Pax2a is expressed in oocytes and is responsible for early development and oogenesis in zebrafish

Thesis

Pax2a is expressed in oocytes and is responsible for early development and oogenesis in zebrafish

Theeranukul Pachoensuk

Graduate school of science and technology
Department of Bioscience
Shizuoka university
Japan

December 2020

LIST OF ABBREVIATIONS **3**

ABSTRACT **5**

CHAPTER I GENERAL INTRODUCTION **7**

GENERAL INTRODUCTION 8

Oocyte maturation *8*

Ovulation *11*

Selection of ovulation inducing genes in vivo *12*

CHAPTER II **20**

INTRODUCTION 21

MATERIAL AND METHODS 24

Experiment fish *24*

Zebrafish reproduction *24*

Genome editing *24*

DNA extraction *25*

Polymerase chain reaction (PCR) *26*

Heteroduplex mobility assay (HMA) *26*

DNA sequencing *27*

Mutant lines generation and phenotype observation *28*

Western blot analysis *29*

Immunohistochemistry in whole body *29*

Immunochemistry in eggs *30*

RNA extraction *30*

Complementary DNA (cDNA) synthesis *31*

Quantitative PCR (qPCR) *31*

Recombinant Pax2a *32*

Histological observation *33*

Statistical analysis *34*

RESULTS 35

Genome editing *35*

Mutant lines generation and phenotype observation *40*

Western blot, immunohistochemistry and immunochemistry analysis *45*

Quantitative PCR (qPCR) *47*

DISCUSSION 49

CHAPTER III CONCLUSION AND PERSPECTIVE 51

CONCLUSIONS 52

PERSPECTIVE 53

REFERENCES 54

ACKNOWLEDGEMENTS 60

LIST OF ABBREVIATIONS

A	Adenine
T	Thymine
G	Guanine
C	Cysteine
17α, 20β-DHP	17α, 20β-dihydroxy-4-pregnen-3-one
mPR	Membrane-bound progestin receptor
nPR	Nuclear progesterone receptor
DES	Diethylstilbestrol
EtOH	Ethanol
Tes	Testosterone
CRISPR	clustered regularly interspaced short palindromic repeats
Cas9	CRISPR associated protein 9
sgRNA	single guide RNA
tracrRNA	trans-activating CRISPR RNA
qPCR	Quantitative polymerase chain reaction
GVBD	Germinal vesicle breakdown
LH	luteinizing hormone
GTH	gonadotrophic hormone
MIH	maturation-inducing hormone
MPF	maturation promoting factor
cDNA	complementary DNA
mRNA	messengerRNA
FSH	Follicle stimulating hormone
LH	Luteinizing hormone
pax2a	paired box2a
pax2a$^{+/-}$	*pax2a* heterozygous mutant
pax2$^{-/-}$	*pax2a* homozygous mutant
stm	starmaker
WT	wild type
HMA	heteroduplex mobility assay
rpm	round per minute
PCR	polymerase chain reaction

LB	Luria-Bertani
dNTP	deoxy ribonucleotide phosphate
HE	hematoxylin and eosin
EDTA	Ethylenediaminetetraacetic acid
TE	Tris and EDTA
TBSN	NP-40 in PBS
PBS	Phosphate-buffered saline
TPBS	Tween20 in PBS
RT-PCR	reverse transcriptase PCR
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
DDW	double distilled water
rPax2a	recombinant Pax2a

ABSTRACT

Oocyte maturation and ovulation are two important processes that can produce the next generation and maintain the species. For a long time, many molecular biologists have attempted to study two mechanisms of oocyte maturation and ovulation in many species, including the fish model zebrafish. A large part of the molecular system of genes that control these two processes remains unclear. The process of oocyte maturation and ovulation is actually related and overlapping. It is almost indistinguishable. The in vivo assay is a new system for discovering only ovulation-inducing genes that do not contain oocyte maturation-inducing genes. Ethanol, diethylstilbestrol (DES), testosterone (Tes), and 17α, 20β-dihydroxy-4-pregnane-3-one (17α, 20β-DHP) were treated in vivo into zebrafish. Ethanol did not alter immature oocyte maturation, but DES and Tes could only induce oocyte maturation, and 17α, 20β-DHP could induce both of oocyte maturation and ovulation. Due to these characteristics, the ovulation-inducing gene was isolated from the oocyte maturation-inducing gene by gene profile comparison using microarray and RNA sequencing. From these experiments, 11 probable ovulation-inducing genes were selected.

Pax2a is an interesting gene found in 11 genes that are likely related to induce ovulation. *Pax2a* has been reported to be involved in the regulation of development of many organs of zebrafish embryos, such as transcription factors in the brain, eye, thyroid and kidney. Nevertheless, the function of *pax2a* in the ovulation process is not understood. In this study, genome editing was used to investigate the function of zebrafish *pax2a*. CRISPR/Cas9 has been applied to edit *pax2a*. Microinjection of CRISPR/Cas9 solution into 1-cell stage embryos. F0 mosaic zebrafish were bred under appropriate conditions, and after adulthood, F0 mosaic was paired with wild type to produce F1 heterozygous zebrafish. HMA (heteroduplex mobility assay) and DNA sequencing were performed to check for mutations. The same F1 heterozygous zebrafish were paired to obtain F2 homozygous mutant zebrafish. Then, ovulation, fertilization, and early developmental phenotypes of the embryo were observed. Histological analysis was used to check oocytes in fish for wild-type and *pax2a* homozygous mutants. In addition, Pax2a expression was checked using Western blotting, quantitative polymerase chain reaction (qPCR), and immunohistochemistry.

This study established a line lacking 6 nucleotides, including the start codon of *pax2a*. This mutation could result in complete knockout of *pax2a* or a shift of the start

codon to the next ATG. The results of expression analysis of *pax2a* using Western blot, qPCR, and immunohistochemistry showed that the *pax2a* homozygous mutant expresses the Pax2a protein. This meant that the start codon of the *pax2a* mutant was shifted to the next ATG and only 12 amino acids were deleted. *Pax2a* homozygous mutant fish were fertile and were able to lay eggs. It means that the *pax2a* is not responsible for ovulation. However, embryos born from homozygotes had a high proportion of unfertilized eggs, and F3 homozygous embryos showed some unusual characteristics. For example, the epidermal embryo becomes oval. The oval embryo then showed other significant abnormalities such as cardiac edema, abnormal tail formation, and abnormal yolk morphology. These abnormalities were found when parents of female *pax2a* homozygous mutants were paired with wildtype or male *pax2a* homozygous mutants. However, when the male homozygous mutant was paired with the wild type, the embryos showed different types of abnormal characteristics. No oval abnormalities were found in embryos from male *pax2a* homozygous mutant zebrafish and female wild-type parents. These results suggest that *pax2a* is a gene whose effects are passed on to the next generation by the maternal effect. Very few homozygous embryos survived to adulthood. The surviving adult F3 homozygotes had very small bodies and the ovaries were barely mature in females. Histological analysis showed that the number of oocytes was extremely low. These results indicate that *pax2a* is involved in oogenesis, fertilization and early development.

The results of this study revealed that *pax2a* is not an ovulation-inducing gene but showed a new role for *pax2a* in its involvement in oogenesis, fertilization, and early embryonic development. These new findings may help us understand the molecular pathways of oogenesis, fertilization, and early embryonic development. It is also expected to help, for example, solve the problem of human infertility.

CHAPTER I
General introduction

GENERAL INTRODUCTION

Oocyte maturation

Oocytes are produced in ovaries. Oogonium, the primordial oocyte, increase the number by mitosis. After that, oocytes started meiosis and stopped at the prophase I for their meiotic cell cycle, called immature oocyte. Oocytes are stimulated by hormone to resume the meiosis until metaphase II stages, called mature oocyte. The process that develops the oocyte from immature until mature oocyte is oocyte maturation. During the maturation, there are many morphological changes, such as, chromosome condensation, germinal vesicle breakdown (GVBD), and first polar expulsion. Germinal vesicle (GV), that is oocyte nucleus, is normally located in the center of oocyte, then, for response to hormonal stimulation, GV migrates from the center to the animal pole. After that, GVBD occurs and the oocytes are become transparent from opaque. In the meiosis division, oocyte produces the first polar body after telophase I. Oocyte expels the first polar body to outside, and continue in meiosis division until metaphase II-arrest, now called, mature oocyte (**Figure 1**).

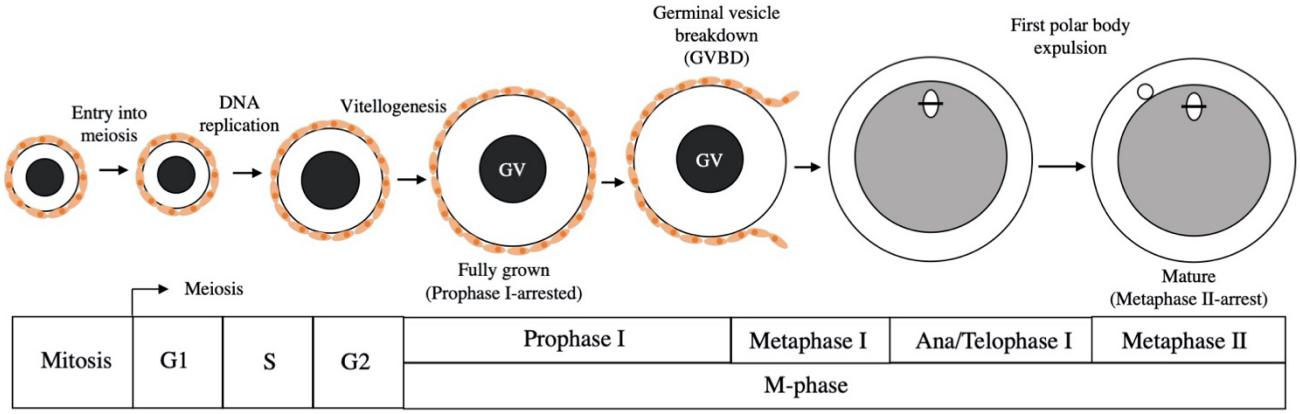

Figure 1 Processes of oocyte growth, oocyte maturation, and meiosis. Oocytes stop their mitosis and enter the meiosis at prophase I. The oocytes start to accumulate the yolk (vitellogenesis). The prophase I-arrested oocytes are called immature, then, resume meiosis process to metaphase II after germinal vesicle breakdown (GVBD) by hormonal stimulation. After that, the oocyte was expelled form follicle layer and produce the fertilization membrane.

Maturation-inducing pathway has been classified to be induced by 3 major protein substances, which are gonadotropic hormone (GTH), maturation-inducing hormone (MIH), and maturation promoting factor (MPF)(**Figure 2**). Pituitary gland

secrets GTH, especially, luteinizing hormone (LH) for oocyte maturation in teleost. LH binds the receptors at follicle cells to synthesize and secrete MIH. 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DHP) is the first identical MIH in amago salmon *Oncorhynchus rhodurus* (Nagahama and Adachi, 1985) and this steroid hormone has been reported to be a natural MIH for many kind of fishes, including, zebrafish *Danio rerio* (Pang and Ge, 2002, Fukada et al., 1994, Haider and Rao, 1992, Petrino et al., 1993, Rahman et al., 2002). 17α, 20β-DHP is received on oocyte membrane and transduced to the cytoplasm. MIH binds to membrane progestin receptor which have been identified, classified, and characterized in fish oocytes. 7 transmembrane domain characteristic of G protein-couple receptor (GPCR) was simulated from the cDNA cloned from the ovary of spotted seatrout (Zhu et al., 2003a, Zhu et al., 2003b). Membrane progestin receptor induced the non-genomic steroid signaling pathway via G-protein coupled receptor. In fish, inhibitory-G protein (G_i) is related the signal transduction pathway (Oba et al., 1997, Yoshikuni and Nagahama, 1994). MIH induces decreasing of intracellular cyclic Adenosine monophosphate (cAMP) (Haider and Chaube, 1995, Cerda et al., 1998). Adenyl cyclase activator can activate the level of cAMP, in contrast, it prevents the oocyte maturation by 17α, 20β-DHP. This experiment suggested that oocyte maturation have been induced by negative role of cAMP (Haider, 2003).

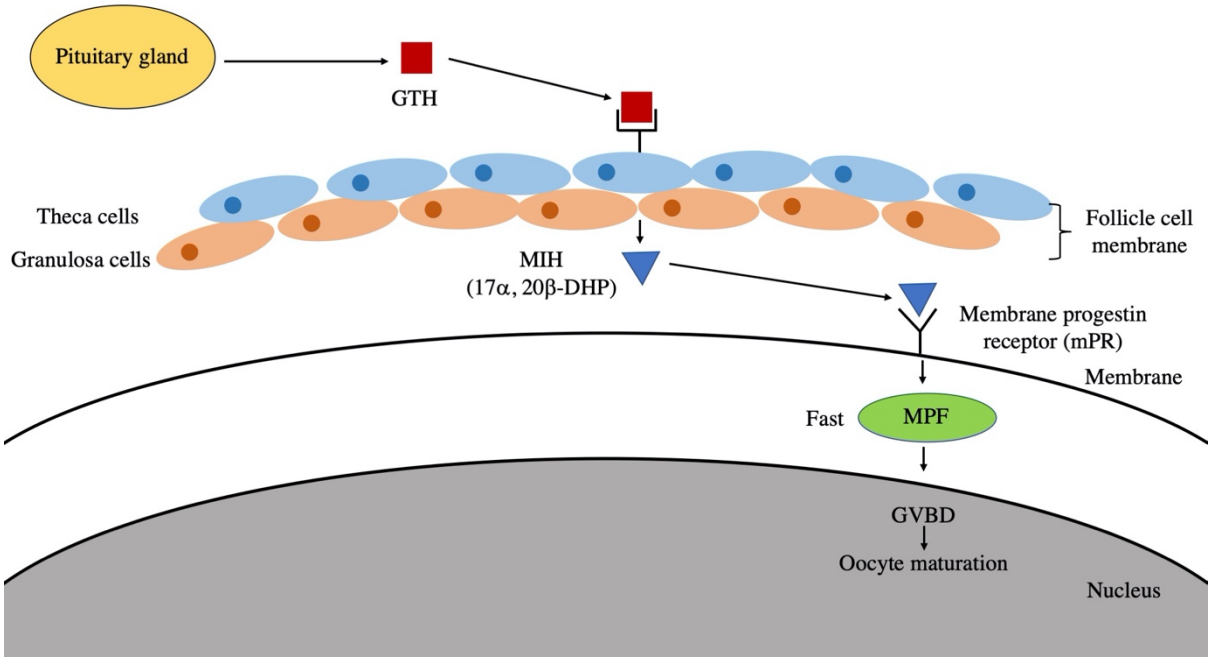

Figure 2 Summary of mechanism of oocyte maturation. Gonadotrophic hormone (GTH) was released from pituitary in brain and went to the receptor on follicle layers

pass through blood system. After that, maturation inducing hormone (MIH) was produced from follicle layer and bind to membrane progestin receptor (mPR) at the oocyte's membrane. This process activated to produce maturation promoting factor (MPF) for germinal vesicle breakdown (GVBD) and finally oocyte maturation was occurred.

MPF is the final major protein for oocyte maturation. In fish, the first MPF was purified in carp *Cyprinus carpio* (Yamashita et al., 1992). Oocyte maturation cannot be induced by directly injected MIH into oocyte cytoplasm. However, the oocyte maturation can be induced by directly injected mature cytoplasm into the new oocyte cytoplasm. This study recommended that MPF is built and activated inside the oocyte cytoplasm after MIH attached the receptor on the membrane. For teleost, oocyte required MPF for maturation and initiated faster in MPF-simulated than LH-stimulated and/or MIH-stimulated oocyte (Kondo et al., 1997). The immunoblotting analysis suggested that the purified MPF were Cdc2 (34 kDa) and Cyclin B (46 or 48 kDa). Cdk and Cyclin B complex, that is the MPF, is activated by Cdk-activating kinase (CAK) and inducing germinal vesicle breakdown (GVBD), finally, the oocyte maturation occurs (**Figure 3**).

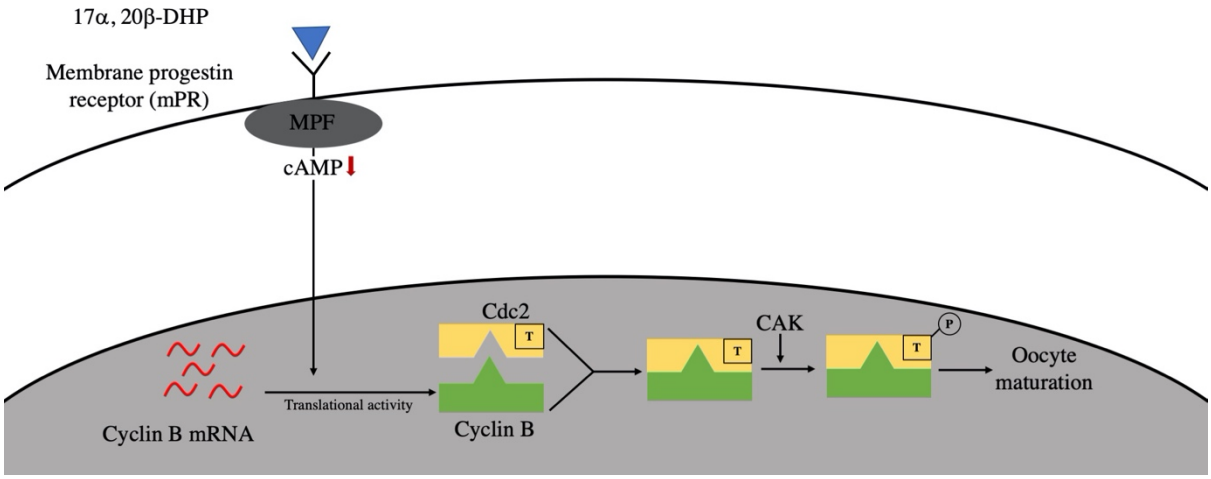

Figure 3 MPF formation and activation in oocytes. Immature oocytes synthesized Cyclin B by translational activation after MIH 17, 20β-DHP stimulation. Cyclin B was combined with Cdc2 to make complex formation and activated oocyte maturation through phosphorylation.

Ovulation

Ovulation is the step to release mature oocyte from their follicular layer. This event occurs subsequent to oocyte maturation. In oocyte maturation, the microvillar connections between follicular cells and oocyte is disrupted, called follicular separation. After that in the ovulation process, the oocyte is expulsed from the follicles through the opening site, called follicular rapture, and produce the fertilization membrane (**Figure 4**). These steps require many molecules for regulation. In some experiment, MIH or 17α, 20β-DHP can induced both oocyte maturation and ovulation *in vitro*, however, some fish species can be induced just only GVBD or oocyte maturation. It demonstrated that ovulation process was more delicate than oocyte maturation. Pinter and Thomas (1999) showed the evidence that ovulation process required the transcriptional activity and new mRNA synthesis, while, oocyte maturation is regulated through nongenomic pathway of MIH. This is more reason why ovulation is more delicate and harder than oocyte maturation. Despite the mechanisms of ovulation have been studied in several species to date, the biochemical pathway still unclear.

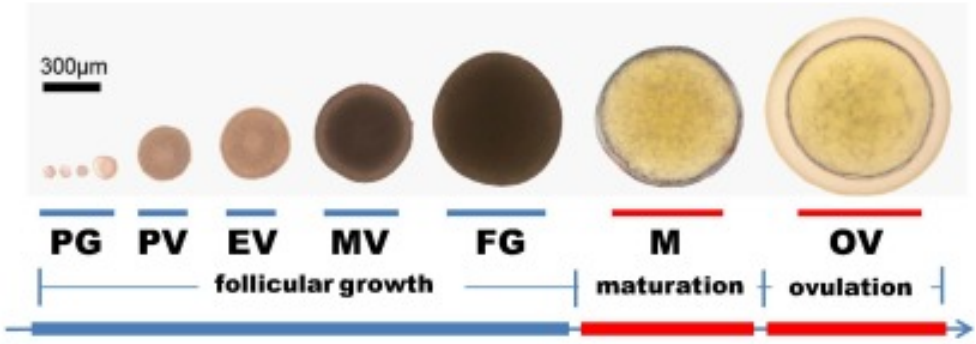

Figure 4 Stage of morphological change in oocyte during follicular growth, oocyte maturation to ovulation. There are indicated in many stages, including, primary growth stage (PG), previtellogenic stage (PV), early vitellogenic stage (EV), full-grown stage (FG), maturation (M), and ovulation (OV).

Selection of ovulation inducing genes *in vivo*

The oocyte maturation and ovulation are two essential mechanisms to produce the next generations and maintain species. Unfortunately, the real mechanisms of 2 crucial steps have been not clear studies yet especially in ovulation step. In bony fish, 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DHP) is the maturation-inducing steroid hormone which activates both oocyte maturation and ovulation pathways. Nevertheless, two different receptors and signal transduction pathways demonstrated that oocyte maturation and ovulation pathway are dissimilar. For oocyte maturation step, 17α, 20β-DHP binds to membrane progestin receptor (mPR) and transduces to the cytoplasm via nongenomic steroid signaling pathway, whereas 17α, 20β-DHP, which is steroid hormone, has ability to passe the oocyte membrane through nuclear membrane and bind nuclear progestin receptor (nPR). This pathway can produce mRNA and activate ovulation via genomic steroid signaling pathway. The mechanisms of oocyte maturation and ovulation are independently induced, however, cooperatively overlapped using same steroid hormone, 17α, 20β-DHP, and hardly distinguished from each other (**Figure 5**).

Diethylstilbestrol (DES), an endocrine-disrupting chemical (EDC), is one kind of non-steroid estrogen that can trigger the oocyte maturation, due to, Cyclin B, which is one of important MPF, and GVBD were detected from the oocyte treated by DES *in vitro* (Tokumoto et al., 2004). Antibody of anti-MIH receptor can blocks oocyte maturation by DES inducing. That implies the interaction between DES and MIH receptors. In addition, the direction between DES and MIH receptor was investigated by steroid binding assay (Tokumoto et al., 2007). DES also was treated the zebrafish *in vivo* to stimulate the oocyte maturation without ovulation (Klangnurak et al., 2018, Klangnurak and Tokumoto, 2017).

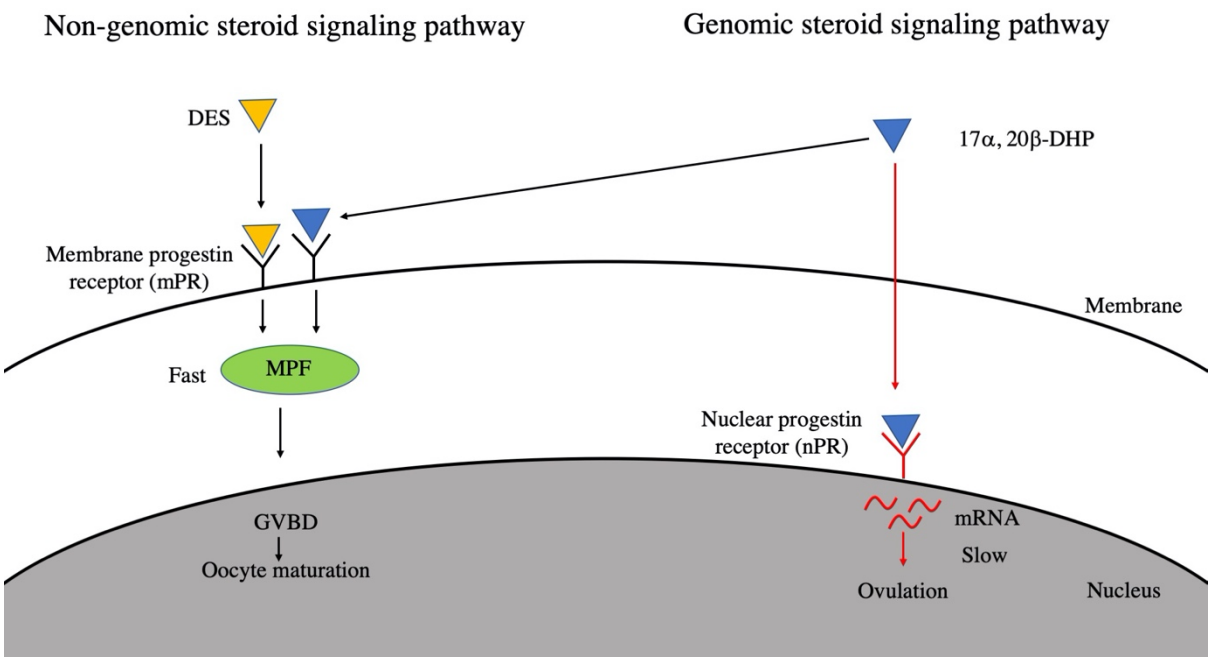

Figure 5 Genomic and non-genomic steroid signaling pathways. For the classic mechanisms of steroid action, 17, 20β-DHP binds to nuclear progestin receptor (nPR) and acts through the genomic steroid signaling pathway (slow process) for ovulation, in addition, can bind to a membrane progestin receptor (mPR) and activate the non-steroid signaling pathway (fast process) to induce oocyte maturation, called alternative mechanism of steroid action. Despite, DES can also bind mPR and active non-steroid signaling pathway to induce oocyte maturation, it cannot mediate the ovulation process pass through steroid signaling pathway.

In vivo assay is the technique that induce oocyte maturation and ovulation in the living zebrafish (Tokumoto et al., 2011). This assay can study the effect of all biological process in living organisms or cell. DES were studied the effects in the surrounding aquatic environment. The easiest method is adding the chemical into water. For oocyte maturation and ovulation experiments, endocrine disrupting chemicals (EDCs) or steroid hormones are added into the water that have zebrafish (**Figure 6**). These external applies could induce or prevent oocyte maturation and/or ovulation in living zebrafish. The environmental chemicals showed effect both genomic actions via nuclear receptor and non-genomic through membrane action. *In vivo* assay represents new system to discover genes essential for ovulation.

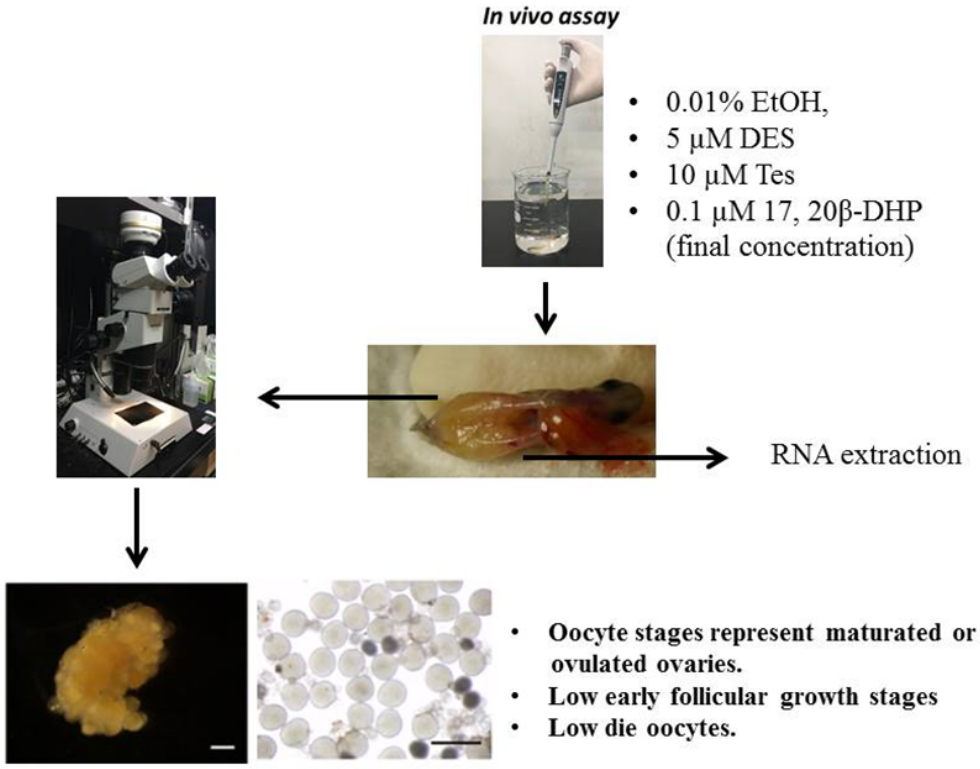

Figure 6 *In vivo* assay. Female zebrafish were treated with EtOH, DES, Tes, and DHP by dropping these chemicals in the water surrounding zebrafish, then incubate at 28.5°C for 3 hours. Fish were killed by spinal severance and dissected their body cavity. The lobe of ovary was checked the stage under stereomicroscope.

Zebrafish is the one of popular model of organisms for understanding cellular basis, including, genetics, development and reproduction. Zebrafish have rapidly developing transparent embryos, which is well suited to early stage development *in vivo*. Many characteristics can be observed, for example, cell shape changes, cell proliferation, cell migration, and tissue formation. However, the limitation of study in zebrafish is only in early stage embryos. Zebrafish will produce the pigments while they are growing up. These pigments are the obstacle for observation the changing in living embryo. *Roy orbison* (*roy*) strain of zebrafish were established by removing pigments in zebrafish (**Figure 7**). This strain was spontaneous mutant and was complete lack of iridophores, uniformly pigmented eyes, low scattered of melanocyte, and transparency of skin. This unique phenotype is convenient to observe the characteristic inside fish body, especially, numbers and stage of ovary, in living zebrafish.

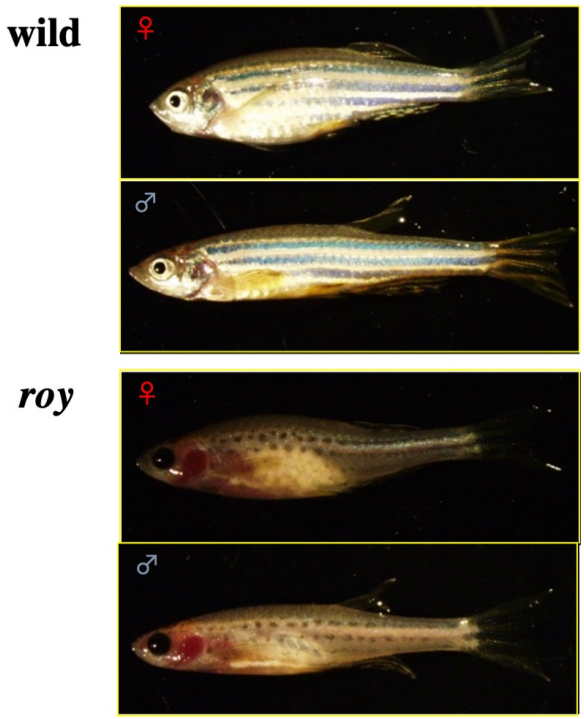

Figure 7 Comparison between normal zebrafish and *roy orbison* (*roy*) strain. Normal zebrafish is opaque with white and black stripes liked zebra, while *roy* strain was established by removing the iridophores that are silver color. It affected that *roy* strain has transparent body and easily observe inside their body.

Klangnurak and Tokumoto (2017) and Klangnurak et al. (2018) performed *in vivo* assay to selected the high possibility of ovulation inducing genes without oocyte maturation inducing genes. 17α, 20β-DHP were treated to living zebrafish to demonstrate the group of oocyte maturation and ovulation. Furthermore, DES were applied to the living zebrafish to demonstrate only oocyte maturation, while, ethanol (EtOH) referred to non-activated group that was not activated both oocyte maturation and ovulation. The gene that up-regulate in the DHP group (oocyte maturation and ovulation) and low expression in the DES group (only oocyte maturation) were the target genes of ovulation inducing gene (**Figure 8**). After treated living zebrafish with all chemicals, RNA from the oocytes were extracted RNA using ISOGEN (Nippon Gene, Tokyo, Japan), then, complementary DNA (cDNA) were performed by Illustra Ready-To-Go RT-PCR Beads (GE Healthcare Life Sciences, Buckinghamshire, UK). Microarray and RNA sequencing were used to predict the expression. The gene expression in each group were compared and the up-regulated genes during induction of ovulation were selected by Subio Platform. The genes that up-regulated in 17α, 20β-DHP more 1.8 folds greater than other groups were selected for microarray technique, however, genes showed more than 2 folds higher expression in 17α, 20β-DHP than other group were chosen for RNA sequencing. Venn diagrams analysis was operated to select the overlapping genes among all groups. Statistical analysis, ANOVA, was calculated the significantly high possibility of ovulation inducing genes. All of statistically selected genes were confirm using quantitative polymerase chain reaction (qPCR).

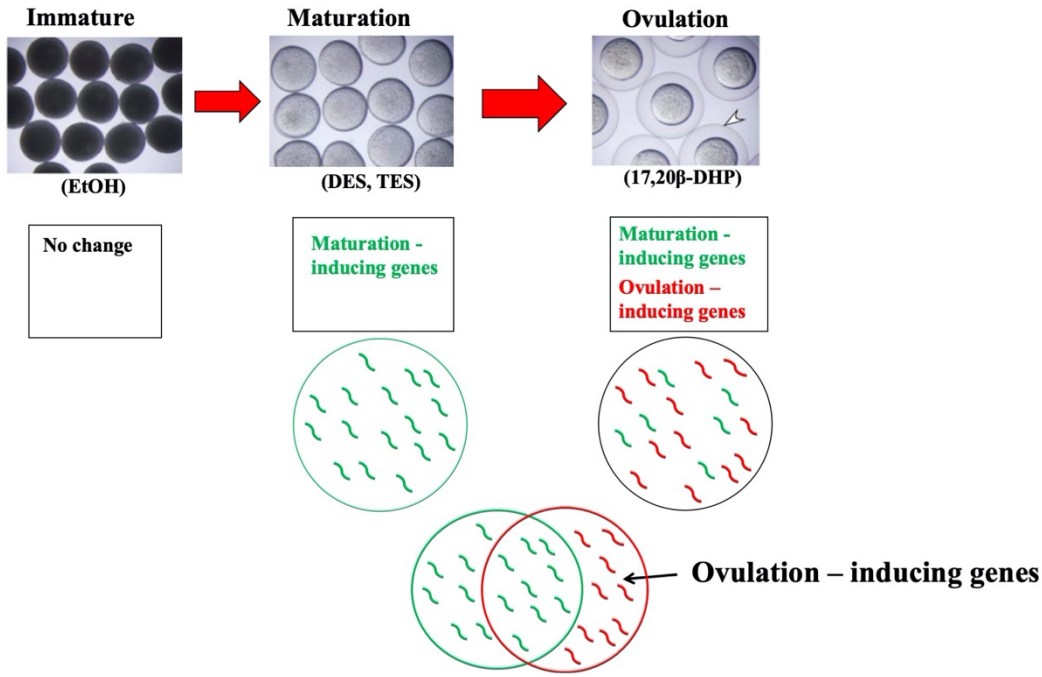

Figure 8 Idea to select only ovulation-related genes. The oocyte stopped the development in the maturation stage (treated with DES and Tes) and the oocyte in ovulation stage that have both oocyte maturation and ovulation (treated with 17, 20β-DHP). Up-regulated genes expressed in ovaries treated by 17, 20β-DHP but down-regulated for DES and Tes treatment were candidate genes for ovulation induction.

From these experiments, 11 high possibility ovulation inducing candidate genes, 3 genes from microarray and 8 genes from RNA sequencing, were selected (**Figure 9**). For microarray analysis, solute carrier family 37 (the glucose-6-phosphate transporter) member 4a (*slc37a4a*) and zebrafish gene collection number 65811 (*zgc:65811*) were selected from statistical selection protocol, moreover, zebrafish gene collection number 92184 (*zgc:92184*) was selected from non-statistical selection procedure. For RNA sequencing analysis, cathepsin Bb (*ctsbb*), salt inducible kinase1 (*sik1*), paired box protein 2a (*pax2a*), starmaker (*stm*), a disintegrin-like and metalloproteinase domain with thrombospondin-15 motifs (*adamts-15*), RNA binding motif protein 47 (*rbm47*), chymotrypsinogen B1 (*ctrb1*), and protease, serine, 59, tandem duplicate 1 (*prss59.1*) were selected as the high possibility ovulation inducing gene. In addition, prostaglandin-endoperoxide synthase 2a (*ptgs2a*) was added for the positive control in ovulation inducing gene. From these 11 gene candidates, there were several molecular signaling pathway while the fish ovulation, such as, related gene of apoptosis, related genes of maintain fertilization and new plyers in ovulation inducing pathway.

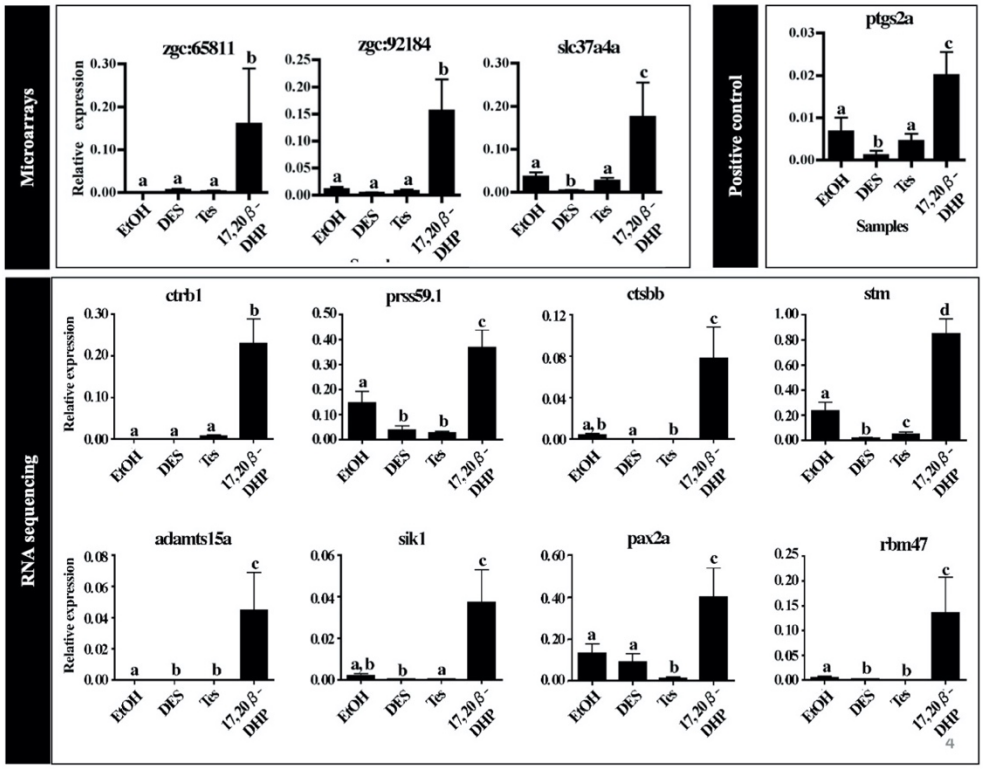

Figure 9 Gene expression profiles of candidates of ovulation-related genes using microarray and RNA sequencing protocol. The qPCR was used to analyzed and confirmed the high possible genes which related to ovulation comparing with elongation factor 1α (EF1α). 11 genes were selected, consist of 3 genes from microarray technique, 8 genes from RNA sequencing analysis, and 1 gene for positive control.

CHAPTER II

INTRODUCTION

Ovulation process that maintain species and produce the next generation. It is the phenomenon that matured oocyte ruptures from follicle layer and set the fertilization membrane. This process is mediated by nuclear progestin receptor (nPR) through genomic steroid signaling pathway in zebrafish. Study in various species suggest the ovulation-associate genes are overlapping with oocyte-maturation-associate genes and hardly to distinguish from each other, liming the study of ovulation associate. 17α, 20β-dihydroxy- 4-pregnen-3-one (17, 20β-DHP), maturation-inducing hormone (MIH), can induce both the maturation and ovulation process, binding to a membrane progestin receptor (mPR) via non-genomic steroid signaling pathway and binding to a nuclear progestin receptor (nPR) via genomic steroid signaling pathway, respectively.

However, the method to investigate only the ovulation-associate gene without maturation-associate gene was established using induction *in vivo* (Tokumoto et al., 2011). Diethtlstilbestrol (DES), a non-steroidal estrogen, can bind the membrane progestin receptor (mPR) and activate only oocyte maturation through non-genomic steroid signaling pathway via *in vivo*. DES directly input to the surrounding water, after that several hours, only oocyte maturation is induced. On the other hand, 17α, 20β-dihydroxy- 4-pregnen-3-one (17α, 20β-DHP) induced both oocyte maturation and ovulation. From this assay, it might be possible to distinguish the ovulation-associate gene without oocyte-maturation associate gene.

Pax2a (paired box2a) is the one of highly possible candidate of ovulation-inducing genes in zebrafish selected by using RNA sequencing analysis (Klangnurak et al., 2018). *Pax2a* is the gene in *pax* proteins which encode transcription factors, which are controlled by both activation and inhibitory in C-terminal regulatory region (Dorfler and Busslinger, 1996). Pax proteins contain highly conserved DNA-binding domain, called paired domain (PD), consisting of two conserved helix-turn-helix motifs. In many Pax proteins, the paired domain is fused to a second DNA binding domain of paired-like homeobox family. From the crystal structure resolution of the paired domain bound to DNA, the results demonstrated that the paired domain is consisted of 2 subdomains, including, N-terminal (PAI) and C-terminal (RED) subdomain. Each form has a three α-helical fold. In addition, PAI subdomain encompassed the N-terminal β-sheet, participating in DNA-binding activity. Pax protein have the sequence of

octapeptide that are comprised of 8 peptides. This region can induce the fit mechanisms for the binding activity. Another sequence site to attach the DNA is DNA binding homeodomain. This site is located after the octapeptide and also have 3 α-helix folds. At the last of Pax2a protein next to C-terminus, there is the transactivation/inhibitory domain. This domain containing binding sites for other proteins which are the transcription factors, or transcription regulators. These binding have not only the activation functions, but also inhibitory functions. However, these are frequently referred to as activation functions (Treisman et al., 1991) (**Figure 10**).

Many *pax* genes produce alternative RNA transcripts. *In vivo* bioassay, the Pax proteins show the diverse of functions, related to the ability of alternatively splicing gene products to different structure. Moreover, the generally important role is the DNA binding regions (Underhill, 2012). The paired domain includes 2 subdomains and each subdomain can bind major grooves of the DNA helix (Xu et al., 1995).

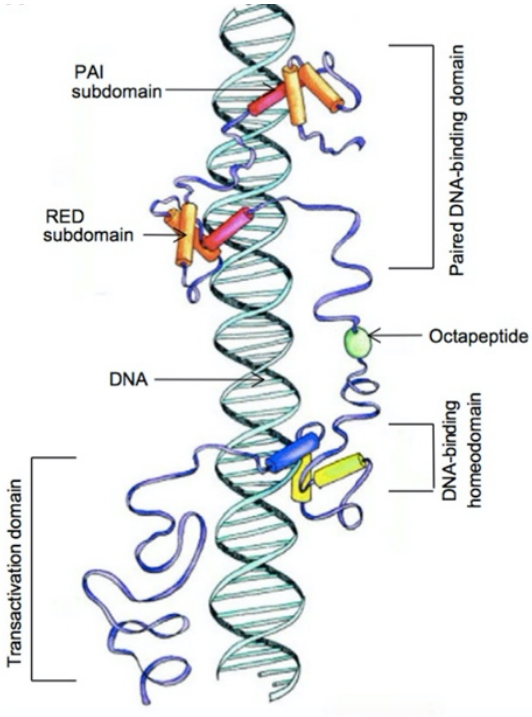

Figure 10 Structure of Pax2a protein. Pax2a protein contains a transactivation domain and a DNA-binding domain known as the paired domain (PD) that is consist PAI and RED subdomains. Pax2a contains partial homeodomain and/or octapeptide region. (Blake and Ziman, 2014)

Pax family are included in the body pattern of development in organisms, early specification of cell fate and morphogenesis in many kinds of tissues and organs, including Pax2 proteins, that are essential for development, differentiation, growth, survival, and morphogenesis (Mansouri et al., 1999, Mansouri et al., 1996, McCarroll et al., 2012). In the development of embryo, *pax2a* plays role in eye, ear, kidney, thyroid, and central nervous system (CNS) (Mackereth et al., 2005, Porazzi et al., 2009, Picker et al., 2002, Riley et al., 1999, Pfeffer, 1998). *Pax2* is the earliest *pax* gene, which is expressed for control the neural tube in embryo. Midbrain-hindbrain boundary (MHB), controlling the development of midbrain and cerebellum is required Pax2 for formation (Pfeffer et al., 2002). Expression of *pax*2 is related with *pax*5 and *pax*8 for control MHB. Furthermore, *pax2a* acts in the anterior-posterior pattern formation, which is the process of regionalization in specific areas of cell differentiation along the anterior-posterior axis (Picker et al., 2002). In early embryogenesis of zebrafish, Pax2a maintains and differentiates of thyroid follicle (Wendl et al., 2002). Productions of follicle stimulating hormone (FSH) and luteinizing hormone (LH) are controlled by the triiodothyronine (T3) and Tetraiodothyronine (T4) from thyroid gland. FSH and LH were induced, despite, the low concentration of T3 and T4 (Liu et al., 2011).

However, the function of *pax2a* in eggs, especially, ovulation process, is not reported. In this study, *pax2a* knock out was performed in zebrafish using CRISPR/Cas9 system. The novel functions, in term of, ovulation, fertilization, and early development, were investigated in eggs and ovulated embryos, using phenotypic analysis and protein expression of homozygous mutant fish. This innovated method can produce the *pax2a* homozygous mutant eggs.

MATERIAL AND METHODS

Experiment fish

The *roy orbison* (*roy*) strain of zebrafish (*Danio rerio*), which have the transparent body, were used in this study. The specific characteristic of this strain is highly transparent, so, the oocytes are easily observed. Zebrafish were cultivated following the standard protocol (Westerfield, 1995) and were raised in the flow-system maintain at 28.5°C for 14 hours light: 10 hours dark cycle. Zebrafish were fed with Paramesium spp. in the larva period about 1 month, after that, were fed with live brine shrimp in the morning and instant food (Tetra Guppy, Tetra GmbH, Melle, Germany) in the evening. All experiments were conducted by approved (approval number. 2020F-4) by the Institutional Ethics Committee of Shizuoka University, Japan.

Zebrafish reproduction

Adult male and female *roy* zebrafish, which ready to breed, were used to reproduce the new fish. From the unique characteristic of *roy* that their body is very transparent. So, we can observe the abundant and maturity of ovary by normal eye visual. Male and female were put in the pairing tank that is the small tank in the big tank. The bottom of small tank is the net that the net size the big enough for the fertilized egg can pass through the net for protect parents eat their eggs. The pair tank was set up in the evening time. Fertilized eggs were collected from the success pairs in the morning of next day and put in 6 centimeters of Petri dish containing the water with methylene blue. The fertilized eggs were incubated 28.5°C for 5 days, after that, the hatched fly that can swim is released to the big tank.

Genome editing

CRISPR/Cas9 system was used to edit genome for zebrafish in this experiment. The 1-cell stage embryos were set up in the 1% agarose gel. The needle that were made from glass capillaries and was connect to the air pump injector. The sgRNA (10μM), Cas9 (500ng/ml), and tracrRNA (20μM) were mixed and load into the needle. The 1-cell stage embryos were rotated in the same position that vegetal pole was face to the needle, whereas animal pole was opposite with needle. The needle was moved by rotating. The tip of needle punctured the chorion and passed to the animal pole of embryo through yolk of embryo (**Figure 11**). Total 4 nl of mixture CRISPR/Cas9 was

microinjected using gas operated Narishige IM300 microinjector (Narishige Scientific Instrument Lab, Tokyo, Japan) under microscope. The edited larva, called F0, were incubated at 28.5°C for 5 days and were released to the cultured tank with the suitable condition after they started to swim. The experimental zebrafish were checked genotype using HMA and DNA sequencing method (**Figure 12**).

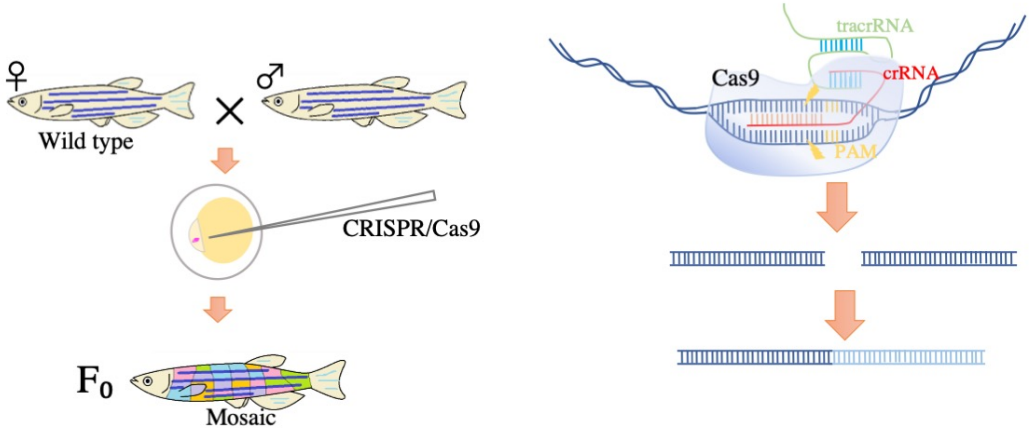

Figure 11 Summary of genome editing in zebrafish. Microinjection process to produce *pax2a* knock out zebrafish using CRISPR/Cas9

DNA extraction

Genome DNAs were extracted from the small part of caudal fin using Phenol-Chloroform protocol. The small part of caudal fin was clipped and put into the 1.5 ml Eppendorf containing 200 µl of SDS Lysis buffer and 0.5 µl of Proteinase K. Sample was incubated in 50°C at least 2 hours. 100 µl Phenol-chloroform was added to the tube and mixed well. The tubes were centrifuged at 15,000 rpm (round per minute) in 4°C for 5 minutes. 150 µl of supernatant was kept to the new 1.5 ml Eppendorf tube. 100 of Chloroform was added into the supernatant and mixed well. The tubes were centrifuged at 15,000 rpm in 4°C for 5 minutes. 110µl of new supernatant was moved to new 1.5 ml Eppendorf tube. 475 µl of absolute ethanol and 25 µl of 4M of NaCl were added into the new supernatant. The tubes were centrifuged at 15,000 rpm in 4°C for 10 minutes. After centrifuge, liquid in the tube was carefully removed by vacuum machine. 200 µl of 70% ethanol was added, then, Eppendorf tubes were centrifuged at 15,000 rpm in 4°C for 10 minutes. Liquid in the tube was carefully removed by vacuum machine. Cover of tube was opened in the room temperature to dry the ethanol about 1 hour. 35 of DDW was added and kept in -30°C.

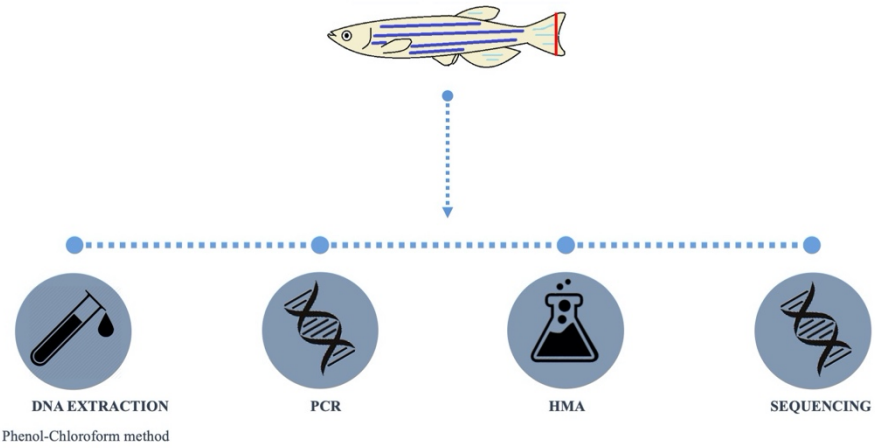

Figure 12 Molecular method to find the genotyping to genome editing zebrafish, including, DNA extraction using phenol-Chloroform method, Polymerase change reaction (PCR), Heteroduplex mobility assay (HMA), and DNA sequencing

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed to amplify the target site of editing genome using specific primers. Specific primers for *pax2a* gene, covering target site of sgRNA in editing genome, were designed by Primer3 software (Untergasser et al., 2012). A 25 µl PCR solution consisted of 0.1 µl (0.5 U) of Ex *Taq* DNA polymerase; 2 mM dNTP mix (Takara Bio Inc., Shiga, Japan); each 2.5 µl of 10X Ex *Taq* butter (MG^{2+}-plus), and both forward (5'-GGCGACCTCAGTCGATTATCT-3') and reverse (5-ACAAAGCCAGCTCAACGTAAA-3') primers; and 4µl of genomic DNA template, was amplified. PCR reactions were carried out (Takara Bio Inc., Shiga, Japan) under the following conditions: 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, and finally at 72°C for 10 minutes. PCR products were elctrophoresed through 1.5% (w/v) agarose gel, stained with Midori Green Advance DNA stain (Nippon Genetics Europe, Dueren, Germany), for visualization.

Heteroduplex mobility assay (HMA)

Heteroduplex mobility assay (HMA) is the pre-sequencing technique. HMA was applied to investigate the genotyping of mutant fish (**Figure 13**). Each 5 µl of PCR product was heated at 95°C for 5 minutes, after that immediately sunk in the ice. Total 5µl of samples were mixed with 3 µl of 10X loading buffer and run through 15% polyacrylamide gels (150 V, 30 mA, for 1 hour) in 1xTBE buffer by power station

(ATTO, Tokyo, Japan). One gel of 15% polyacrylamide gel was made from 3.75 ml of 40% polyacrylamide, 5.25 ml of DDW, 1 ml of 10xTBE, 50 µl of 10% APS, and 10 µl of TEMED. The loaded gel was stained with ethidium bromide for 3 minutes and wash with 1xTBE for 5 minutes, after that, were visualized under an UV transilluminator. To investigate the homozygous mutant fish, moreover, 2.5µl of each mutant PCR product was mixed with 2.5 µl of wild-type fish PCR product.

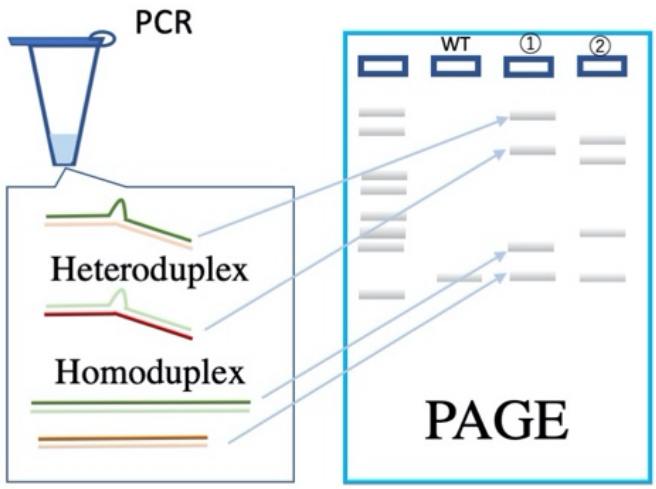

Figure 13 Mechanism of heteroduplex mobility assay (HMA) to find the heterozygous and homozygous knock-out zebrafish

DNA sequencing

Mutant genomic DNAs were amplified, which covering site of start codon of *pax2a* gene and editing genome of sgRNA, using new specific primers. Total 25µl of reaction mixtures were mixed, contained 0.5 µl (0.5 U) of KOD-Plus DNA polymerase, 2.5µl of 10X Buffer for KOD -Plus, 2.5µl of 2 mM dNTP mix, 1µl of 25 mM $MgSO_4$ (TOYOBO CO., LTD., Osaka, Japan), each 0.75µl of 10 µM forward (5'-TCGTGTCTTCGTTGAATGGA-3') and reverse primer (5'-TGAAATGTAACCAG CGGACA-3'), and 4µl of genomic DNA template. PCR were performed by these conditions; 94°C for 2 minutes, 3 steps for 35 cycles of 94°C for 15 seconds, 66°C for 30 seconds, and 68°C for 1 minute, and finally at 68°C for 10 minutes. PCR products were purified by Alkaline phosphatase(AP) and ExonucleaseI (GE Healthcare Life Science, IL, USA). 5µl of DDW, each 0.05µl of AP and exonuclease, and 10µl of PCR product. The mixtures were performed in 37°C and 80°C each 15 minutes. Sequencing reactions were carried out from Sanger sequencing protocol (Sanger, 1988).

Mutant lines generation and phenotype observation

The *pax2a* knock out (KO) fish were established to observe the physiological and anatomical function in term of oocyte maturation, ovulation, fertilization, and early development of embryos. To generate mutant fish, the 1-cell stage embryos were microinjected by CRISPR/Cas9. The F0 zebrafish were investigate the mutation using HMA. Heterozygous mutant zebrafish of *pax2a* (*pax2a*$^{+/-}$) were established by pairing F0 mutant zebrafish and wild type. The same strain of *pax2a*$^{+/-}$ were inbreeded to produce homozygous mutant (*pax2a*$^{-/-}$). Phenotypes, including, oocyte maturation, ovulation, fertilization, and early development of embryos, of *pax2a*$^{-/-}$ were investigated (**Figure 14**).

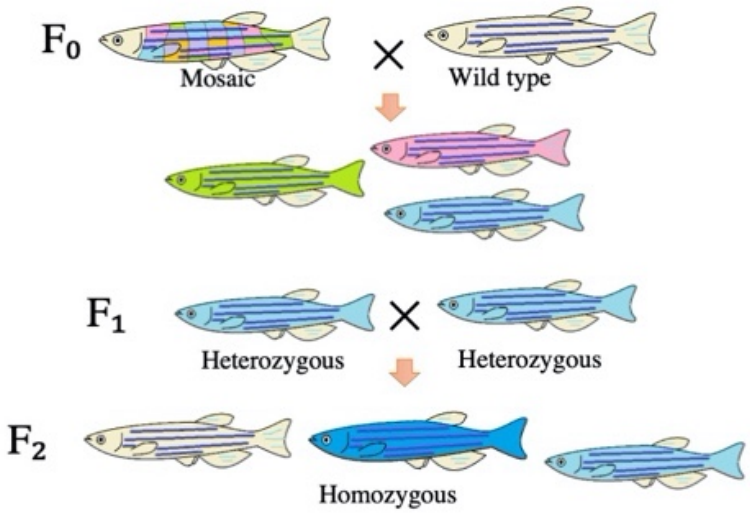

Figure 14 Mutant line generation to produce the *pax2a* homozygous zebrafish (*pax2a*$^{-/-}$). F0 (mosaic) fish were paired with wild type to produce F1 heterozygous zebrafish (*pax2a*$^{+/-}$). The same genotypic F1 heterozygous zebrafish (*pax2a*$^{+/-}$) male and female were paired to produce *pax2a* homozygous zebrafish (*pax2a*$^{-/-}$)

Spawning and fertility rate of pairs of *pax2a*$^{-/-}$, *pax2a*$^{-/-}$ female x WT, *pax2a*$^{-/-}$ x WT, and pairs of WT were investigate. In the evening, males and female of *pax2a*$^{-/-}$ were set in the breeding tank which have the small cage inside the large tank. The bottom of small tank has the net which the fertilized eggs can pass trough to the floor for preventing adult fish eating them. The next morning the embryos were collected to the preti dish from the large tank. The embryos were counted and check the morphological characteristics in case of unfertilized, abnormal and survived embryos

under stereo-microscope. The fry (5dpf) were released to the small tank and fed *paramecium spp*. until 2 months.

Western blot analysis

Eight of intact follicles and embryos in each stages, including, 1,000-cell stage, 18-somite, prim-22, and pec fin stage(Kimmel et al., 1995), were transferred to 1.5 ml eppendrof. Samples were washed with zebrafish's Ringer for 2 times, then, were crushed with plastic pestle in zebrafish's Ringer for 20µl. Mixture were centrifuged at 15,000 rpm for 10 minute in 4°C. The supernatant was transferred to the new eppendrof tube and were mixed with 20µl of 2x SDS PAGE sampling buffer, but the precipitate was mixed with 40µl of 2x SDS PAGE sampling buffer. After that, the samples were incubated in 95°C for 5 minutes. Totally 10µl of were loaded in 12% acrylamide SDS-PAGE gels. The proteins were transferred from gel to nitrocellulose membrane in Western blot analysis. Membrane was incubated in 4°C overnight with blocking solution (5% non-fat milk in TTBS buffer; 50 mM Tris/100 mM NaCl/0.1% Tween 20, pH 7.4). The transferred membrane was washed with TTBS 5 minutes for 3 times, and incubated with Pax2a antibody diluted 2,000-fold in TBS (50 mM Tris/100 mM NaCl, pH 7.4) for 1 hour at room temperature. The membrane was then washed with TTBS 5 minutes for 3 times, incubated with horseradish peroxidase conjugated to goat anti-mouse antibody (Thermo Fisher, MA, USA) for 1 hour at room temperature, and washed again with TTBS 5 minutes for 3 times. The nitrocellulose membrane was treated with enhanced chemiluminescence (Western lightning plus ECL, company) and was taken the photo using LAS-4000 mini machine (GE Healthcare Life Science, IL, USA).

Immunohistochemistry in whole body

Zebrafish eggs were fixed in 4% paraformaldehyde (PFA) in 4°C for overnight and soaked with 30% sucrose for 3 hours or until the samples were sunk. The fixed eggs were transferred to embedding chamber and embedded with Tissue-tek O.C.T. compound. The embedding chamber was dipped into liquid N_2. Next, samples were cut 10µm on cryostat microtome at -20°C (Westerfield, 1995). The cut samples were transferred to slides and PAP pen (Daido Sangyo Co. Ltd.,Tokyo, Japan), which is hydrophobic material, was used to circumscribe the section. The slides were gently washed with TPBS (0.1% tween 20 in PBS solution; 0.8%NaCl, 0.02% KCl, 0.02 M

PO_4, pH7.3) 2 minutes for 3 times. The solution was removed and replace with blocking solution (5% non-fat milk in PBS buffer; 0.8%NaCl, 0.02% KCl, 0.02 M PO_4, pH7.3) for 30 minutes covering with the dark cover with into which distilled water-moistened paper. The slides were washed with TPBS 5 minutes for 3 times, and treated with anti-Pax2a antibody diluted 100-folds in PBS at 4°C for overnight in the dark cover. For negative control, anti-Pax2a was pre-adsorbed with recombinant Pax2a for 1 hour with rotation, then, treated as the same with normal method that was dilute in PBS at 4°C for overnight in the dark cover. The samples were washed with TPBS 10 minutes for 3 times, and replaced with Alexa Fluor 555-conjugate anti-mouse luminacent (Cell Signaling Technology, MA, USA) for 1 hour at room temperature in the dark cover. The experimental slides were washed with TPBS 10 minutes for 3 times, treated with prolong reagent, and observed under confocal laser microscope LSM700 machine (Carl Zeiss, Jena, Germany).

Immunochemistry in eggs

Eggs were treated extraction solution containing 0.5% Tx-100 and 0.1 M PBS-Na pH7.2 for 2 minutes on ice, after that, fixed with 3.7% formaldehyde in 0.1M PBS-Na at room temperature with agitation overnight. Fixed samples were cut equatorially using fine surgery blade, washed with TBSN (1% of NP-40 in TBS) at 6 hours at room temperature, or overnight at 4°C, incubated in primary antibody, anti-Pax2a, 200 times-fold dilution in TBSN for 24 hours with agitation in 4°C. For negative control, anti-Pax2a was pre-adsorbed with recombinant Pax2a for 1 hour with rotation, then, treated as the same with normal method that was dilute in TPBS at 4°C for 24 hours. After that, the samples were washed for 24 hours with 5 changes of TBSS. The samples were incubated in 1,000 times-fold dilution of Alexa Fluor 555-conjugate anti-mouse luminacent (Cell Signaling Technology, MA, USA) in TBSN for 24 hours with agitation in 4°C, washed with TBSN for 5 changes 24 hours, dehydrated with absolute methanol 2 times, and mounted in clearing solution (benzylalcohol: benzyl-benzoate (1:2)). The confocal laser microscope LSM700 machine (Carl Zeiss, Jena, Germany) was performed to investigate the expression of Pax2a protein.

RNA extraction

The samples, ovulated eggs and 8-cells stage embryos, among 3 groups, *pax2a*$^{-/-}$, *pax2a*$^{+/-}$, and WT, were used to extract the total RNA. The samples were put

in 1.5 ml Eppendorf tube, frozen in the liquid nitrogen for 10 seconds, and kept in -80°C. Frozen samples were extracted put in 750 µl of ISOGEN-LS RNA extraction kits (Nippon Gene, Tokyo, Japan). High speed homogenizer was cleaned by methanol, 70% ethanol, and DDW, respectively. The samples were homogenized until the samples were fine pulverize and ISOGEN-LS solution was clear. Every new samples had to clean the machine with same steps. Homogenized solutions were kept in room temperature for 5 minutes. 200 µl of chloroform was added to the homogenized samples, shanked with vortex machine for 15 seconds for each sample, and kept in room temperature for 3 minutes. Mixed samples were centrifuged at 11,500 rpm in 4°C for 15 minutes. Clearance supernatant was moved to new Eppendorf tube. 500 µl of isopropanol was admixed to RNA supernatant, gently mixed, and kept in room temperature for 5 minutes. The samples were centrifuged at 11,500 rpm in 4°C for 10 minutes. Liquid was removed from the tube. 1ml of 70% ethanol was added to the tube and gently mixed. The tubes were centrifuged at 11,500 rpm in 4°C for 5 minutes. All liquids were delicately removed from the tube and beware the gel-like RNA at the bottom of the tube. The cover of tube was opened to dry all alcohol on the ice for 5 minutes. 20 µl of DEPC-treated water was added. Dissolve RNA was diluted for 50 times, then measured the concentration using spectrophotometer (GeneQuant II; Pharmacia Biotech, Stockholm, Sweden).

Complementary DNA (cDNA) synthesis

Each 1µg of RNA was used for cDNA synthesis using Illustra Ready-To-Go RT-PCR Beads (GE Healthcare Life Sciences, Buckinghamshire, UK). Total 50 µl of mixture, that was 0.5 µl of poly T, RNA 1ug, and other was DDW, was added to the tube of Illustra Ready-To-Go RT-PCR Beads that have beads. The mixture was slightly mixed without bubbles. Tubes were performed at 42°C for 20 minutes and 95°C for 5 minutes, respectively. Total 50 µl of cDNA was stored in -30°C.

Quantitative PCR (qPCR)

The quantitative PCR was conducted to investigate the expression level of *pax2a* in each sample. The 20µl of mixture, containing, 1µl of 10µM forward primer (5'-TAATGCTTGCGGTCCCTTAAATATG-3'), reverse primer (5'-ATCAGTCCA TTCAACGAAGACACG-3'), 10µl of SYBR green PCR master mix, 5µl of 10-fold diluted cDNA. The qPCR was performed by LightCycler Nano System (Roche Applied

Science, Mannheim, Germany), following this conditions; 95°C for 5 minutes, 45 cycles of 95°C for 15 seconds, 61°C for 15 seconds, and 72°C for 20 second, then 65°C and 95°C each for 20 seconds for final melting curve analysis. Each sample was performed 3 times for replication. Expression level of mRNA was normalized with expression level of a common reference gene, Elongation factor1α (*EF1α*). The relative abundance of *pax2a* normalized with EF1α was reported in the mean and standard error (mean ± SE).

Recombinant Pax2a

Total RNAs were extract from ovary of zebrafish by ISOGEN-LS RNA extraction kits (Nippon Gene, Tokyo, Japan). Total cDNA (complementary DNA) was performed by Illustra Ready-To-Go RT-PCR Beads (GE Healthcare Life Sciences, Buckinghamshire, UK). The specific primers were designed containing the sequence of restriction site of EcoRI and XhoI at the 5' and 3' -end, respectively. Entire open reading frame of *pax2a* of zebrafish was amplified by PCR using the designed specific primers. Amplified DNA and pET27b (Novagan) were cut with restriction enzyme, then, the cut PCR fragments were inserted into the pET27b, that contained 33 extra amino acids at N-terminus and a penta-histidine tag at the C-terminus. The recombinant pET27b vectors were transformed into *E. coli* strain BL21. 40 µl of competence cell of *E. coli* (BL21) was added to the 1.5 ml Eppendorf tube with the recombinant plasmid 10 µl. The mixtures were kept on ice with water for 30 minutes, then, the mixture tubes were sunk in 42°C of water for 1 minute. The mixture tubes were stored on ice again for 2 minutes. 500 µl of SOC buffer was added into the tube of mixture and cultured with shaking for 1 hour in 37°C. The cultured mixtures were plated on the agar blot containing Ampicillin, an antibiotic. Colonies of *E. coli* containing recombinant *pax2a* in pET27b were cultured in Luria-Bertani (LB) broth with Ampicillin (30 µg/ml). LB broth were incubated in 37oC with shaking about 114 rpm for overnight. *E. coli* were check the success of insertion of recombinant by restriction enzyme cutting and PCR. Plasmids were extracted from *E. coli*. 1.5 ml of Eppendorf tube containing 1 ml of LB with culture pET27b *E. coli* were centrifuge 15,000 rpm at 4°C for 10 seconds. Supernatant of LB was removed. 100 µl of solution I was added into the tube, then, mixed by vortex and kept on ice for 5 minutes. 200 µl of solution II was added, gently

mixed, and kept on ice for 5 minutes. 250 μl of solution III was added, mixed by vortex, and kept on ice for 5 minutes. 20 μl of chloroform was added and mixed by vortex. The mixture was centrifuged 15,000 rpm at 4°C for 5 minutes. 400 μl of supernatant was kept to the new 1.5 ml Eppendorf tube, after that, 1 ml of ethyl alcohol was added. The mixture was kept on ice for 5 minutes and centrifuged 15,000 rpm at 4°C for 10 minutes. Supernatant was discharged. 500 μl of 70% ethyl alcohol were added and centrifuged again with 15,000 rpm at 4°C for 10 minutes. Supernatant was discarded and dried. 20 μl of TE buffer was added to each tube. The samples were check the quantity of plasmid DNA by gel electrophoresis. For restriction enzyme cut experiment, 20 μl of extracted plasmid was mixed with 5 μl of 10x H buffer, 1 μl of NdeI restriction enzyme, 1 μl of XhoI restriction enzyme, 1 μl of RNase, and 19 μl of DDW. The mixture was incubated at 37°C for overnight. The mixture was loaded and checked by agarose gel electrophoresis. For PCR experiment, 25 μl PCR solution consisted of 0.1 μl (0.5 U) of Ex *Taq* DNA polymerase; 2 mM dNTP mix (Takara Bio Inc., Shiga, Japan); each 2.5 μl of 10X Ex *Taq* butter (Mg^{2+}-plus), and both forward and reverse primers; and 4μl of genomic DNA template, was amplified. PCR reactions were carried out (Takara Bio Inc., Shiga, Japan) under the following conditions: 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, and finally at 72°C for 10 minutes. PCR products were elctrophoresed through 1.5% (w/v) agarose gel, stained with Midori Green Advance DNA stain (Nippon Genetics Europe, Dueren, Germany), for visualization. The successfully inserted colonies were purified by Ni-NTA agarose column chromatography. Polyclonal antibodies specific for Pax2a were raised in a mouse against purified recombinant Pax2a.

Histological observation

The fish were fixed in Bouin's solution (75 ml of saturated solution of picric acid, 25 ml of 40% formaldehyde, and 5 ml of glacial acetic acid) for overnight at the room temperature with agitation, after that, kept in 70% ethanol at 4°C for preservation. The fixed sample was embedded in paraffin in the aluminum foil case. The embedded sample was cut about 8 μm by microtome. The sections were floated on the warm water and then attached the glass slide. The slides that had the sectioned samples were removed the paraffin by Xylen 10 minutes for 2 times. Hematoxylin and Eosin (HE),

standard histological technique, was used to observe. The deparaffinized samples were rehydrated in the graded ethanol series (100% ethanol 5 minutes for 2 times, 90% ethanol 5 minutes, and 70% ethanol 5 minutes), after that, DDW for 5 minutes. After rehydration, the sample were stained with Eosin for 3 minutes and dehydrated with grade ethanol series (70% ethanol 3 minutes, 90% ethanol 3 minutes, and 100% ethanol 3 minutes for 2 times), then, soaked in Xylen for 10 minutes. Hematoxylin staining was performed. The samples were soak in Hematoxylin for 3 times and rinsed with DDW. The slides were rinse with the tap water to remove the excess Hematoxylin. The slides were covered with coverslip using permount.

Statistical analysis

All summary data are represented in the mean ± S.D. One-way analysis of variance (one-way ANOVA) was calculated to investigate the significantly difference among each group of experiment comparing with wild type using GraphPad Prism San (Diego, CA). Data were considered significant at P-value < 0.05 (*).

RESULTS

Genome editing

Pax2a gene knockout fish were established using CRISPR/Cas9. F0 and F1 adults were checked before pairing using HMA analysis. In successful *pax2a* editing F0, called mosaic, the HMA result showed several bands in the gel electrophoresis. That was the successful of genome editing by CRISPR/Cas9. Each band indicated the editing and randomly repairing themselves in cell processes (**Figure 15**). Edited F0 adults were crossed to WT to produce F1 *pax2a*$^{+/-}$. The same pattern of HMA result indicated the same mutation (**Figure 16**). DNA sequencing were checked to confirmed and checked the editing that were insertion and/or deletion in that strain. For the *pax2a*$^{+/-}$, DNA sequencing diagrams showed the double peaks in the same position that indicated one peak from WT and one peak from F0 editing genomic fish (**Figure 17**). After that, homozygous *pax2a* mutant (*pax2a*$^{-/-}$) were generated by inbreeding using the same mutation of heterozygous mutant (*pax2a*$^{+/-}$). Homozygous *pax2a* mutant (*pax2a*$^{-/-}$) were searched by HMA. The first HMA was processed with the same condition with F1 generation. For searching F2 *pax2a*$^{-/-}$, the second HMA was done by adding amplicons of WT with that of identified homozygous amplicon into the samples (1:1). The samples which showed homoduplex in the first HMA and heteroduplex in the second HMA (mixed with WT) were homozygous mutant fish (**Figure 18**). DNA sequencing were done after preliminary checked by HMA. DNA sequencing diagram of *pax2a*$^{-/-}$ showed only one peak, however, the mutations were detected comparing with alignment with WT. There were 3 strains, strain A, B, and C, of mutant that were 5 deletions (-5), 2 deletions and 11 insertions (-2,+11), and 6 deletions (-6), respectively. Despite, the mutations were insertion or/and deletion, the mutation in all of strains occurred at the start codon (ATG) of *pax2a* gene (**Figure 19**.). It might possible that all of strains were *pax2a* completely knock out, or the start codon of *pax2a* shifted to next ATG, which were 12 amino acids deletion (**Figure 20**) from 410 amino acids in normal wild type. The 12 amino acids deletion is between the N-terminus of Pax2a and the conserved N-terminal subdomain (PAI subdomain) of pair domain, that are start with β-hairpin structure of Pax2a (**Figure 21**).

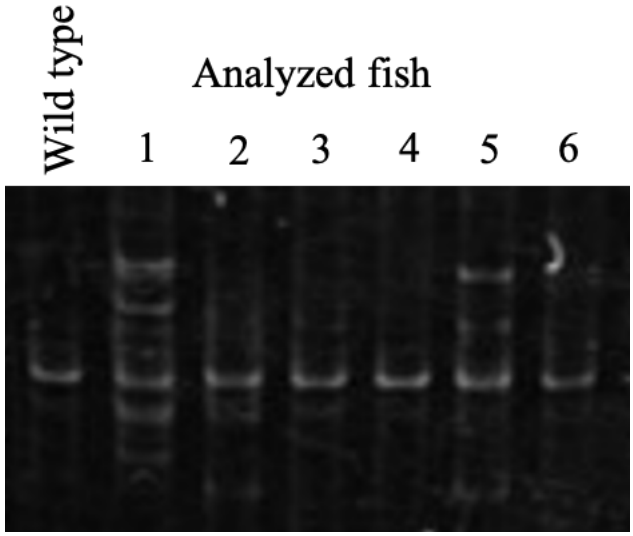

Figure 15 HMA pattern of F0 microinjected zebrafish. Adults of F0 zebrafish which were microinjected by CRISPR/Cas9 to edit *pax2a* gene. Individual fish is showed in each lane. The successful mutants were showed in the several bands (No. 1 and 5), however, the single band like WT indicated unsuccessful (No. 2, 3, 4, and 6).

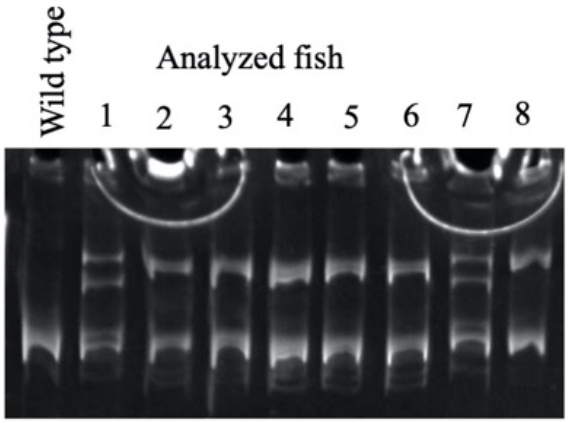

Figure 16 HMA pattern of F1 generation of zebrafish. F1 zebrafish from the successful F0 editing founders were investigated. Two pattern of bands are showed in HMA result, including, group1 (No.1 and 7) and group2 (No. 2-6 and 8). The different patterns of HMA analysis is implied the different type of mutations.

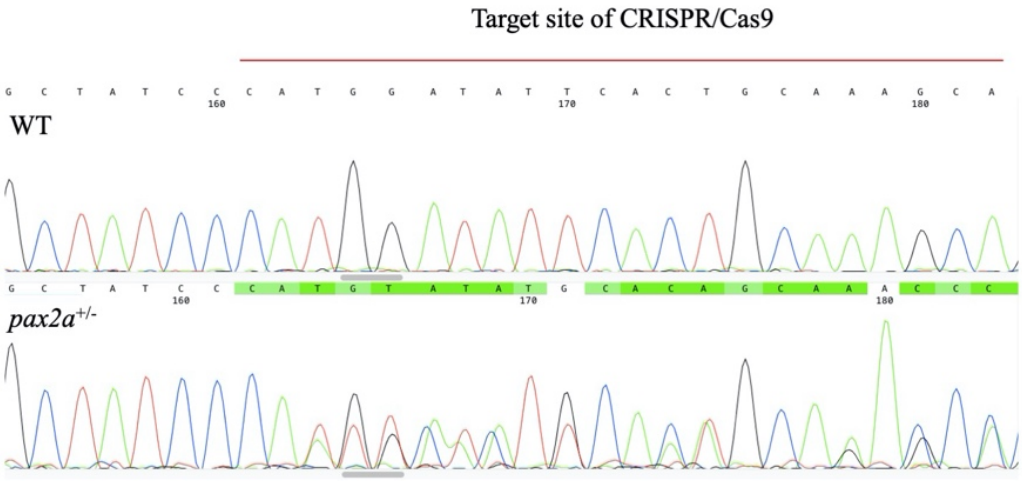

Figure 17 DNA sequencing result of F1 heterozygous zebrafish (*pax2a*$^{+/-}$). The peak of DNA sequencing of wild type showed only one peak, while *pax2a* heterozygous zebrafish (*pax2a*$^{+/-}$) appeared the double peaks started from the target site of CRISPR/Cas9

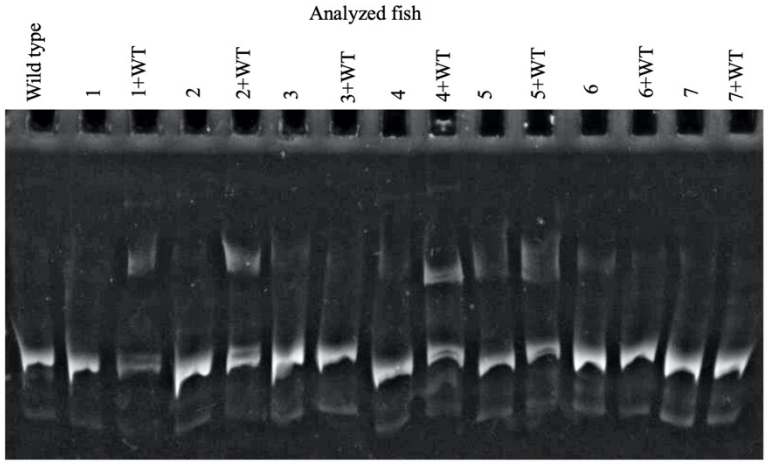

Figure 18 HMA pattern of F2 generation of zebrafish. F2 zebrafish of strain C generated from F1 *pax2a*$^{+/-}$. The first HMA, which were No. 1-4, were loaded and, for comparison, next right lane was the second HMA (mixed F2/WT) that labelled as No. (1+WT) – (4+WT). Homozygous zebrafish were sample No. 1, 2 and 4 which were showed the single band in the first HMA and showed several bands in the second HMA.

Figure 19 Nucleotide sequencing of WT and *pax2a* mutant fish induced indel mutation. The deleted and inserted nucleotides in DNA alignment are indicated by dash and bold, respectively. The number of deleted and inserted bases are represented. There were 3 strains, strain A, B, and C, of mutant that were 5 deletions (-5), 2 deletions and 11 insertions (-2,+11), and 6 deletions (-6), respectively (A). Amino acid alignment of the predicted protein that start codon was shifted to the next ATG. There were 12 amino acids deletion (B).

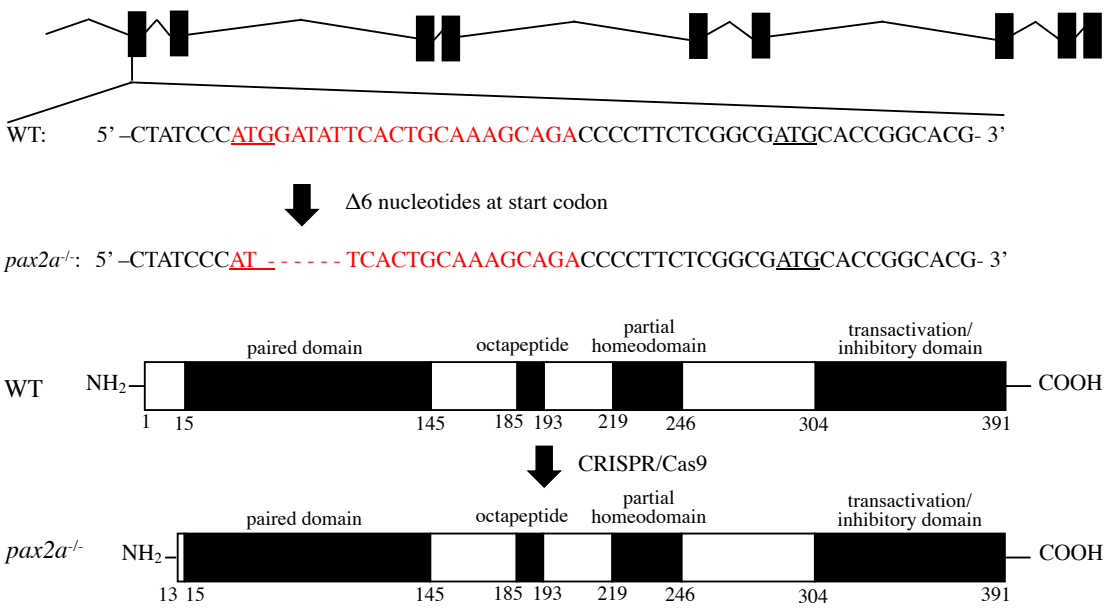

Figure 20 Genome structure and DNA sequence of the target site for the genome editing of *pax2a*. DNA sequences around the target site (in red) for CRISPR/Cas9 digestion in wild type (WT) and *pax2a*$^{-/-}$ mutants are indicated. A 6-nucleotide deletion were induced in the target site in the selected mutant(A). The predicted protein structures of the wild type (WT) and the *pax2a*$^{-/-}$ mutant are indicated. It is expected that a peptide of 12 amino acids deletion at the N-terminus (B).

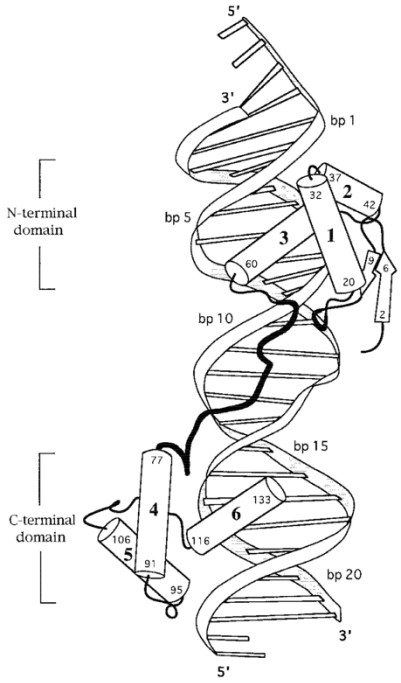

Figure 21 Overview of the Pax protein-DNA complex. Cylinder represents α-helix of paired domain in N-terminal domain (PAI domain) and C-terminal domain (RED domain), consisted of 3 α-helixes in each domain. Arrows show β-hairpin (A). (Xu et al., 1999).

Mutant lines generation and phenotype observation

F2 *pax2a* homozygous zebrafish were establish from same *pax2a* homozygous zebrafish. F2 *pax2a* homozygous male number10 and female number3 were paired to produce the next generation (F3) and observe the phenotype (**Figure 22**).External morphology of *pax2a*$^{-/-}$ mutants in F2 of strain C showed no different from wild type (WT).However, F2 pairing of male and female *pax2a*$^{-/-}$ mutants showed significantly higher percentage of unfertilized eggs comparing with WT pairing (**Figure 25A**). Moreover, the embryos (F3) from F2 male and female *pax2a*$^{-/-}$ mutants showed some abnormal characteristics. The oval-shape that is the earliest detectable morphological abnormality of *pax2a*$^{-/-}$ embryos was showed in epiboly stage about 9 hours post-fertilization (hpf). After that, the oval-shape embryos developed to be more crucial characteristics, including, yolk abnormality, tail formation abnormality, and heart edema. These abnormal phenotypes showed the same characteristics with the *pax2a*$^{-/-}$ female x WT and the pairs of *pax2a*$^{-/-}$(**Figure 23**).

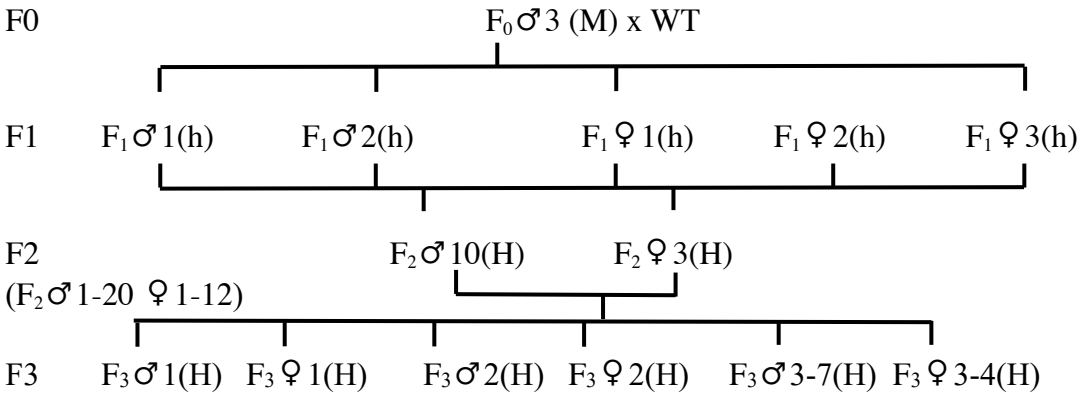

Figure 22 Family tree of *pax2a* knock out zebrafish strain C (-6)

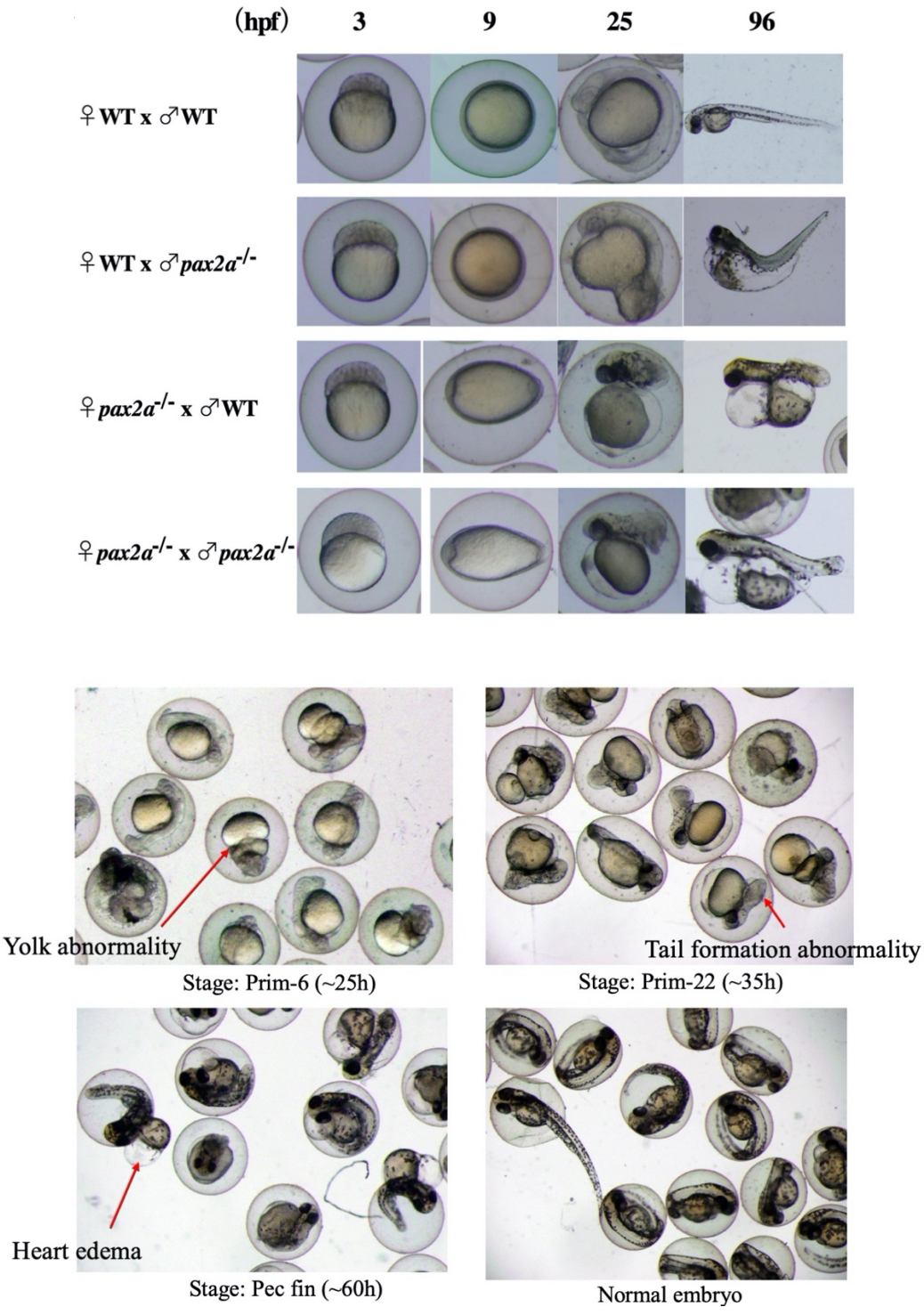

Figure 23 Abnormal morphologies and rate of *pax2a* mutant development. Abnormal embryos from mutant pairs (*pax2a*$^{-/-}$ male x *pax2a*$^{-/-}$ female), *pax2a*$^{-/-}$ female x WT, and *pax2a*$^{-/-}$male x WT comparing with wild type in the 3, 9 25 and 96 hpf. The oval shape appeared in embryos from mutant pairs (*pax2a*$^{-/-}$ male x *pax2a*$^{-/-}$ female), *pax2a*$^{-/-}$ female x WT in epiboly (9 hpf), then, the abnormal become more severe which were yolk abnormality, tail formation abnormality, and heart edema.

Ratio of the major and minor embryonic axes were used to quantify the abnormal of embryos in epiboly (**Figure 24A**). Embryos from mutant pairs (*pax2a*$^{-/-}$ male x *pax2a*$^{-/-}$ female) and *pax2a*$^{-/-}$ female x WT exhibited oval shape and their ratio of the major and minor axes, which were 1.33 and 1.29, also showed significantly longer than wild type, however, embryos from *pax2a*$^{-/-}$ male-xWT was the almost same ratio of normal epiboly (1.04 and 1.02) (**Figure 24B**).

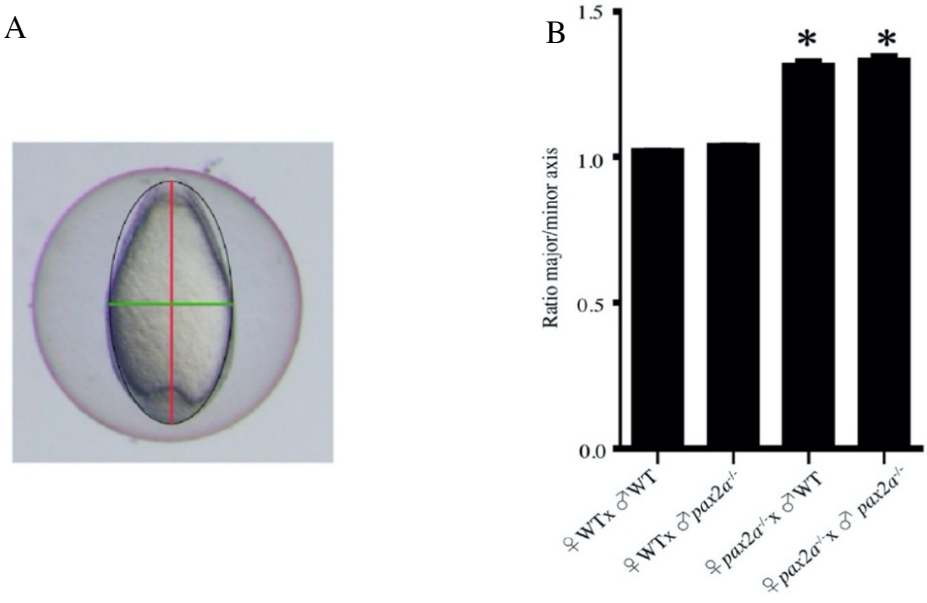

Figure 24 Ratio of major (red line) and minor (green line) axes of embryos in epiboly stage were examined (A). The average of ratio in pax2a mutant were compared with wild type (B).

The percentage of abnormal embryos of *pax2a*$^{-/-}$ mutant pairs and *pax2a*$^{-/-}$ female x WT were significantly higher than pairs of wild type (**Figure 25B**). Other than that, the survived embryos of F3 from the F2 *pax2a*$^{-/-}$ mutant pairs and *pax2a*$^{-/-}$ female x WT were significantly low (**Figure 25C**). Few numbers of F3 embryos can growth to be adult fish. F3 juveniles tended to die during the cultivation, consequently, lower numbers of juveniles developed to adults. Only 11 F3 adult fish were obtained from total 425 eggs from F2 generation (2.6%).

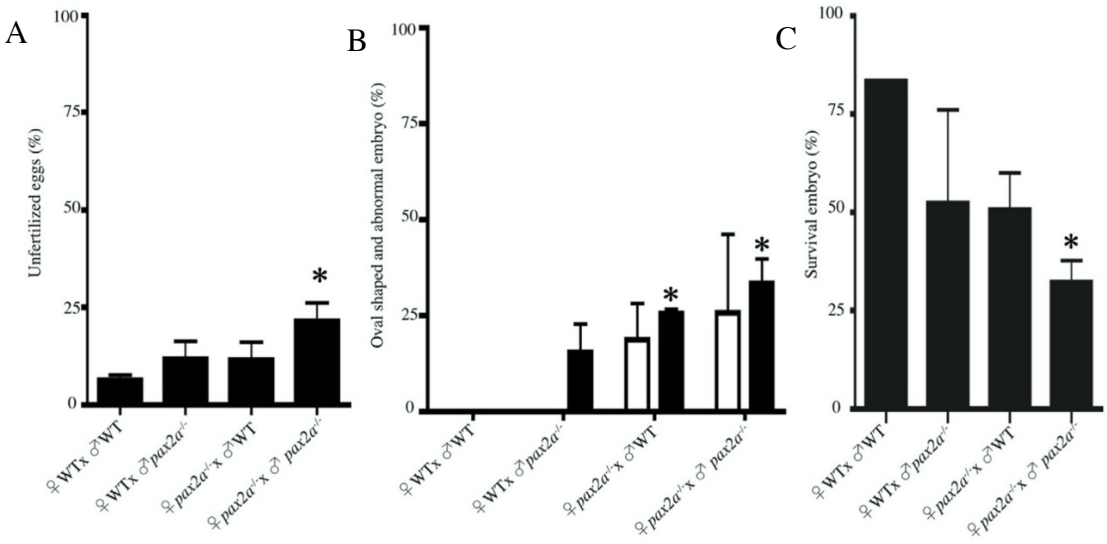

Figure 25 Percentage of unfertilized eggs, abnormal embryos, and survival embryos. Percentage of unfertilized eggs in the eggs obtained from embryos in four groups of pairing, including, wild type pairs (WT x WT), mutant pairs (*pax2a*$^{-/-}$ male x *pax2a*$^{-/-}$ female), *pax2a*$^{-/-}$ female x WT, and *pax2a*$^{-/-}$male x WT(A). Percentage of abnormal embryos obtained from embryos in four groups of pairing, including, wild type pairs (WT x WT), mutant pairs (*pax2a*$^{-/-}$ male x *pax2a*$^{-/-}$ female), *pax2a*$^{-/-}$ female x WT, and *pax2a*$^{-/-}$male x WT. White bar referred to oval shape embryo and black bar was abnormal embryos including oval shape embryo (B). Percentage of survival embryos obtained from embryos in four groups of pairing, including, wild type pairs (WT x WT), mutant pairs (*pax2a*$^{-/-}$ male x *pax2a*$^{-/-}$ female), *pax2a*$^{-/-}$ female x WT, and *pax2a*$^{-/-}$male x WT (C).

The F3 adult *pax2a*$^{-/-}$ zebrafish from the survived embryos slowly grown and dwarf, furthermore, the F3 *pax2a*$^{-/-}$ female seem like the male (**Figure 26D**). Ovaries of *pax2a*$^{-/-}$ zebrafish were produced less than *pax2a*$^{+/-}$ and WT, respectively (**Figure 26A-C**) From the histological results, it showed that gonads of *pax2a* F3 homozygous mutants were not normally well developed comparing with WT. There was only stage I ovaries in the *pax2a* homozygous mutant female, it meant that their ovaries stopped the development at the stage I (**Figure 27C-D**). In contrast, there were stage I, II, and III in normal wild type (**Figure 27A-B**). Moreover, mating ability of these fish were deficiency, including mating behavior and fertilization. The fertilization rate and number of spawning eggs of F3 female *pax2a*$^{-/-}$ were low which were 3 from 16 times

(18.75%) and total spawning eggs from 3 successful times were only 29 eggs. These results were indicated that *pax2a* might effect to oocyte maturation, fertilization, and early development of embryos.

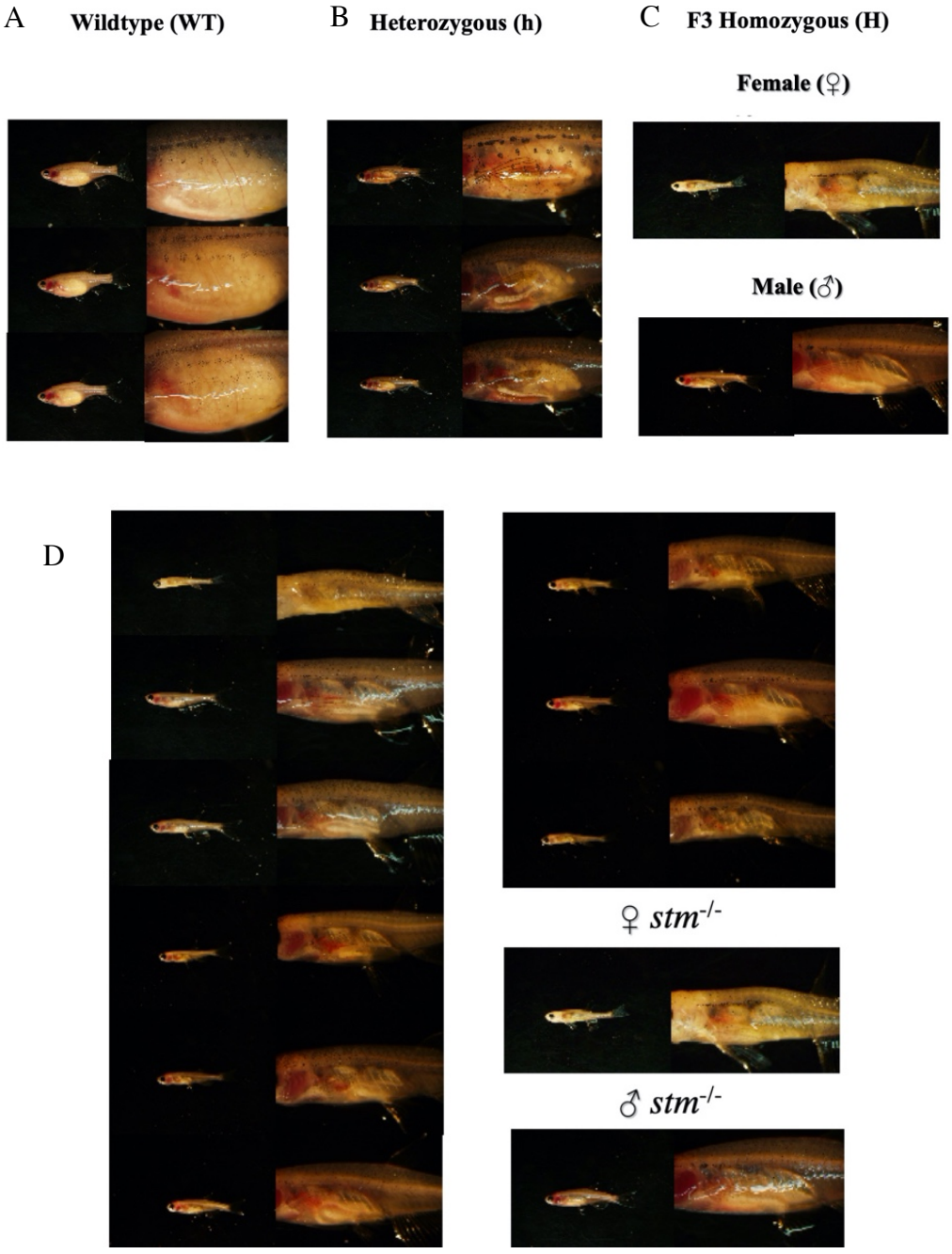

Figure 26 Comparison of ovaries and external appearance of wild type, heterozygous (*pax2a*$^{+/-}$), and homozygous mutant (*pax2a*$^{-/-}$). Ovaries of WT (A). Ovaries of heterozygous (*pax2a*$^{+/-}$) (B). Ovaries of homozygous mutant (*pax2a*$^{-/-}$) (C). External appearance of F3 homozygous mutant (*pax2a*$^{-/-}$) which were survived from abnormal characteristics of F2 *pax2a*$^{-/-}$male and *pax2a*$^{-/-}$ female pairing (D).

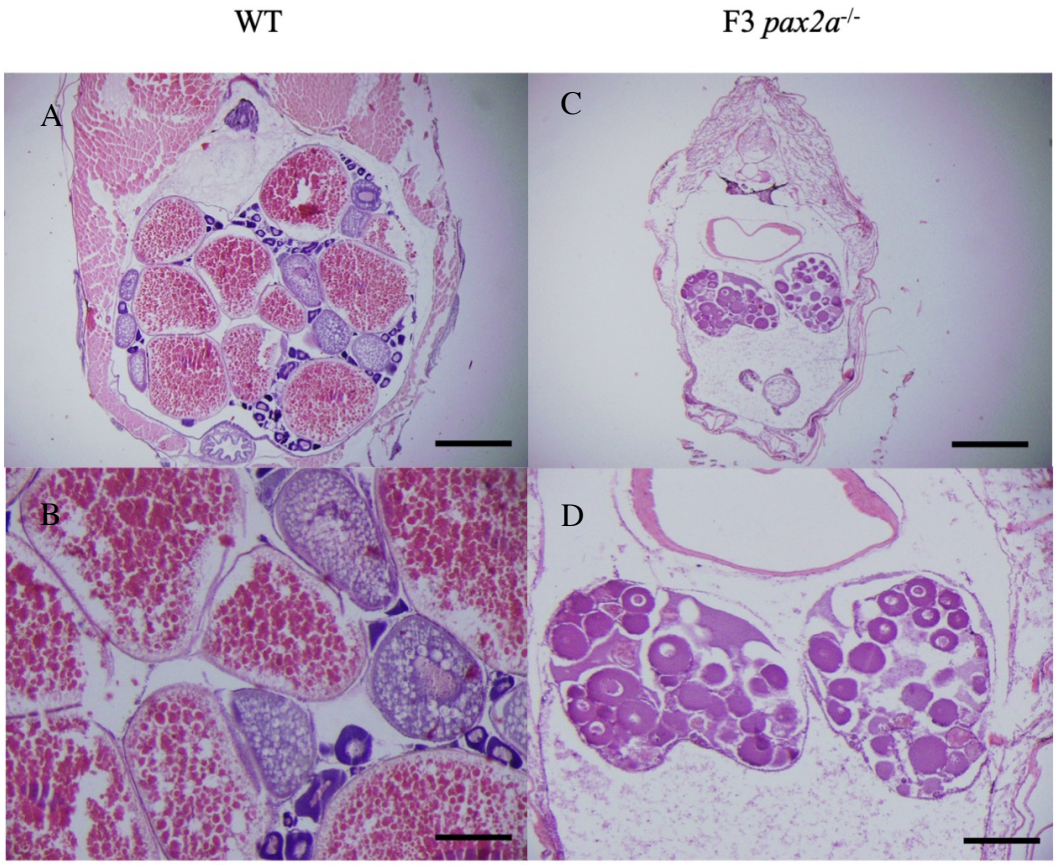

Figure 27 Histological result of transverse section of wild type and female F3 homozygous mutant zebrafish (*pax2a*$^{-/-}$), that indicated the successful pairing, using Hematoxylin and Eosin (HE). The oocyte in the wild type body showed stage I, II, and III (A) and enlarge picture of wild type (B). The oocyte of F3 homozygous mutant zebrafish (*pax2a*$^{-/-}$) had only stage I (C) and enlarge picture of F3 homozygous mutant zebrafish (*pax2a*$^{-/-}$) (D).

Western blot, immunohistochemistry and immunochemistry analysis

Mature oocyte and early stage of embryos (3, 11, 36, and 60 hpf) were investigated the expression of Pax2a. The results showed that Pax2a expressed the high level in the mature oocyte and gradually decrease in each stage of early stage of embryos (3, 11, 35 and 60 hpf, respectively) (**Figure 28B**). Pax2a expressed in the eggs and decreased after 11 hpf (18 somite stage) during development. However, Pax2a was not detected at 35 hpf (Prim-22 stage) and 60 hpf (pec-fin stage). These results suggested that Pax2a was produced as a maternal protein in oocyte before ovulation, whereas Pax2a was not produced during early development.

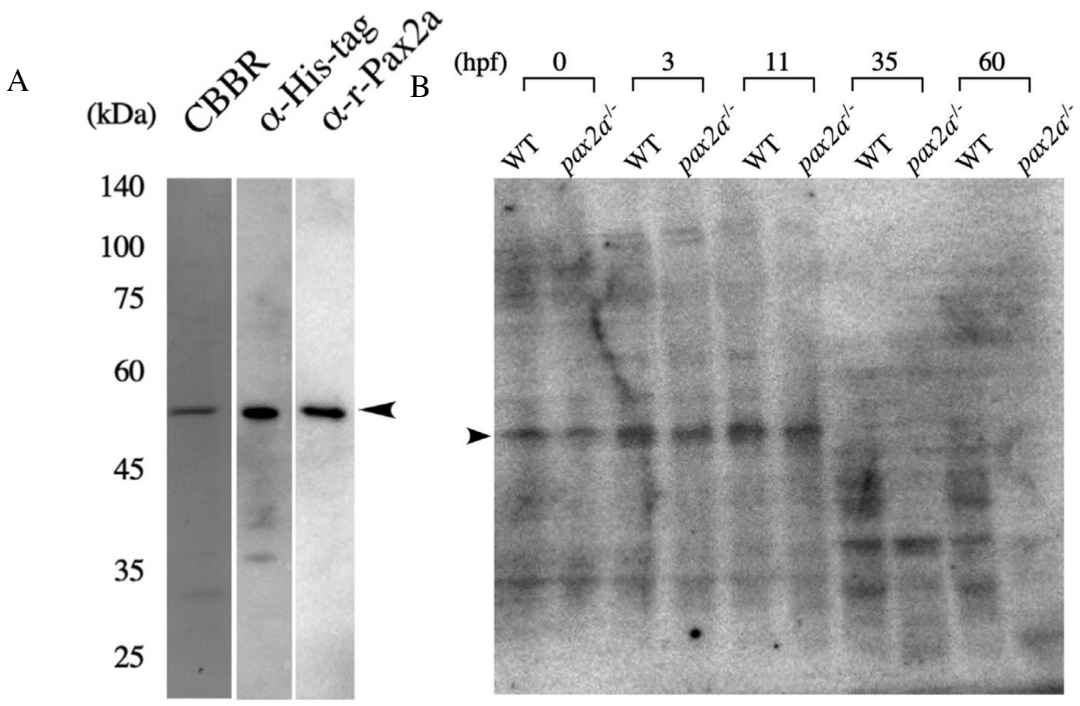

Figure 28 Western blot analysis of expression of Pax2a in zebrafish. Band approximately 45 kDa is observed throughout development of zebrafish. Eight of intact follicles (0 hpf) and embryos in each stages, including, 1,000-cell stage (3 hpf), 18-somite (11 hpf), prim-22 (35 hpf), and pec fin stage (60 hpf) were loaded per lane. Same stage of wild type and *pax2a*$^{-/-}$ was compared. Pax2a recombinant (rPax2a) in *E. coli* was used as positive control.

This result was confirmed by the immunohistochemistry analysis that signal of Pax2a express in the layer of fertilization membrane (**Figure 29A**). Expression of Pax2a showed in all stages of ovary, but in the lower stage, for example, stage I expressed higher than later stage. The expression of Pax2a in the membrane layer of ovaries similar with expression of Pax2a in the eggs that highly expressed near the membrane (**Figure 29B**). The expression of Pax2a appeared both in wild type and *pax2a* homozygous mutant from the western blot and immunohistochemical results. These data indicated that the *pax2a* mutant strain established in this study exhibited a shift in the start codon producing an N-terminal 12 amino acid deletion. These results suggested that the *pax2a* was activated before the ovulation and slightly decrease in the early development.

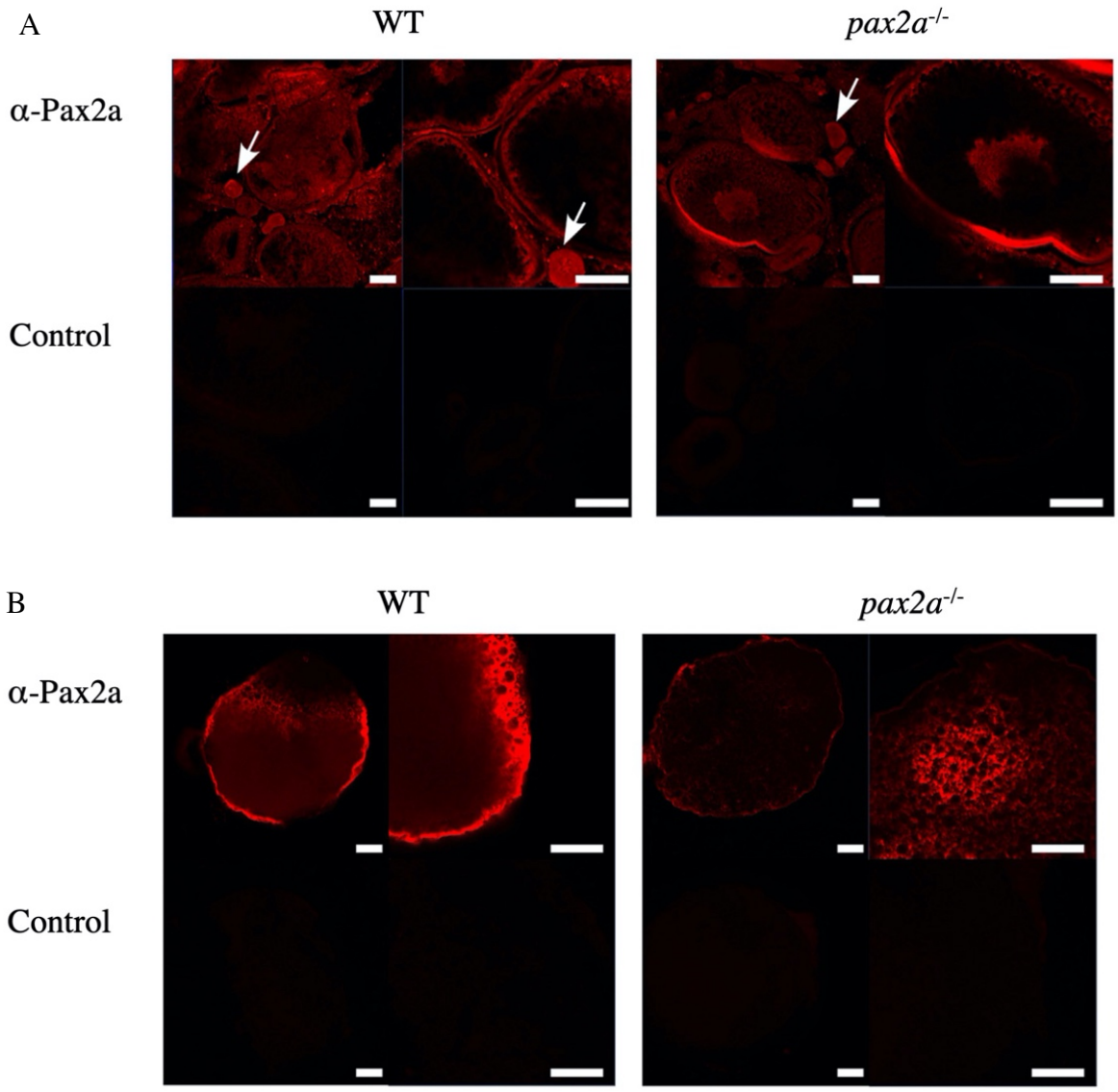

Figure 29 Expression of Pax2a protein was performed using immunohistochemical analysis for wild type (WT) and *pax2a* homozygous mutant (*pax2a*$^{-/-}$). Frozen sections of whole bodies of female for observing oocyte(A) and eggs (B) of wild type (WT) and *pax2a* homozygous mutant (*pax2a*$^{-/-}$) were section and stained with anti-Pax2a. Anti-Pax2a pre-adsorbed with recombinant Pax2a (control). The scale bars indicated 200 μm in A and 100 μm in B.

Quantitative PCR (qPCR)

Relative expression of *pax2a* in ovulated ovaries and 8-cells stage embryos were compared in 3 groups, including *pax2a*$^{-/-}$, *pax2a*$^{+/-}$, and WT. The mRNA abundance was investigated triplicate for each sample. All data were normalized with the expression of Elongation factor 1α (*EF1α*). The results indicated that relative

expression level of *pax2a* in ovulated eggs of *pax2a*$^{-/-}$ was significantly decreased comparing with *pax2a*$^{+/-}$ and WT (**Figure 30A**). Embryos expressed Pax2a lower than ovulated eggs (**Figure 30B**), consistence with results of western blot that Pax2a expressed in the ovulated egg and slightly decrease in embryo. This data suggested the *pax2a*$^{-/-}$ lowly expressed the *pax2a* gene.

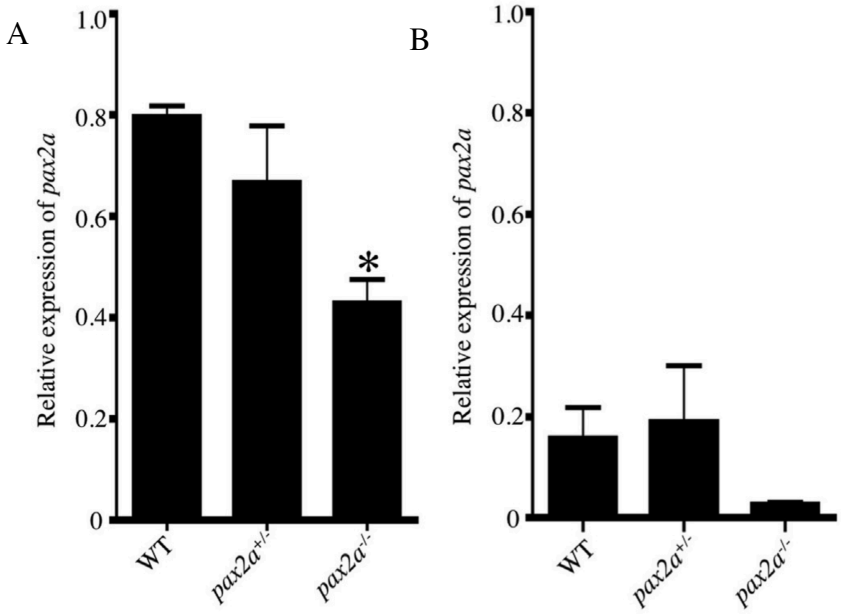

Figure 30 Expression of *pax2a* of among wild type (WT), *pax2a* heterozygous mutants, and *pax2a* homozygous mutant using qPCR analysis. Eggs (A). 8 cell stage embryos (B).

DISCUSSION

In vivo, *pax2a* knockout zebrafish were established using CRISPR/Cas9 system in this experiment. Despite there have been studies of *pax2a* in early development of zebrafish and other species, this study provides the first report to establish *pax2a* knockout zebrafish and investigate the functions in oocyte maturation, ovulation, and early development. *Pax2a* is the one of high possible candidate gene of ovulation-inducing gene (Klangnurak et al., 2018). Normally, its main role related to be transcription factor responded to develop some organs, for example, boundary of mid-hind brain, kidney and thyroid follicles in zebrafish (Mackereth et al., 2005, Porazzi et al., 2009, Picker et al., 2002, Riley et al., 1999, Pfeffer, 1998). However, the role of *pax2a* to oocyte maturation and ovulation are rarely investigated. Consequently, we focus on the effect of *pax2a* to interrupt to oogenesis, oocyte maturation, ovulation, fertilization, and early development. The homozygous mutant zebrafish of *pax2a* (*pax2a*$^{-/-}$) showed significantly highly unfertilized eggs comparing wild type. This result related to starmaker (*stm*) gene knock out fish we reported previously. (Klangnurak et al., 2018). This result showed that the unfertilized rate of *pax2a* similar with *stm* knock-out mutant. *Pax2a* is an up-regulate gene of *stm* (Bajoghli et al., 2009).

In addition, the embryos from the *pax2a*$^{-/-}$ parents demonstrated the abnormal characteristic embryos. The oval-shape that is the earliest detectable morphological abnormality of *pax2a*$^{-/-}$ embryos was showed in epiboly stage about 9 hours post-fertilization (hpf). The same phenotype with oval shape was reported by injected RNA of NRAS. The effected of NRAS injection caused Ras MAPK pathway signal transduction (Runtuwene et al., 2011, Anastasaki et al., 2009). After that, the crucial abnormal characteristics appeared after oval-shape phenotype in gastrulation defects, including, heart edema, yolk abnormality, and tail formation abnormality. These abnormal characteristics were similar to *wnt*, *sox*, and BRAFs knockout zebrafish (Okuda et al., 2010, Gao et al., 2016, Jindal et al., 2017).

Pax2 acts the upstream of the expression of *wnt4/5*. *Pax2* siRNA treatment showed the low expression of *pax2*, *wnt4*, and *wnt5* in catfish, in addition, *pax2* silenced catfish also demonstrated the lower mRNA of *sox9b* (Prathibha and Senthilkumaran, 2016). In catfish, decreased expression of *sox9b* is important to differentiate the ovary (Raghuveer and Senthilkumaran, 2010). For zebrafish, *pax2a* is the upstream of sox9a/b in the maintenance the optic placode (Hans et al., 2004). It might be possible that *pax2a*

and sox9a/b are related in ovarian development of zebrafish. Nowadays, ovarian functions of nodal, which is well-known gene for early development, was demonstrated in zebrafish (Zayed et al., 2020). For zebrafish, roles of nodal were suggested to make the process of follicle growth, steroidogenesis, and oocyte maturation. *Pax2a* might have the role of same process of nodal gene.

Embryonic lethal was showed in *pax2* knock-out mice (Torres et al., 1996). This result supported that *pax2a* plays a crucial role in early development. The mutants were expected to become lethal in zebrafish. It is highly possible that viable non-serious mutant strain was established by designing the target sequence at the position containing the start codon in our study. Despite the deletion of only 12 amino acids before the N-terminal paired box subdomain, F3 generation of mutants' strain showed high abnormal incidence. It was impossible to maintain the strain by mating of male and female homozygous mutants. There was research demonstrated that one missense mutation of the β-hairpin motif was predicted as probably damaging the kidney and caused the Focal segmental glomerulosclerosis (FSGS), kidney injury, in human (Barua et al., 2014). In addition, Wheat et al. (1999) mentioned that the missense mutations, which occurred in or nearby the β-hairpin motif, lead to the serious developmental anomalies results. In our knock outed *pax2a*, the mutants produced Pax2a that had 12 amino acid deletion near the β-hairpin. This is presumed that the deletion of the N-terminus for 12 amino acids may partially block or effect the paired domain to DNA.

From the results and discussions that mentioned above, we suggested the novel roles of *pax2a* in oogenesis, fertilization, and early development.

CHAPTER III
Conclusion and perspective

CONCLUSIONS

Pax2a editing zebrafish were established in this study. Observed phenotype of *pax2a* homozygous showed new function of *pax2a* through oogenesis, oocyte development, and early development. However, *pax2a* did not directly affect on ovulation. The *pax2a* homozygous mutant zebrafish can spawn the eggs but the number of eggs and fertilization rate were low comparing with wild type. The embryos from F2 *pax2a* homozygous zebrafish appeared a lot of abnormal phenotypes, starting with oval shape in epiboly stage. After that, oval shape abnormal embryo developed other abnormalities, including, heart edema, tail formation abnormality, and yolk abnormality. These abnormal phenotypes affected to the survival rate of *pax2a* homozygous mutant embryos. Survival rate of *pax2a* homozygous embryos was significantly lower than wild type. However, some F3 normal embryos were obtained to continue the mutant strain. F3 *pax2a* homozygous mutant were dwarf comparing with wild type size. Furthermore, the F3 *pax2a* homozygous female appeared only few numbers of stage I developed oocyte in their body. There were no stage II and III of oocyte in their body. This result indicated that *pax2a* related to oogenesis and oocyte development.

PERSPECTIVE

Although the novel function of *pax2a* for oogenesis, oocyte development, and early development of embryo were found, the role of *pax2a* in these functions still unknown. The mechanism of *pax2a* have to study to clear the new functions of *pax2a* for the oogenesis, oocyte development, and early development of embryo. In addition, other genes, that were high possibility ovulation inducing genes, have to investigate, including, solute carrier family 37 (the glucose-6-phosphate transporter) member 4a (*slc37a4a*), zebrafish gene collection number 65811 (*zgc:65811*), zebrafish gene collection number 92184 (*zgc:92184*), cathepsin Bb (*ctsbb*), salt inducible kinase1 (*sik1*), starmaker (*stm*), a disintegrin-like and metalloproteinase domain with thrombospondin-15 motifs (*adamts-15*), RNA binding motif protein 47 (*rbm47*), chymotrypsinogen B1 (*ctrb1*), and protease, serine, 59, tandem duplicate 1 (*prss59.1*). Especially starmaker (*stm*), that had been shown the previous studies that *stm* was a downstream gene of *pax2a*. *Stm* normally be understood as the gene for otolith formation, nevertheless, role of *stm* during ovulation still unknown. It might be possible that *pax2a* and *stm* were cooperatively work on ovulation process.

REFERENCES

ANASTASAKI, C., ESTEP, A. L., MARAIS, R., RAUEN, K. A. & PATTON, E. E. 2009. Kinase-activating and kinase-impaired cardio-facio-cutaneous syndrome alleles have activity during zebrafish development and are sensitive to small molecule inhibitors. *Human Molecular Genetics,* 18**,** 2543-2554.

BAJOGHLI, B., RAMIALISON, M., AGHAALLAEI, N., CZERNY, T. & WITTBRODT, J. 2009. Identification of Starmaker-Like in Medaka as a Putative Target Gene of Pax2 in the Otic Vesicle. *Developmental Dynamics,* 238**,** 2860-2866.

BARUA, M., STELLACCI, E., STELLA, L., WEINS, A., GENOVESE, G., MUTO, V., CAPUTO, V., TOKA, H. R., CHAROONRATANA, V. T., TARTAGLIA, M. & POLLAK, M. R. 2014. Mutations in PAX2 associate with adult-onset FSGS. *J Am Soc Nephrol,* 25**,** 1942-53.

BLAKE, J. A. & ZIMAN, M. R. 2014. Pax genes: regulators of lineage specification and progenitor cell maintenance. *Development,* 141**,** 737-751.

CERDA, J., REICH, G., WALLACE, R. A. & SELMAN, K. 1998. Serotonin inhibition of steroid-induced meiotic maturation in the teleost Fundulus heteroclitus: role of cyclic AMP and protein kinases. *Mol Reprod Dev,* 49**,** 333-41.

DORFLER, P. & BUSSLINGER, M. 1996. C-terminal activating and inhibitory domains determine the transactivation potential of BSAP (Pax-5), Pax-2 and Pax-8. *Embo Journal,* 15**,** 1971-1982.

FUKADA, S., SAKAI, N., ADACHI, S. & NAGAHAMA, Y. 1994. Steroidogenesis in the Ovarian Follicle of Medaka (Oryzias-Latipes, a Daily Spawner) during Oocyte Maturation. *Development Growth & Differentiation,* 36**,** 81-88.

GAO, R., MA, L. Q., DU, X. G., ZHANG, T. T., ZHAO, L., LIU, L. H., LIU, J. C., GUO, F. J., CHENG, Z. & HUANG, H. Z. 2016. Rnf25/AO7 positively regulates wnt signaling via disrupting Nkd1-Axin inhibitory complex independent of its ubiquitin ligase activity. *Oncotarget,* 7**,** 23850-23859.

HAIDER, S. 2003. Cyclic AMP level and phosphodiesterase activity during 17alpha,20beta-dihydroxy-4-pregnen-3-one induction and theophylline inhibition of oocyte maturation in the catfish, Clarias batrachus. *Comp Biochem Physiol A Mol Integr Physiol,* 134**,** 267-74.

HAIDER, S. & CHAUBE, S. K. 1995. Changes in total cAMP levels during oocyte maturation in the catfish, Clarias batrachus. *Comparative Biochemistry and Physiology a-Physiology,* 112**,** 379-385.

HAIDER, S. & RAO, N. V. 1992. Oocyte Maturation in Clarias-Batrachus .3. Purification and Characterization of Maturation-Inducing Steroid. *Fish Physiology and Biochemistry,* 9**,** 505-512.

HANS, S., LIU, D. & WESTERFIELD, M. 2004. Pax8 and Pax2a function synergistically in otic specification, downstream of the Foxi1 and Dlx3b transcription factors. *Development,* 131**,** 5091-102.

JINDAL, G. A., GOYAL, Y., YAMAYA, K., FUTRAN, A. S., KOUNTOURIDIS, I., BALGOBIN, C. A., SCHUPBACH, T., BURDINE, R. D. & SHVARTSMAN, S. Y. 2017. In vivo severity ranking of Ras pathway mutations associated with developmental disorders. *Proceedings of the National Academy of Sciences of the United States of America,* 114**,** 510-515.

KIMMEL, C. B., BALLARD, W. W., KIMMEL, S. R., ULLMANN, B. & SCHILLING, T. F. 1995. Stages of Embryonic-Development of the Zebrafish. *Developmental Dynamics,* 203**,** 253-310.

KLANGNURAK, W., FUKUYO, T., REZANUJJAMAN, M. D., SEKI, M., SUGANO, S., SUZUKI, Y. & TOKUMOTO, T. 2018. Candidate gene identification of ovulation-inducing genes by RNA sequencing with an in vivo assay in zebrafish. *Plos One,* 13.

KLANGNURAK, W. & TOKUMOTO, T. 2017. Fine selection of up-regulated genes during ovulation by in vivo induction of oocyte maturation and ovulation in zebrafish. *Zoological Letters,* 3.

KONDO, T., YANAGAWA, T., YOSHIDA, N. & YAMASHITA, M. 1997. Introduction of cyclin B induces activation of the maturation-promoting factor and breakdown of germinal vesicle in growing zebrafish oocytes unresponsive to the maturation-inducing hormone. *Dev Biol,* 190**,** 142-52.

LIU, C. S., ZHANG, X. W., DENG, J., HECKER, M., AL-KHEDHAIRY, A., GIESY, J. P. & ZHOU, B. S. 2011. Effects of Prochloraz or Propylthiouracil on the Cross-Talk between the HPG, HPA, and HPT Axes in Zebrafish. *Environmental Science & Technology,* 45**,** 769-775.

MACKERETH, M. D., KWAK, S. J., FRITZ, A. & RILEY, B. B. 2005. Zebrafish pax8 is required for otic placode induction and plays a redundant role with Pax2 genes in the maintenance of the otic placode. *Development,* 132**,** 371-382.

MANSOURI, A., GOUDREAU, G. & GRUSS, P. 1999. Pax genes and their role in organogenesis. *Cancer Research,* 59**,** 1707s-1709s.

MANSOURI, A., HALLONET, M. & GRUSS, P. 1996. Pax genes and their roles in cell differentiation and development. *Current Opinion in Cell Biology,* 8**,** 851-857.

MCCARROLL, M. N., LEWIS, Z. R., CULBERTSON, M. D., MARTIN, B. L., KIMELMAN, D. & NECHIPORUK, A. V. 2012. Graded levels of Pax2a and Pax8 regulate cell differentiation during sensory placode formation. *Development,* 139**,** 2740-2750.

NAGAHAMA, Y. & ADACHI, S. 1985. Identification of maturation-inducing steroid in a teleost, the amago salmon (Oncorhynchus rhodurus). *Dev Biol,* 109**,** 428-35.

OBA, Y., YOSHIKUNI, M., TANAKA, M., MITA, M. & NAGAHAMA, Y. 1997. Inhibitory guanine-nucleotide-binding-regulatory protein alpha subunits in medaka (Oryzias latipes) oocytes--cDNA cloning and decreased expression of proteins during oocyte maturation. *Eur J Biochem,* 249**,** 846-53.

OKUDA, Y., KONDOH, H. & KAMACHI, Y. 2010. B1 SOX coordinate cell specification with patterning and morphogenesis in the early zebrafish embryo. *Developmental Biology,* 344**,** 490-491.

PANG, Y. F. & GE, W. 2002. Gonadotropin and activin enhance maturational competence of oocytes in the zebrafish (Danio rerio). *Biology of Reproduction,* 66**,** 259-265.

PETRINO, T. R., LIN, Y. W. P., NETHERTON, J. C., POWELL, D. H. & WALLACE, R. A. 1993. Steroidogenesis in Fundulus-Heteroclitus-V - Purification, Characterization, and Metabolism of 17-Alpha, 20-Beta-Dihydroxy-4-Pregnen-3-One by Intact Follicles and Its Role in Oocyte Maturation. *General and Comparative Endocrinology,* 92**,** 1-15.

PFEFFER, P. L., GERSTER, T., LUN, K., BRAND, M. AND BUSSLINGER, M. 1998. Characterization of three novel members of the zebrafish Pax2/5/8 family:

dependency of Pax5 and Pax8 expression on the Pax2.1 (noi) function. *Development,* 125**,** 3063-3075.

PFEFFER, P. L., PAYER, B., REIM, G., DI MAGLIANO, M. P. & BUSSLINGER, M. 2002. The activation and maintenance of Pax2 expression at the mid-hindbrain boundary is controlled by separate enhancers. *Development,* 129**,** 307-18.

PICKER, A., SCHOLPP, S., BOHLI, H., TAKEDA, H. & BRAND, M. 2002. A novel positive transcriptional feedback loop in midbrain-hindbrain boundary development is revealed through analysis of the zebrafish pax2.1 promoter in transgenic lines. *Development,* 129**,** 3227-3239.

PINTER, J. & THOMAS, P. 1999. Induction of ovulation of mature oocytes by the maturation-inducing steroid 17,20 beta,21-trihydroxy-4-pregnen-3-one in the spotted seatrout. *General and Comparative Endocrinology,* 115**,** 200-209.

PORAZZI, P., CALEBIRO, D., BENATO, F., TISO, N. & PERSANI, L. 2009. Thyroid gland development and function in the zebrafish model. *Molecular and Cellular Endocrinology,* 312**,** 14-23.

PRATHIBHA, Y. & SENTHILKUMARAN, B. 2016. Involvement of pax2 in ovarian development and recrudescence of catfish: a role in steroidogenesis. *Journal of Endocrinology,* 231**,** 181-195.

RAGHUVEER, K. & SENTHILKUMARAN, B. 2010. Isolation of sox9 duplicates in catfish: localization, differential expression pattern during gonadal development and recrudescence, and hCG-induced up-regulation of sox9 in testicular slices. *Reproduction,* 140**,** 477-487.

RAHMAN, M. A., OHTA, K., YOSHIKUNI, M., NAGAHAMA, Y., CHUDA, H. & MATSUYAMA, M. 2002. Characterization of ovarian membrane receptor for 17,20 beta-dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in yellowtail, Seriola quinqueradiata. *General and Comparative Endocrinology,* 127**,** 71-79.

RILEY, B. B., CHIANG, M. Y., FARMER, L. & HECK, R. 1999. The deltaA gene of zebrafish mediates lateral inhibition of hair cells in the inner ear and is regulated by pax2.1. *Development,* 126**,** 5669-5678.

RUNTUWENE, V., VAN EEKELEN, M., OVERVOORDE, J., REHMANN, H., YNTEMA, H. G., NILLESEN, W. M., VAN HAERINGEN, A., VAN DER BURGT, I., BURGERING, B. & DEN HERTOG, J. 2011. Noonan syndrome gain-of-function mutations in NRAS cause zebrafish gastrulation defects. *Disease Models & Mechanisms,* 4**,** 393-399.

SANGER, F. 1988. Citation Classic - DNA Sequencing with Chain-Terminating Inhibitors. *Current Contents/Life Sciences***,** 23-23.

TOKUMOTO, T., TOKUMOTO, M., HORIGUCHI, R., ISHIKAWA, K. & NAGAHAMA, Y. 2004. Diethylstilbestrol induces fish oocyte maturation. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 3686-3690.

TOKUMOTO, T., TOKUMOTO, M. & THOMAS, P. 2007. Interactions of diethylstilbestrol (DES) and DES analogs with membrane progestin receptor-alpha and the correlation with their nongenomic progestin activities. *Endocrinology,* 148**,** 3459-67.

TOKUMOTO, T., YAMAGUCHI, T., II, S. & TOKUMOTO, M. 2011. In Vivo Induction of Oocyte Maturation and Ovulation in Zebrafish. *Plos One,* 6.

TORRES, M., GOMEZ-PARDO, E. & GRUSS, P. 1996. Pax2 contributes to inner ear patterning and optic nerve trajectory. *Development,* 122**,** 3381-91.

TREISMAN, J., HARRIS, E. & DESPLAN, C. 1991. The Paired Box Encodes a 2nd DNA-Binding Domain in the Paired Homeo Domain Protein. *Genes & Development,* 5**,** 594-604.

UNDERHILL, D. A. 2012. PAX Proteins and Fables of Their Reconstruction. *Critical Reviews in Eukaryotic Gene Expression,* 22**,** 161-177.

UNTERGASSER, A., CUTCUTACHE, I., KORESSAAR, T., YE, J., FAIRCLOTH, B. C., REMM, M. & ROZEN, S. G. 2012. Primer3-new capabilities and interfaces. *Nucleic Acids Research,* 40.

WENDL, T., LUN, K., MIONE, M., FAVOR, J., BRAND, M., WILSON, S. W. & ROHR, K. B. 2002. Pax2.1 is required for the development of thyroid follicles in zebrafish. *Development,* 129**,** 3751-60.

WESTERFIELD, M. 1995. *The Zebrafish Book: a Guide for the laboratory Use of Zebrafish (Danio rerio),* Univ. of Oregon Press, Eugene, OR.

WHEAT, W., FITZSIMMONS, D., LENNOX, H., KRAUTKRAMER, S. R., GENTILE, L. N., MCINTOSH, L. P. & HAGMAN, J. 1999. The highly conserved beta-hairpin of the paired DNA-binding domain is required for assembly of Pax-Ets ternary complexes. *Mol Cell Biol,* 19**,** 2231-41.

XU, H. E., ROULD, M. A., XU, W., EPSTEIN, J. A., MAAS, R. L. & PABO, C. O. 1999. Crystal structure of the human Pax6 paired domain-DNA complex reveals specific roles for the linker region and carboxy-terminal subdomain in DNA binding. *Genes Dev,* 13**,** 1263-75.

XU, W. G., ROULD, M. A., JUN, S., DESPLAN, C. & PABO, C. O. 1995. Crystal-Structure of a Paired Domain-DNA Complex at 2.5-Angstrom Resolution Reveals Structural Basis for Pax Developmental Mutations. *Cell,* 80**,** 639-650.

YAMASHITA, M., FUKADA, S., YOSHIKUNI, M., BULET, P., HIRAI, T., YAMAGUCHI, A., LOU, Y. H., ZHAO, Z. & NAGAHAMA, Y. 1992. Purification and Characterization of Maturation-Promoting Factor in Fish. *Developmental Biology,* 149**,** 8-15.

YOSHIKUNI, M. & NAGAHAMA, Y. 1994. Involvement of an inhibitory G-protein in the signal transduction pathway of maturation-inducing hormone (17 alpha,20 beta-dihydroxy-4-pregnen-3-one) action in rainbow trout (Oncorhynchus mykiss) oocytes. *Dev Biol,* 166**,** 615-22.

ZAYED, Y., MALIK, R., QI, X. & PENG, C. 2020. Nodal regulates ovarian functions in zebrafish. *Mol Cell Endocrinol,* 511**,** 110821.

ZHU, Y., BOND, J. & THOMAS, P. 2003a. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. *Proc Natl Acad Sci U S A,* 100**,** 2237-42.

ZHU, Y., RICE, C. D., PANG, Y., PACE, M. & THOMAS, P. 2003b. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc Natl Acad Sci U S A,* 100**,** 2231-6.

ACKNOWLEDGEMENTS

I am very grateful to my supervisor, Professor Dr. Toshinobu Tokumoto. He gave me a lot of suggestion of research and lifestyle in Japan and opportunity to conduct my doctoral course in Shizuoka university. I had a lot of experience for attending the conferences both in Japan and aboard (Singapore) by his help. He invested a lot of energy to advise me to finish my research in Shizuoka university. I had opportunity for learning the novel machines and methods from his suggestion.

In addition, I would also thank my thesis committees for their insightful comments, and useful questions: Professor Dr. Taketomo Fujiwara from Department of Environment and Energy Systems, Graduate School of Science and Technology, Shizuoka University, who was my thesis defense chairman, Professor Dr. Takashi Ushimaru from Department of Bioscience, Graduate School of Science and Technology, Shizuoka University, who was a member of my thesis committee, Professor Dr. Shinyo Kodani from Department of Applied life sciences, Faculty of agriculture, Shizuoka university, who was a member of my committee of Thesis Defense, and Professor Dr. Tohru Kobayashi from Institute for Environmental Sciences, Graduate Division of Nutritional and Environmental Sciences, University of Shizuoka who was a member of my committee of Thesis Defense

I would like to thanks Japanese government (Monbukagukusho:MEXT) scholarship for financial support for my life, thesis, and opportunity to attend the conferences in Japan and Singapore.

My sincere thanks also go to all lab members for their suggestion useful guidance and support for all experiments in this thesis and taught me many new experimental methods.

Finally, I gave special thanks to my family who really deserve my appreciation for their full support, encouragement and inspiration throughout these years.