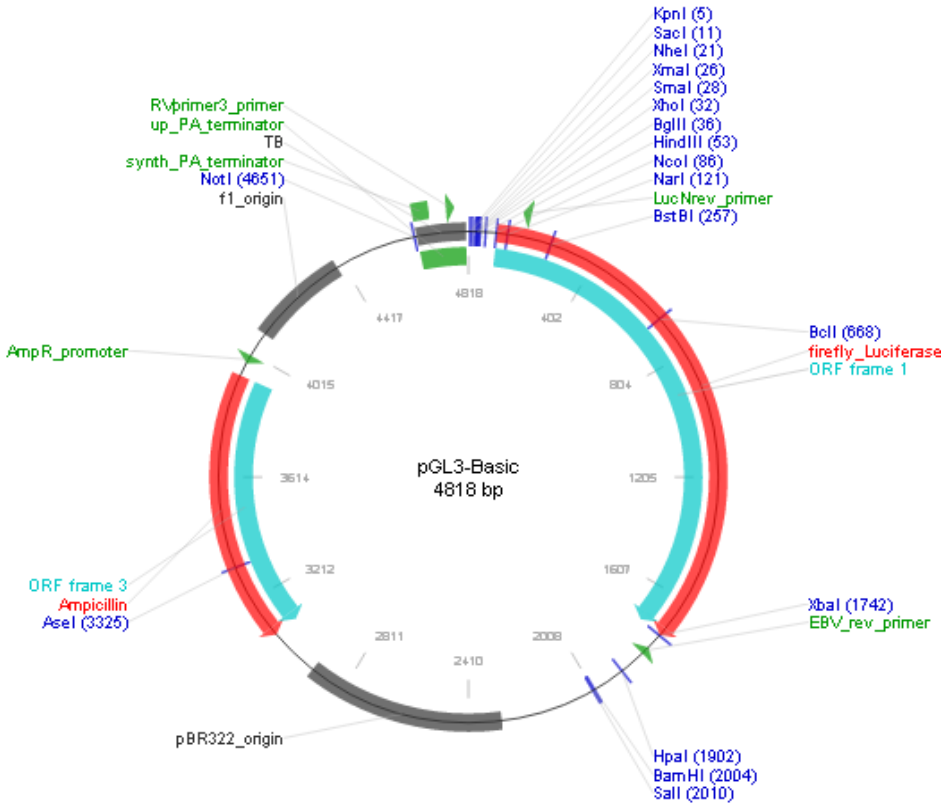


Figure 6.1 pGL3 basic vector map. pGL3 basic vector contains firefly luciferase coding region and various restriction sites to facilitate the insertion of promoter while creating promoter reporter construct.

6.2.5 Transient transfection of promoter-reporter constructs in HepG2 cell lines

To examine the activity of promoter reporter constructs, they were transiently transfected together with empty pGL3 basic vector (used as control) in HepG2 cell lines known to internally express PPARγ (Han C. *et al*., 2002). For cell culture and passage please refer to section 3.4.3 of chapter 3. Cell seeding was done one day before transfection. 500 µl of 2 X 10^4 cells/ml were seeded in 6 welled plate. On the day of transfection, cells were at 60% confluence. Before proceeding with transfection all required solutions were prepared. First 2 µl of Lipofectin transfection reagent (Invitrogen) was mixed with 100 µl of OptiMEM Serum Free Medium (GIBCO) and incubated for 45 minutes at room temperature. Another solution was prepeared consisting of 1 µg of each promoter reporter construct and 0.1µg of pRL-TK plasmids in 25 µl of Sereum Free Medium. pRL-TK works as internal control in the experiment. Both the solutions were mixed and kept at room temperature for 20 minutes to allow the formation of DNA-lipofectin complex. Lipofectin facilitates the transfer of DNA inside the cells. 200 µl of Serum Free Medium was later on added to the mixture.

Growth medium was discarded from the cells grown in 6 well plates. Cells were washed twice with pre warmed 1XPBS. Subsequently 2 ml of transfection mixture was layed over these cells for 3 hours. The transfection mix was discarded and cells were washed again. Complete MEM was mixed with 10% fetal calf serum and added to the cells. Cells were then incubated overnight before the luciferase assay.

6.2.6 Co-transfection of HepG2 cell lines with promoter reporter construct and pSVsport-PPARγ vector

To further confirm the PPARγ mediated transcriptional regulation of COX-1 promoter the promoter reporter construct along with pRL-TK plasmids was co-transfected with pSVsport-PPARγ vector into the HepG2 cells using the same method which was mentioned in section 6.2.5 of this chapter.

6.2.7 Luciferase assay

Before measuring the luciferase activity, cells were lysed. Briefly, complete MEM medium was removed from the cells and they were washed with 1x PBS twice. 500 µl of 1X passive lysis buffer was then added to the cells. Cells were gently shaken on the orbital shaker for 15 minutes to facilitate lysis. The lysate was transferred in a microcentrifuge tube and centrifuged at 1000g for 30 seconds at 4˚C. Lysate was then transferred in the fresh tube.

20 µl of the lysate was added to a 100 µl of Luciferase Assay Buffer II (LARII) (Promega). This mixture was subjected to the first luminescence reading by placing it in TD-20/20 Turner Designs luminometer (Turner Designs USA). The first luminescence reading was taken to measure the activity of internal control Renilla luciferase. After which 100 µl of Stop and Glo® Reagent (Promega) was added to the transfection mixture and the second reading were taken for the firefly luciferase activity. Readings obtained from firefly luciferase activity were

normalized against the reading of Renilla luciferase. The experiments were performed in triplicate.

Table 6.1 Primers to generate deletion construct of COX-1 promoter. Forward primers (F) and reverse primer (R) used for PCR amplification of zebrafish COX-1 promoter fragments. Letters in bold represent the restriction sites incorporated in the primers (AAGCTT for HindIII and AGATCT for BglII)

Name	Sequence 5'-3'
A (+134) R	CAT**AAGCTT**GCTCACCTCTCATTGTAGTTTTGAA
A1 (-428/+134)F	CAT**AGATCT**AGACCAAACGCACTTACAAA
A2 (-1067/+134)F	CAT**AGATCT**TGCACTCGAATTGATATGTTGG
A3 (-1369/+134)F	CAT**AGATC**TCCAAAGTCCACAAACAAACACA
A4 (-1952/+134)F	CAT**AGATCT**CATTTTTCCCAGCAAGCATT
A5 (-2573/+134)F	CAT**AGATCT**GATTAGGGTTGTAAAGAAGGTTGTGTTC

6.2.8 Extraction of nuclear protein from zebrafish ovary and synthesis of probes

Nuclear protein was extracted from zebrafish ovary using NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce Biotechnology) following manufacturer's specifications. Protein was immediately stored in -80˚C till further use. Concentration of protein was determined as described in 4.2.6.

25 bases of Oligonucleotide sequence containing PPRE site was deduced from the zebrafish COX-1 promoter. The forward (5'ATAGGGTGGGGGAGCGGTCAGAG 3') and its reverse complement oligo (5'CTCTGACCGCTCCCCCACCCTAT3') were subjected to biotin labeling and used as probes for EMSA. Biotin labeling of these probe was done using Biotin 3'-End DNA Labeling Kit (Pierce Biotechnology). To generate a double stranded biotin labeled probe both forward and reverse oligos were mixed in equal volume and heated at 90° C for 1 minute and cooled down at room temperature for 30 minutes. Double stranded biotin labeled probe was kept in -20° C until further use. Similarly double stranded unlabeled probe was also prepared.

6.2.9 Electrophoretic Mobility Shift Assay (EMSA)

In order to exhibit the functionality of PPRE site, binding ability of the PPARγ present in nuclear protein of zebrafish ovary with probes containing PPRE from zebrafish COX-1 promoter was tested. Three separate reactions were set using Light Shift Chemiluminescent EMSA Kit (Thermo Scientific, USA).

To facilitate the binding of biotin labeled probes with the protein present in nuclear extract, 20 µg of nuclear protein, together with 1µM of probe was mixed with 2µl of 10x binding buffer and 1 µl of each of 50% (v/v) glycerol, 1% (v/v) NP-40, 1 µg/µl poly (dI-dC), 100 mM MgCl2, 1 M KCl, 200 mM EDTA. Binding buffer and all other reagents were provided with the kit. The reaction was incubated for 20 minutes on room temperature.

To ascertain the specificity of DNA protein complex 50µM excess of unlabeled probe was mixed in the above mentioned binding reaction and reaction

was incubated for 20 minutes. This process is known as cold competition and causes the fading or disappearance of specific DNA-protein complex.

To confirm the identity of the DNA Protein binding complex, supershift assay was performed, in which 5 µl of PPARγ antibody was mixed in the binding reaction. The mix was incubated for 30 minutes at room temperature. After incubation, 5 µl of 1X loading buffer was added in all the reaction mixes mentioned above and the mixes were subjected to gel electrophoresis.

6.2.9.1 Gel electrophoresis of binding complexes

DNA-protein complexes were separated on 6% non denaturing polyacrylamide gel which was prepared using 40% 29:1 Acrylamide/bisacrylamide in 5x TBE buffer. Electrophoresis was performed at 4˚C in PROTEAN II Slab Electrophoresis Cell (Bio-Rad, USA) at 100V for 1 hour.

6.2.9.2 Transfer of DNA-protein complex on membrane by blotting

A Hybond-N+ membrane (Amersham, USA) was soaked in 0.5% TBE buffer. The transfer of DNA-protein complex was carried out exactly as described in section 5.2.5 of chapter 5.

6.2.9.3 UV cross-linking of DNA-protein complex on the membrane

To permanently fix the DNA-protein complexes on the membrane UV cross-linking was done. Membrane was taken out from the transfer apparatus and dried on a paper towel. It was placed in the UV-light cross-linker instrument (Hoefer, USA) making sure that the DNA-protein complex transferred side of the membrane is facing upwards. The cross-linking was done at 120 mJ/cm for 1 min.

6.2.9.4 Chemiluminescent detection of DNA –protein complex

Membrane was blocked in blocking buffer for 1 hour with gentle agitation and incubated in Streptavidin Horseradish Peroxidase Conjugate (1:300 dilutions in blocking buffer) for 30 minutes. Membrane was washed 4 times for 10 minutes with 1x washing buffer. Membrane was then incubated in Substrate Equilibration Buffer for 30 minutes. Membrane was then subjected to Chemiluminescent Substrate Working Solution for 5 minutes without any agitation. X-ray development of membrane was done to detect the DNA-protein complexes. All the reagents mentioned above were provided with Light Shift EMSA Kit (Pierce).

6.3 Results

6.3.1 Expression of PPARγ in vitellogenic and mature oocyte

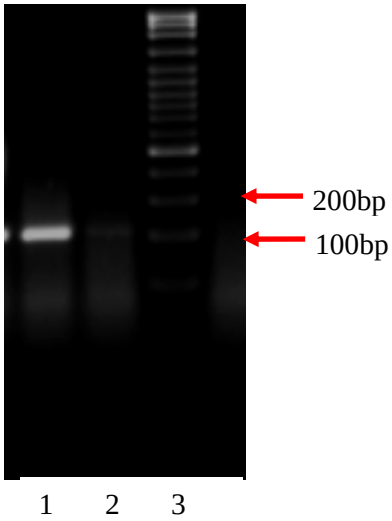
In order to find out the possibility of inverse relationship between the expression pattern of PPARγ and COX, mRNA expression of PPARγ in EMV, LV and M oocytes was obtained through semi-quantitative real time PCR. The expression of PPARγ was then compared with the expression of COX-1 and COX-2 in EMV, LV and M oocytes attained in the chapter 4 of this study. First 153 bp region of PPARγ was PCR amplified. It separated as sharp band on 1.5% agarose gel. The amplified fragment sequence exhibited its identity with published zebrafish PPARγ sequence (Figure 6.2 A and B).

Real time PCR analysis showed very low mRNA expression of PPARγ in EMV and LV oocytes (Figure 6.3). Significant reduction in mRNA expression was observed from LV to mature oocytes. Comparison of PPARγ expression with COX-

1 mRNA expression in EMV, LV and M oocytes showed an inverse relationship. While COX-1 expression was upregulated from full grown to mature oocytes, PPARγ demonstrated strong suppression from full gown to mature oocytes. Collectively these results indicate that since an inverse expression pattern is observed between PPARγ and COX-1, a possibility of PPAR mediated downregulation of COX-1 cannot be denied. However to confirm it further, it is necessary to explore the promoter of COX-1 for the presence of PPRE the specific sequence on which PPARγ binds to directly regulate the transcription of a gene.

COX-2 demonstrated reduced expression throughout the samples and was further suppressed during maturation, hence it did not demonstrate inverse relationship with PPARγ. However to confirm further if COX-2 is also directly regulated by PPARγ, zebrafish COX-2 promoter should be explored for the presence of PPRE.

A



B

```
Query 1     GTGGAAGGCGAGCAGATGATCAACTGCAGATACATGCCGCTCCACGAGCACCGGCGCTCA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 920   GTGGAAGGCGAGCAGATGATCAACTGCAGATACATGCCGCTCCACGAGCACCGGCGCTCA  979

Query  61   GATCTGGGCATCATGCATGAGGTGGAGCTGCGCTTCTTCCACAGCTACCAGTCCAGATCT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  980  GATCTGGGCATCATGCATGAGGTGGAGCTGCGCTTCTTCCACAGCTACCAGTCCAGATCT  1039

Query  121  GCCGAGGCCATCAGCGAAGTCACCGAATTCGCC  153
            |||||||||||||||||||||||||||||||||
Sbjct  1040 GCCGAGGCCATCAGCGAAGTCACCGAATTCGCC  1072
```

Figure 6.2 Amplification and sequencing of PPARγ. 153bp amplicon of PPARγ resolved on 1.5% w/v agarose gel (A). BLAST alignment of PPARγ amplicon sequence. Query represents PPARγ amplicon and subject represents published zebrafish sequence (B).

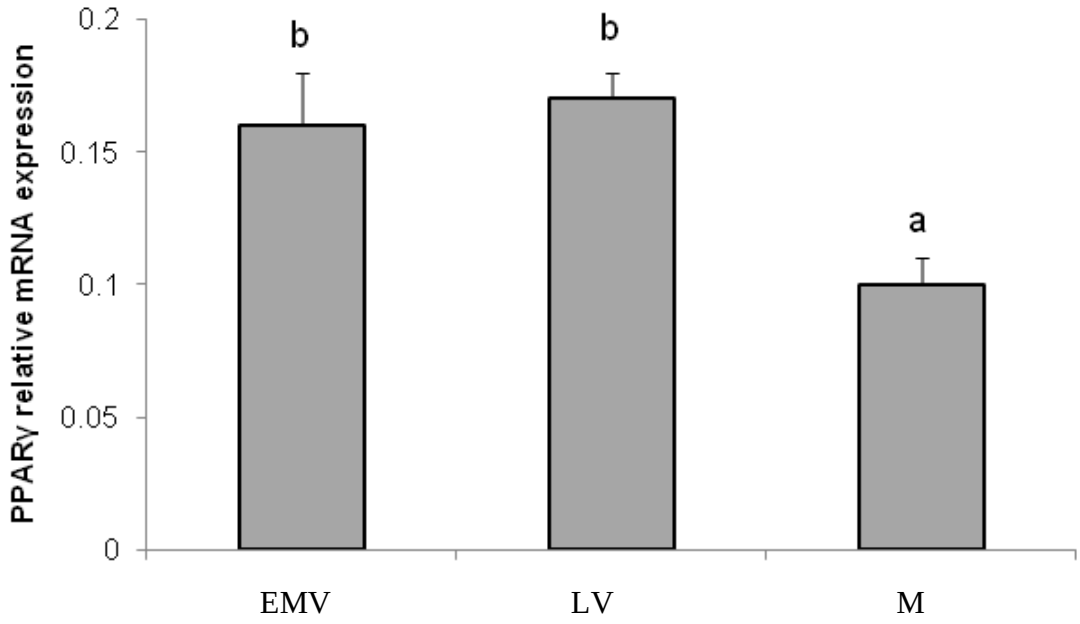


Figure 6.3 mRNA Expression of PPARγ in oocytes mRNA expression of PPARγ in early and mid-vitellogenic (EMV), Full grown (LV) and matured (M) zebrafish follicles**.** Mean values with different letters are significantly different ($P<0.05$).

6.3.2 *In silico* detection of PPRE sequence on COX-1 and COX-2 promoter of zebrafish

Previously in chapter 4, chapter 5 and section 6.3.1 of this chapter, it was established that COX-1 was necessary for oocyte maturation and it shared an inverse mRNA expression pattern with the known cyclooxygenase regulator PPARγ. Collectively these findings indicated the possibility of PPARγ mediated transcriptional regulation of COX-1 during oogenesis. If the possible transcriptional control is a direct transcriptional control then the PPAR specific binding site, PPRE, has to be present on the promoter of COX-1. In order to confirm the possibility of

direct transcriptional regulation, firstly presence of PPRE was checked with the help of *in silico* analysis.

A putative PPRE "GGGGGAgCGGTCA" at position -2470 to -2458 (Figure 6.4) was detected during *in silico* analysis of 3 kb COX-1 promoter, suggesting the direct possible transcriptional control of COX-1 by PPARγ . However *in vivo* confirmation of PPARγ mediated transcription of COX-1 is required along with COX-1 promoter cloning and sequencing, in order to verify the presence and function of this site. At the same time 3 kb COX-2 promoter was also scanned for the detection of PPRE site, but there was no site located. Based on previous COX-2 related results and this analysis, the involvement of COX-2 was ruled out from the current scope of this study at this point.

Figure 6.4 Position of PPRE on COX-1 A5 promoter sequence A5 sequence was aligned with zebrafish genomic DNA, it showed the sequence similarity with 5' flanking region of zebrafish COX-1 present on chromosome 5. PPRE site was found at position -2470 to -2458 denoted by red letters in the sequence. The query represent sequence of A5 and the sbjct represents zebrafish genomic DNA.

6.3.3 The role of PPARγ in regulating the transcriptional activity of zebrafish COX-1 promoter

In order to directly confirm PPARγ mediated transcriptional regulation of COX-1, a detailed analysis of COX-1 promoter activity *in vivo*, in the presence of PPARγ protein was carried out. To achieve this target, five 5' deletion fragments, A1 (-428/+134), A2 (-1067/+134), A3 (-1369/+134), A4 (-1952/+134) and A5 (-2573/+134) of COX-1 promoter were generated using PCR amplification strategy. Details about the size and position of these fragments are provided in table 6.2.

The amplification of these fragments was confirmed by resolving them on 0.7% agarose gel, where they exhibited size based separation in the form of sharp bands (Figure 6.5). All the five fragments were cloned in pGL3 basic vector to generate promoter reporter constructs. The sequence verification was done by performing a BLAST search using the sequences of these fragments. The fragment sequences confirmed their identity with the 5' flanking region of COX-1 in zebrafish genomic DNA at chromosome number 5. Sequencing of A5 revealed the presence of sequence GGGGGAgCGGTCA" at position -2470 to -2458 (Figure 6.4) which was claimed as putative PPRE site by *in silico* analysis done in section 6.3.2.

Table 6.2 Position and sizes of five deletion constructs derived from zebrafish COX-1 promoter. First base of start codon is numbered as +1, bases upstream to start codon are numbered as minus (-) and downstream are numbered as plus (+)

Name of deletion construct	Position on Zebrafish COX-1 promoter	Size in base pairs (bp)
A1	-428 /+134	562
A2	-1067 /+134	1201
A3	-1369 / +134	1503
A4	-1952/+134	2086
A5	-2573/+134	2707

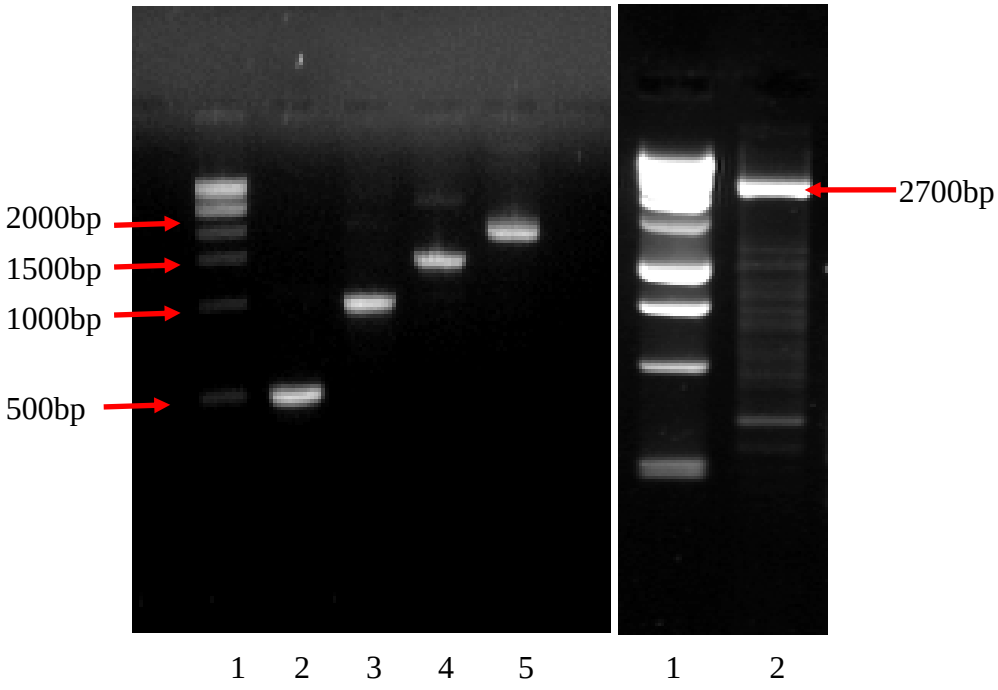


Figure 6.5 Amplification of COX-1 promoter constructs. Deletion constructs A1 (-428/+134), A2 (-1067/+134), A3 (-1369/+134), A4 (-1952/+134) and A5 (2573/+134) resolved on 0.7% agarose gel.

The promoter reporter constructs were transfected in HepG2 cell lines and their promoter activities were examined in the presence of endogenously expressed PPARγ (Figure 6.6). HepG2 cell lines were selected as *in vivo* model system, because they are known to express PPARγ endogenously.

Luciferase activity of deletion construct A5 was drastically inhibited compared to other deletion constructs. It exhibited 10 folds suppression from the luciferase activity of A1 which was maximum out of the five deletion constructs. Luciferase activity of promoter fragment A4 was 7 fold higher than A5 in HepG2 cell lines. Between A5 and A4 there was a loss of -2573 and -1951 bp region where

according to *in-silico* and sequence analysis a PPRE site lies, specifically at position -2470 to -2458. Significantly enhanced activity of A4 confirmed that in the absence of PPRE binding site, endogenous PPARγ protein was unable find a suitable binding space on A4. Hence the transcriptional suppression of COX-1 promoter by PPARγ which was seen in A5 did not occurred in A4. This observation also exhibited that inhibition in the activity of A5 is because of the direct transcriptional control of PPARγ happening due to the presence of specific PPRE on it. The strength of transcriptional inhibitory control exerted by PPARγ on COX-1 promoter can be realized by the 7 fold activation in promoter activity of A4 compared to A5.

Luciferase activity of A3 was approximately a fold higher than A4. However Deletion construct A2 exhibited two fold suppression in activity when compared with the activity of A3. This indicated presence of binding sites corresponding to the suppression of promoter activity between -1369 and -1067 bp length of promoter that was lost between the two deletion constructs. However the suppression was not as significant as the suppression which was seen in the activity of A5 construct, again proving that PPARγ puts forth a very strong direct inhibitory transcriptional control on COX-1 promoter activity. Promoter activity of A1 deletion construct was maximum of all the promoter constructs, which signifies that -428/+134 base pair region is the maximum promoter length, for the highest promoter activity.

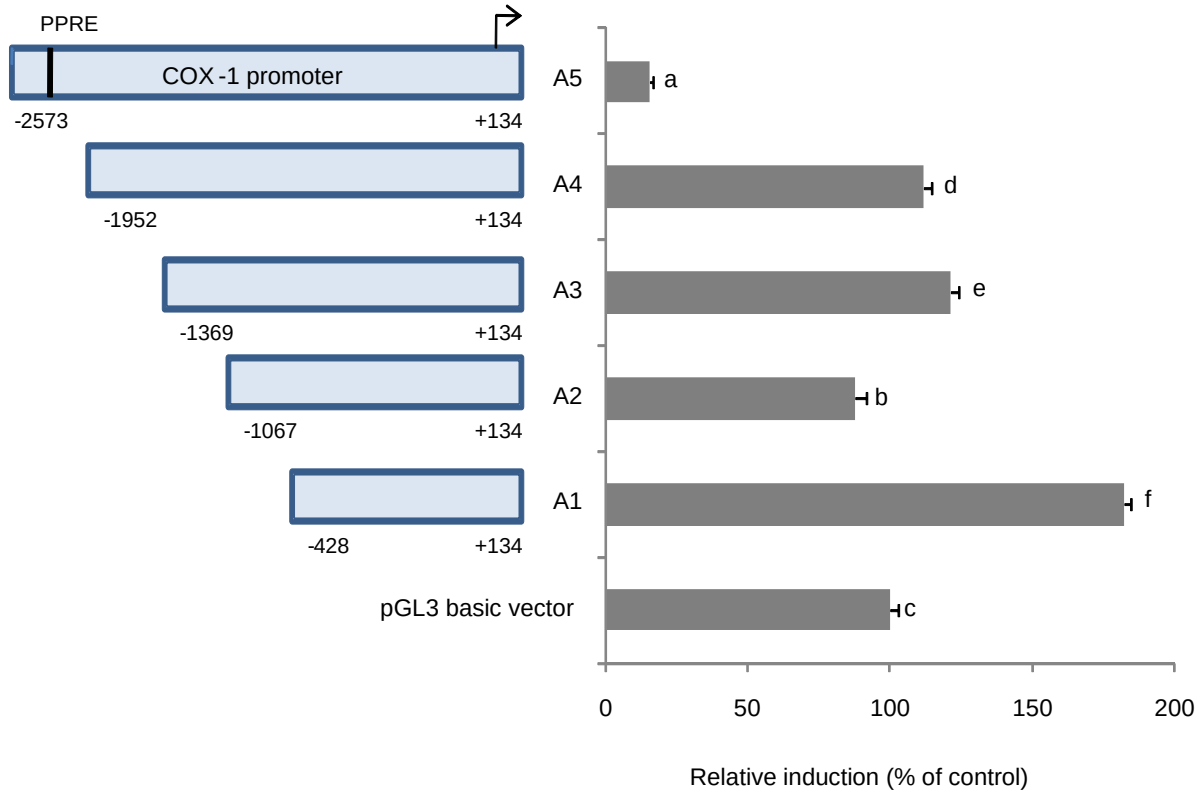


Figure 6.6 Transfection of COX-1 promoter reporter constructs in HepG2 cells. Graphical representation of luciferase activities of zebrafish COX-1 promoter reporter constructs A1, A2, A3, A4 and A5 transfected in HepG2 cells with the empty pGL3 vector. Mean values with different letters are significantly different ($P<0.05$) and presented as percentage of induction as compared to empty pGL3 basic vector as control (100%).

To confirm the specificity of PPARγ mediated direct transcriptional control of COX-1, deletion construct A5 which has PPRE site was cotransfected in HepG2 cell lines with pSVsport-PPARγ vector which expresses PPARγ protein. The intention of cotransfection was to examine the activity of A5 when pSVsport-PPARγ vector starts overexpressing PPARγ in HepG2 cell lines causing increase in PPARγ amount in the cells. As expected, activity of A5 co-transfected with pSVsport-PPARγ vector was further significantly suppressed due to the over expression of

PPARγ protein. Compared to the A5 transfected without pSVsport-PPARγ vector, there was a 2.5 fold decrease in the activity of A5 due to the PPARγ overexpression (Figure 6.7).

Collectively, the findings of these experiments suggested that PPARγ downregulates the expression of COX-1 by controlling its transcription. Deletion construct analysis also demonstrated that the inhibitory control of PPARγ does not take place when the PPRE site present at position -2470 to -2458 was deleted, approving the requirement of this site for PPARγ protein binding to happen on COX-1 promoter. To examine the binding capability of putative PPRE site further EMSA was performed.

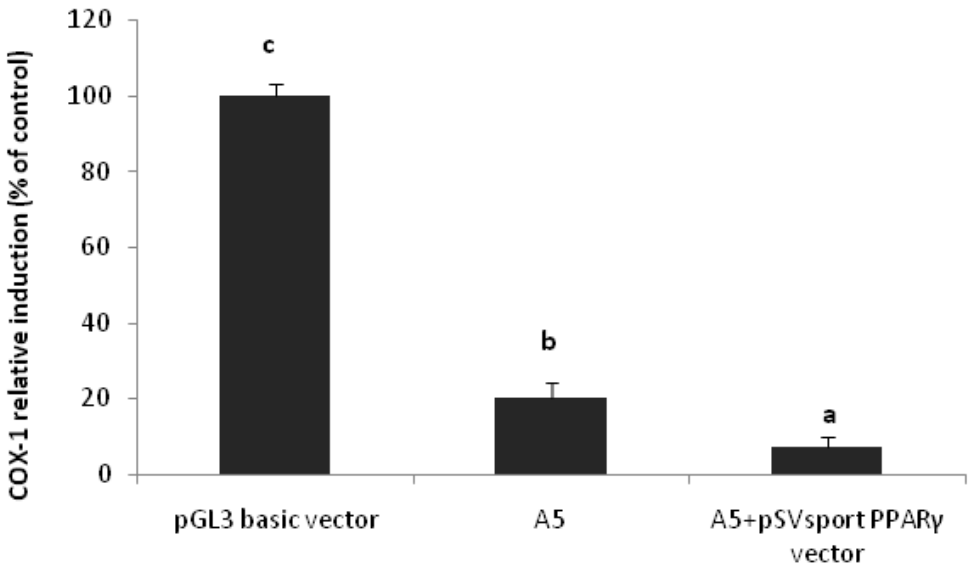


Figure 6.7 Co-transfection of A5 in HepG2 cells with pSVsport PPARγ expression plasmid. Graphical representation of the activity of A5 promoter reporter construct of zebrafish COX-1 promoter, when transfected alone in HepG2 cells and when co-transfected with pSVsport PPARγ expression plasmid. Mean values with different letters are significantly different ($P<0.05$).

6.3.4 PPARγ protein from zebrafish ovary binds to the putative PPRE site of COX-1 promoter

To examine the functionality of PPRE like *cis*-element found on zebrafish COX-1 promoter at position -2470 to -2458 (6.3), EMSA was performed by incubating nuclear extract proteins taken out from zebrafish ovary and synthetic oligo probes containing putative PPRE sequence derived from COX-1 promoter. DNA-protein complexes obtained by EMSA were assessed in the form of band on x-ray film (Figure 6.8)

Incubation of nuclear extract with labeled probes derived from COX-1 promoter containing PPRE site, resulted into the formation of two DNA-protein complexes C1 and C2 (Figure 6.8 lane 3) indicating the binding of PPRE site containing probe with nuclear protein of zebrafish. Cold competition with 50x molar excess of unlabeled probes resulted in the fading of both the complexes (Figure 6.8 lane 2). Cold competition assay establishes that the complexes seen in lane 3 are formed due to the specific binding of probe and nuclear protein. Incubation of PPARγ specific antibody with the zebrafish nuclear protein and probe resulted into bulkier partly shifted complex due to the antibody protein and DNA interaction, showing that PPARγ specific antibody binds to the DNA protein complexes (Figure 6.8 lane 3). This shows that the protein present in DNA Protein complex is PPARγ. Taken together these findings ascertain that PPRE site, present on probe derived from COX-1 promoter, is functional and binds to PPARγ protein present in nuclear extract.

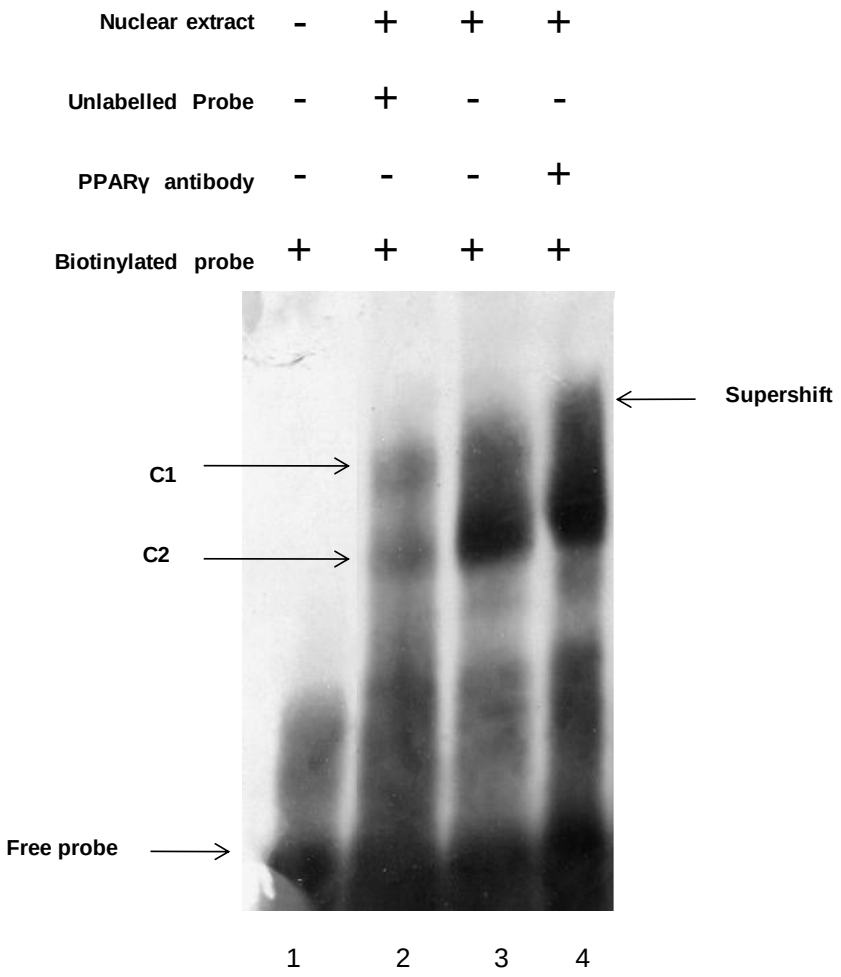


Figure 6.8 Functional verification of putative PPRE present on COX-1 promoter. Biotin labeled PPRE incubated with zebrafish ovary nuclear extract showed two DNA-protein complexes, C1 and C2 (lane 3). Binding specificity was confirmed by cold oligo competition using 50x molar excess of unlabeled probe (lane 2) and super shift assay (lane 4).

6.4 Discussion

Based on the literature review done in chapter 2, it can be derived that PPARγ controls various aspects of female fertility largely by regulating the genes involved in the pathways of oogenesis. As mentioned in section 6.1, existing literatures support the transcriptional regulation of COX by PPARγ. In this study the similar possibility was explored from zebrafish oogenesis point of view.

In this chapter it was demonstrated that mRNA expression pattern of PPARγ is in contrast to COX- 1 during vitellogenesis and maturation. PPARγ mRNA expression was lowest during oocyte maturation, while COX-1 was highest, assuring the inverse relationship between PPARγ and COX-1 expression. This finding was in agreement with the fact that other PPARγ targeted genes are also in inverse expression relationship with PPARγ during oogenesis (Keller *et al*., 1995; Komar & Curry, 2003; Yee *et al*., 1997).

Further, the suppression of COX-1 promoter activity by endogenously expressed PPARγ in HepG2 cell line was established in this chapter. The reporter construct that contained PPRE binding site, showed the strong inhibition of promoter activity which was not seen in other constructs where the putative PPRE site was deleted. It was also observed that over-expression of PPARγ caused by the introduction of external PPARγ expressing plasmid vector in HepG2 cells, resulted in additional significant inhibition of COX-1 promoter activity. The potential PPRE site sequence exhibited *in vitro* binding affinity with PPARγ protein ensuring its functional binding properties. All together these findings corroborate strong direct transcriptional regulation of zebrafish COX-1 by PPARγ.

Previously the presence of PPRE has been reported on human COX-2 promoter and there is a report of transcriptional control of PPARγ on COX-2 in human epeithelial cell line (Meade *et al*., 1999). However mRNA expression of COX-2 was also very low during zebrafish oocyte maturation and there was no inverse relationship seen in case of COX-2 and PPARγ expression patterns in oocytes. Even the in-silico analysis did not reveal any PPRE on COX-2 promoter. If there is any possibility of regulation of COX-2 by PPARγ during zebrafish oogenesis it needs a great deal of exploration. Also COX-2 is induced during ovulation, hence its role and regulation during ovulation needs to be investigated separately.

To our knowledge, this is the first report showing the transcriptional regulation of COX-1 isoform by PPARγ binding. Several studies have reported the downregulation of PPARγ mRNA levels at the advent of LH surge in granulosa cells that marks maturation. (Banerjee and Komar, 2006; Minge *et al*., 2006). Increment in production of prostaglandins in ovarian cells is also reported with LH surge. As this study shows a direct transcriptional regulation of COX-1 by PPARγ, it is possible that LH maintains low level of PPARγ and the regulatory relationship between COX-1 and PPARγ in turn causes the rise in COX-1 activity to allow the production of prostaglandins during vitellogenesis and maturation.

CHAPTER 7

CONCLUSION AND FUTURE STUDIES

7.1 Conclusion

COX-1 and COX-2 are the two isoforms of COX present in zebrafish, both isoforms are required in oogenesis. While COX-2 is induced during ovulation, specific distinct role of these isoforms has not been examined during oocyte growth and maturation before. Objective of this study was to establish the functional role of COX during oocyte growth and maturation and to determine its regulation by PPARγ. In present study, using zebrafish as model system, it was established that out of the two isoforms of cycloooxygenase, COX-1 is more likely to be responsible for the oocyte development and maturation. This study also demonstrates, that the presence of COX-1 is critical for the successful maturation of the oocytes and it's transcription is regulated by master regulator of female fertility, PPARγ.

In chapter 4, real time analysis of COX-1 and COX-2 in vitellogenic and mature oocytes, established the importance of COX-1 over COX-2. In chapter 5 the knockdown of COX-1 caused maturation failure, while oocytes injected with mismatch morpholino matured normally. By combining the observations of chapter 4 and chapter 5 the functional importance of COX-1 was demonstrated during vertebrate oocyte maturation.

Based on the literature review done for this study, strong possibilities were seen regarding the transcriptional regulation of COX by PPARγ during oogenesis. As PPARγ and its targeted genes tend to have an inverse expression mRNA pattern,

in chapter 6 the possibility of the similar connection was explored. mRNA expression of PPARγ was obtained in vitellogenic and mature oocytes by real time PCR. PPARγ expression was drastically reduced during maturation, confirming its inverse expression pattern with COX-1. The finding indicated the possibility of PPARγ mediated downregulation of COX-1 during oogenesis. At the same time, COX-2 expression did not show such correlation.

PPARγ binds to the target gene on the specific sequence present on the promoter of the gene known as PPAR response element (PPRE) and controls its transcription. Presence of PPRE on a gene promoter strongly indicates its direct transcriptional regulation by PPARγ. *In-silico* analysis of 3 kilobase promoter region of COX-1 and COX-2 revealed the presence of PPRE on COX-1 promoter at position -2470 to -2458 bp; which was further confirmed by cloning and sequencing of COX-1 promoter fragment. This PPRE was shown to be functional, as the probes containing the PPRE of COX-1, bind with the PPARγ of nuclear protein of zebrafish ovary.

Five deletions constructs from 2.7 bp COX-1 promoter were prepared and transiently transfected in HepG2 cell line. The construct with the PPRE binding site showed very strong suppression in response to the internal PPARγ protein present in HepG2 cell lines. Further confirmation of suppression of COX-1 activity came by co transfecting PPARγ expression vector with this construct. PPARγ expression vector caused the over expression of PPARγ protein in HepG2 cell lines. Further stronger suppression of COX-1 promoter was observed in this condition. Taken together, findings of chapter 6 establish that PPARγ transcriptionally downregulates COX-1 expression by binding on putative functional PPRE site present on COX-1 promoter. Based on the literature survey, this is the first study, which demonstrates the

functional role of COX-1 during vertebrate oocyte maturation and regulation of the COX-1 isoform by PPARγ binding.

7.2 Future direction

By establishing the role of COX-1 in vertebrate oocyte development, this study opens up a possibility to explore the involvement of this isoform in various other biological processes. To date, studies have reported the induction of COX-2 during ovulation. However there are no reports available to prove the direct involvement of COX-1 in vertebrate oocyte maturation before this study. The study performed here is done on zebrafish; similar studies can be done in various other model systems to confirm if involvement of COX-1 in maturation of oocytes is a fish specific process or it is conserved across various species. Requirement of COX-1 homologue in drosophila oocyte maturation and presence of COX-1 in human oocytes provide support to this hypothesis.

Contrary to COX-1, COX-2 was either absent or present at a very low level, during vitellogenesis and maturation of oocytes. It was induced during ovulation in zebrafish. It points out the possibility of transition between the COX isoforms at ovulation. However existence of the transition and its regulation requires further exploration. Similarly, in order to learn more about the cyclooxygenase isoform preference in biological processes, conditions and factors that influence the COX preferences need to be examined.

It has been previously shown that COX-2 knocked down zebrafish embryos were able to survive. It can be perceived, that COX-1 derived prostaglandin was able

to rescue the COX-2 deficiency. The knock down of COX-1 in this study resulted in oocyte maturation arrest. Since COX-2 is expressed at very low level in zebrafish oocytes and the COX-1 is knocked down, the rescue doesn't seem to be possible. Further research is required to confirm the possibility of prostaglandin derived from one cyclooxygenase isoform being able to rescue the absence of other isoform.

The classic question which is being asked over and over again is; why there is a requirement of two isoforms of COX required for production of prostaglandins? Both COX-1 and COX-2 produce PGs. There is no difference in their substrate or the product. They work through the similar reaction mechanism. Scientists are looking into the origin of cyclooxygenases to find out the answers, but a concrete answer of this question is yet come.

This study shows direct downregulation of COX-1 by PPARγ. Similar studies can be conducted in humans and other complex vertebrate systems in order to confirm if this regulation is fish specific or conserved in other vertebrates as well. In human, PPARγ binding site is found on COX-2 promoter, which was not seen in zebrafish COX-2 promoter. Still, the indirect regulation of COX-2 by PPARγ in zebrafish cannot be denied and needs further investigation.

It was also discussed that during maturation, at the advent of LH surge, PPARγ gets downregulated and prostaglandin production gets upregulated in granulose cell. In this study it was demonstrated that PPARγ trascriptionally downregulates COX-1, but there is a likelihood of LH being the upstream regulator of the entire process during maturation, which needs investigation.

PPARγ and its ligands are considered to use in cancer therapy and Alzheimer's. Role of PPARγ is well known in tumor suppression. It has been now

identified that COX-1 is upregulated in various forms of cancer. COX-1 is the only isoform of COX expressed in ovarian cancer. As direct down regulation of COX-1 by PPARγ in zebrafish oogenesis is shown in this study, similar possibility can be explored in ovarian cancer studies as well. COX-1 is also expressed in Alzheimer's affected human brain. An investigation of direct or indirect regulation of COX-1 by PPARγ in higher vertebrates in such diseases can have medically important outcomes.

PPARγ exerted control on COX-1 expression only covers a part of COX-1 transcriptional regulation, other transcription factors and their effect on COX-1 transcription requires further study. At the same time full characterization of zebrafish COX-1 promoter also needs to be done. This study and some other recent studies demonstrate that COX-1 can be as crucial as COX-2 in vertebrates. However the full potential of this isoform requires further investigation.

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