QATAR UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

UNCOVERING THE EPIGENETIC CONTRIBUTION OF PROTEIN ARGININE METHYLTRANSFERASE 5 (PRMT5) DURING ZEBRAFISH CARDIOMYOGENESIS

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ABSTRACT

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Title: Uncovering the Epigenetic Contribution of Protein Arginine Methyltransferase 5 (PRMT5) during Zebrafish Cardiomyogenesis

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The heart develops through an extremely intricate process mediated by a network of transcription factors initiated by key chromatin modification events. Scientist have implicated the protein arginine methyltransferase 5 (PRMT5) in the regulation of cell-specific gene expression programs. In turn, this drives the correct proliferation and differentiation of different types of cells. We showed her that the knockdown of morpholino antisense oligonucleotide-mediated PRMT5 in zebrafish embryos delays heart development. This was accompanied by a moderate decrease in blood flow (20%), significantly higher decrease in general cardiac output (65%), and reduction in red blood cells count (50%). The reduction of the expression of PRMT5 also resulted in a 4-fold decrease in the rate of hatching and had negative impacts on locomotion and tail flicking in PRMT5 morphants. Conversely, these phenotypes were almost fully rescued by the re-expression of *human PRMT5.* The expression of transcription factors (GATA5, MYF5, and CDC37) which are required in the initial stages of cardiomyogenesis was investigated. As well as the key heart failure markers, *ANP/NPPA* and *BNP/NPPB*. The knockdown of PRMT5 resulted in a decline in

transcription of all the target genes studies 24 and 48 hpf. However, there were restorations or slight increases in the ANP/NPPA and BNP/NPPB mRNAs levels 72hpf relative to the control embryos. Analysis of chromatin immunoprecipitation (ChIP) showed that the promoter region of GATA5 and MYF5 are directly targeted by PRMT5, which are both are required for cardiomyocyte differentiation. From these results, it is inferred that PRMT5 is necessary for the normal heart development in zebrafish. This is because the characteristics of PRMT5 regulate the expression of key cardiac transcription factors.

DEDICATION

For my fabulous Mother, The soul of my Father

Wonderfully supportive Family; lovely husband Fadi, kids Raneem and Abdullah

To the soul of my best friend and sister Hiba Al Azzeh

To my beloved country QATAR

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PhD is an exhausting journey and, in many cases, a frustrating one, as well. Your world can be limited to your lab and your bed. There are times you lose yourself, and many more times that you want to give up in an attempt to get your old self back. However, there are also times that you remember the more significant scope and the highest goals you set. There are times you feel pride in your achievements, your contributions to research and society and times that you realize that your new self is your better version. They say, to survive you must be willing to fail. I say you need to have the right people by your side, the whole time.

During this journey, I experienced many ups and downs. I can safely say that I am a different person now to that which begun this PhD almost four years ago. The highs I enjoyed and the lows that I overcame not only made me a stronger, more confident person but also made me appreciate the importance of the people around me. Without this support network around me, I never would have finished

First, I am deeply grateful to God Almighty, my creator, and source of understanding, knowledge, wisdom, and inspiration. Without whose grace, mercy, and guidance, I could not achieve this milestone.

Many people have supported me throughout this journey from beginning research in the laboratories to finally completing my thesis. Thanks to my experienced supervisors, without their faith in my ability, I would have never had the opportunity to do a PhD. Their guidance and experience were essential factors in helping me finish this thesis.

Mostly Prof. Said Sif, who introduced me to this exciting field, helped me in the transition from academic to laboratory work and was a continuous source of enthusiasm, ideas and problem-solving. Moreover, providing essential guidance during my lab work and for giving me opportunities to present my work and to act as a mentor to others. Additionally, I would like to express gratitude to my co-supervisor Dr. Gheyath Nasrallah for his enormous help in providing laboratory space and instrumentation, which helped develop experimental planning and practical skills, and for his kind advice and critical feedback throughout my research work.

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The Biomedical Research Center (BRC), led by Dr. Asma Al-Thani, and her academic and administrative staff always support the concept of scientific research is an essential pillar for professional experience and academic teaching. I want to express my special thanks to Dr. Asma Al-Thani as a facilitator who generously spends some of her time to empower and encourage students to perform biomedical research in Qatar. My work at the BRC enriched my knowledge of educational and research experience. I want to thank all of the BRC staff (Maria, Ola, Duaa, Mashaeal, Enas) for their technical assistance, advice and make reagents available for us.

My family deserves special praise for keeping me well balanced throughout this PhD. The completion of this PhD journey would not be possible if I did not have my fabulous mother (Nahida Arrar) prayers and encouragement, and the spirit of my father Zaki Zakaria. Special thanks to my lovely husband, Fadi, for his understanding, patience, and encouragement to persevere. And for my princess Raneem and my hero Abdullah, who were always proud of me, I am deeply and forever grateful for their endless love, support, and encouragement. Thanks to my brothers (Ziad, Mohammed, Faisal, Yousef, Ali, Mohammed) and sisters (Zainab, Safa, Marwa, Ayat), niece and nephew for their unremitting support and love which facilitates me to tackle the challenges of work, to be free to enjoy life with them.

Lastly, I would like to thank friends for putting up with me through my PhD degree and beyond. I wish that my friend and soulmate, Hiba Alazzeh, with me now to see the achievement of my goals; she was always supportive and encouraging. I dedicate all my achievements to her soul. Many friends were a vast area of support, in particular;

wonderful friend Balsam, I am greatly indebted to your ample support during the high and low points of the past years, you always had time to go for planning our experiments, give advice and have fun together. Always motivating, inspiring, and uplifting me and always finding ways to bring the smile to my face, even in the hardest times.

To my best friends, thank you for your understanding in all those times of crisis and your limitless patience in our gatherings when the only subject of our discussions was my research project. Thank you, girls. Special thanks to Abeer Abdelmajeed, Enas Alhassan, Rana Naser, Reem Aweidah, Mona Qutaifan, Noor Al Dessi, and Soulaf Alkhateeb for continuous support and help

Last, but by no means least, I would like to thank the zebrafish core staff at the BRC specially Enas Al Absi, I must also recognize the sacrifice of thousands of zebrafish for their essential contributions to my project and the field of biomedical research as a whole.

I am not sure what the future holds but aspire to continue contributing to research knowledge and its applications to improve outcomes in clinical practice.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 4

LIST OF ABBREVIATIONS

%	Percent
℃	Degree Celsius
ADMA	Asymmetric dimethylarginine
ALPM	Anterior lateral plate mesoderm
AMPK	AMP-activated protein kinase
ANOVA	Analysis of Variance
ANP/NPPA	Atrial natriuretic peptide
APS	Ammonium persulfate
BCA	Bicinchoninic acid assay
BMP	Bone morphogenetic protein
BNP/NPPB	natriuretic peptide B
BSA	Bovine serum albumin
CCNCs	Cardiac neural crest cells
CDC73	Cell Division Cycle 73
cDNA	Complementary DNA
CHD	Coronary heart disease
ChIP	Chromatin Immunoprecipitation
Co-IP	Co-immunopricipitation
CpG	Cytosine-guanine dinucleotide
Ct	Cycle threshold
DA	Dorsal aorta

ddH_2O	Double distilled water
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
E2F1	E2 transcription factor 1
E3M	Embryo medium
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to mesenchymal transition
FBS	Fetal bovine serum
FHF	First heart field
FOXO1	Forkhead box O1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA5	GATA binding protein 5
GRNs	Gene regulatory networks
H3 (Me_2) R2	Histone H3 symetrical dimethylarginine 2
H3 (Me_2) R8	Histone H3 symetrical dimethylarginine 8
H4 (Me_2) R3	Histone H4 symetrical dimethylarginine 3
HATs	Histone acetyltransferases
HKMTs	Histone-lysine methyltransferases
HMTs	Histone methyltransferases

Hpf	Hours post fertilization
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IP	Immunoprecipitation
Jak2	Janus kinase binding proteins
Kbp	kilo-base pairs
kDa	Kilodalton
KO	Knockout
MEF2	Myocyte enhancer factor 2
mM	Millimolar
MMA	Monomethyl arginine
MNase	Micrococcalnuclease
MO	Morpholinos
MRF4	Myogenic regulatory factor 4
MRFs	Muscle regulatory factors
mRNA	Messenger RNA
MS	Mass spectrometry
MuSC	Muscle stem cell
MYF5	Myogenic factor 5
MyoD	Myogenic differentiation
OD	Optical Density
p38	Mitogen-activated protein kinase

PAGE	Polyacrylamide gel electrophoresis
Pax3	Pair box transcription factor 3
Pax7	Pair box transcription factor 7
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Posterior Cardinal Vein.
PGC1α	Peroxisome proliferator-activated receptor-γ coactivator-1α
PMSF	Phenyl methylsulfonyl fluoride
PRMT1	Protein arginine methyltransferase 1
PRMT5	Protein arginine methyl transferase 5
PTM	Post-translational modifications
PTU	N-phenylthiourea
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
QU-IACUC	Qatar University's Institutional Animal Care and Use Committee
RioK1	pICln or the Rio domain-containing protein
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPM	Revolutions per minute
RQ	Relative quantity
RT	Reverse transcriptase
RT-qPCR	Real time quantitative polymerase chain reaction

SAM	S-adenosylmethionine
SD	Standard deviation
SDMA	Symmetric dimethylarginine
SDS	Sodium dodecyl Sulfate
SHH	Sonic hedgehog
SWI/SNF	SWItch/Sucrose Non-Fermenting
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with tween-20
TBX1	T-box transcription factor 1
TEMED	Tetramethylethylenediamine
TF	Transcription factor
TFA	Trifluoroacetic acid
THW	Threonine-histidine-tryptophan
TP53	Tumor protein 53
VEGF	Vascular endothelial growth factor
W/V	Weight/Volume
Wnt	Wingless-related integration site
WT	Wild type
ZFEs	Zebrafish embryos

CHAPTER 1: INTRODUCTION

By its continuous pumping action and homeostatic mechanisms, the heart ensures the hemodynamic needs of an organism are met throughout its life. It constantly aligns itself with the altering needs of the organism's developmental stages, from the embryonic phase and through the transitions from fetal to newborn circulation. The heart is actually the first organ that forms in the embryonic development stage as it provides essential functions for survival. Each specific heart development stage requires a highly coordinated series of gene expression to ensure turning on and off of proper genes in different cardiomyocyte differentiation stages. (Vincent et al., 2010; Collins et al., 2016).

Cardiomyogenesis is presided over by the cross-talk and interplay occurring between evolutionarily preserved transcription factors. These include myocyte enhancer factor 2 (MEF2), heart and neural crest-derivatives-expressed proteins (Hand) 1 and 2, and GATA4-6. The factors act together with ATP-dependent chromatin remodelers and histone-modifying enzymes. (Weintraub et al., 1991; Bharathy et al., 2013; Hernández-Hernández et al., 2017). It has been suggested in developmental research into vertebrates such as zebra fish that the synchronized expression of specific myogenic regulatory factors (MRFs) alongside GATA4-6, Hand1 and 2, TBX5, and NKX2-5 enable cardiac progenitor cells from bilaterally paired regions within the anteriorlateral plate mesoderm to move dorsally toward the midline of the embryo. It also induces the expression of cardiogenic genes that start and sustain functional

sarcomeres and fuse to form a linear heart tube. Migration of further cardiac progenitor cells from the second heart field (SHF) facilitates the formation of the definitive zebrafish heart (Weintraub et al., 1991; Lu et al., 2016; Hernández-Hernández et al., 2017). Although several genetic mutations in zebra fish have been explored, no specific one has resulted in a phenotype without a heart. This underpins the complex nature of the molecular mechanisms that trigger cardiogenesis.

The arginine methylation protein is at the core of cell signaling and growth regulatory pathways. However, while the protein arginine methyltransferase (PRMT) family has nine distinct members, their developmental contributions remain largely unknown. The three known types of PRMTs can modify their arginine substrates via asymmetric dimethylation (type I), symmetric dimethylation (type II), or monomethylation (type III) (Pal et al., 2007; Karkhanis et al., 2011). Studies have implicated type II protein arginine methyltransferase 5 (PRMT5) in a wider variety of cellular processes. However, because of its early embryonic lethality in PRMT5 knockout mice, its role in regulating gene expression series during organismal development is unclear (Shailesh et al., 2018; Guccione et al., 2019). It is known to be expressed highly in neonatal rat cardiomyocytes and has the capability to restrain cardiac hypertrophy through methylation and inactivation of GATA4 (Chen et al., 2014). Taking these results into account and the fact that information on PRMT5's role in the early stages of development is scarce, this study used PRMT5-specific morpholino antisense oligonucleotides to focus on assessing the effects of PRMT5 knockdown in zebrafish.

We established that PRMT5 is a vital epigenetic regulator in cardiomyogenesis. Focus was on the contribution of PRMT5 to the necessary transcriptional modulators of heart development and the key mechanisms in the initial stages of cardiomyogenesis, including GATA5, MYF5, and CDC73. Deeper insight into developmental cardiac biology potentially widens existing knowledge of the mechanisms of heart diseases. This would, in turn, enable the detection of the genes affected by PRMT5-mediated methylation and examination into how this potentially affects heart development.

Study Rationale and Hypothesis

The worldwide incidence of heart diseases and heart failure is rising faster than that of any other disease (WHO, 2016; Benjamin et al., 2019). Modification of chromatin by either DNA-dependent chromatin remodeling complexes or chromatin-modifying enzymes has arisen as a crucial regulatory step in several features of control in cellular proliferation, differentiation, and development (Chen et al., 2014). However, limited work has been done to demonstrate the role played by chromatin remodelers in developmental decision-making. More specifically, there is limited information about the mechanisms and gene expression programs influenced by these chromatin remodelers during different growth and developmental stages.

Recent studies have indicated that PRMT5 is involved in a wide assortment of cellular processes including RNA processing, signaling, transcription, RNA processing, and DNA repair (Stopa et al., 2015; Shailesh et al., 2018; Chaturvedi et al., 2019; Lin et al., 2019). However, PMRT5's role in heart development and how it controls cardiomyogenesis in vivo remains poorly understood. We hypothesize that PRMT5, a type II PRMT5 enzyme, serves as an important epigenetic regulator of cardiomyocyte differentiation.

Research Focus and Aims

The objective of this thesis is to analyze the biological function of PRMT5 during heart muscle development, and to test the hypothesis that PRMT5 is a crucial epigenetic regulator for normal heart development**.** The following aims were developed:

- To examine expression of PRMT5 during the different developmental stages of zebrafish.
- To elucidate the role of PRMT5 in heart muscle development at molecular and physiological levels.
- To determine whether the heart muscle specific gene expression program requires PRMT5 activity during different stages of heart muscle development.

Merit of the Proposed Contribution to Science

Understanding genetic determinants of normal heart development is essential to prevent and target therapies for congenital heart diseases. However, genetic factors may not be enough to fully explain the morphogenetic mechanisms by which heart is normally developed. Epigenetic factors are receiving huge attention from medical researchers in attempts to resolve several research questions related to heart malformations. Epigenetics is thought to provide a constricted control of gene expression at the level of both translational and post- translational giving out essential for heart development. Several studies investigated the role of post- translational modification proteins including PRMT5, it is expressed at high levels in heart, skeletal muscle and testis, and is expressed at low levels in across other organs and tissues (Pollack et al., 1999).

It was previously reported that PRMT5 knockout in the heart is fatal in mice and chick embryos. However, the regulatory mechanism by which PRMT5 affects modifies the genetic cardiogenesis is missing in the literature. To fill out this gap, PRMT5 knockdown study is essential. This study is the first of its kind that aims at investigating the impact of PRMT5 knockdown on cardiogenesis by through assessment of cardiac function and on master cardiac transcription factors such as GATA5, MYF5 and CDC73. Results of this research will lead to improved understanding of the underlying mechanisms of congenital heart disease and may open insights toward gene therapies.

Compliance with Ethical Conduct of Research

Animals utilized in research must be cared for and maintained in accordance with applicable laws and requirements outlined by the Qatar University on Animal Care. Approval from the Institutional Animal Care and Use Committee (IACUC) was obtained by submitting a notice of intent to use zebrafish embryos (ZEFs) for less than 72 hours. Additionally, all adult zebrafish breeding was performed under the protection of breeding protocol #QUIACUC039/2017, which was granted to the zebrafish facility at the Biomedical Research Center at Qatar University.

CHAPTER 2: LITERATURE REVIEW

2.1 Mammalian Cardiac Anatomy

The heart is found in the midline of the thorax and is a cone-shaped muscular pump consisting of different compartments which are separated by valves (Figure 2.1). Over the past few years, research on cardiac development has focused on uncovering the genetic mechanisms controlling cardiac differentiation and clarifying the embryonic origin of different cardiac lineages (Sun et al., 2018). Study of the physiological mechanisms behind cardiac function has proven to be instrumental to our understanding of heart disease and the interplay between different cardiac cells.

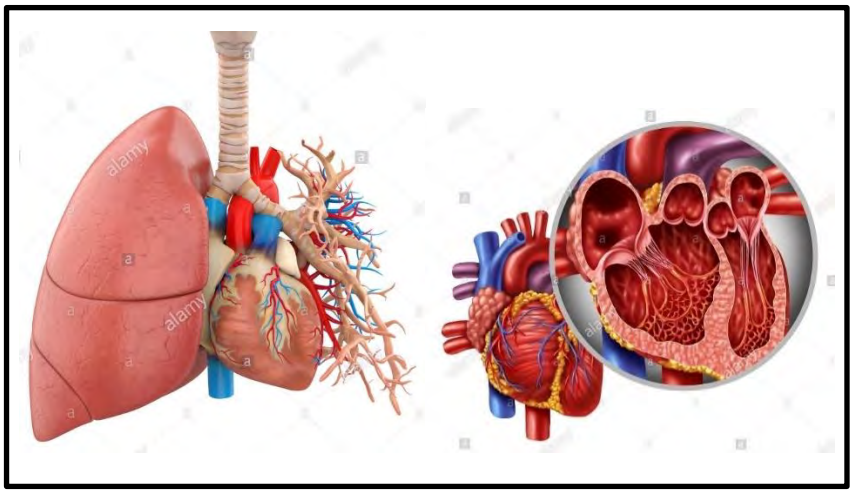


Figure 2.1 Anatomy of the heart. Adapted from *Anatomy of the Human Body* (Gray, 2001).

2.1.1 Vertebrate Heart Development and Maturation

The heart is actually the first organ that forms in the embryonic development stage as it provides essential functions for survival. The precise development of the heart in vertebrate necessitates extremely controlled cellular organization. The differentiation and migration of cardiac precursor cells contribute to the morphology of the heart and its particular compartments (Camp et al., 2011). The occasions driving to heart formation in vertebrates are homologous, and include formation and looping of the heart tube, elongation and growth by the wide addition of cardiac precursor cells, and morphogenesis of the cushions, cardiac chambers, and valves (Vincent et al., 2010; Brade et al., 2013).

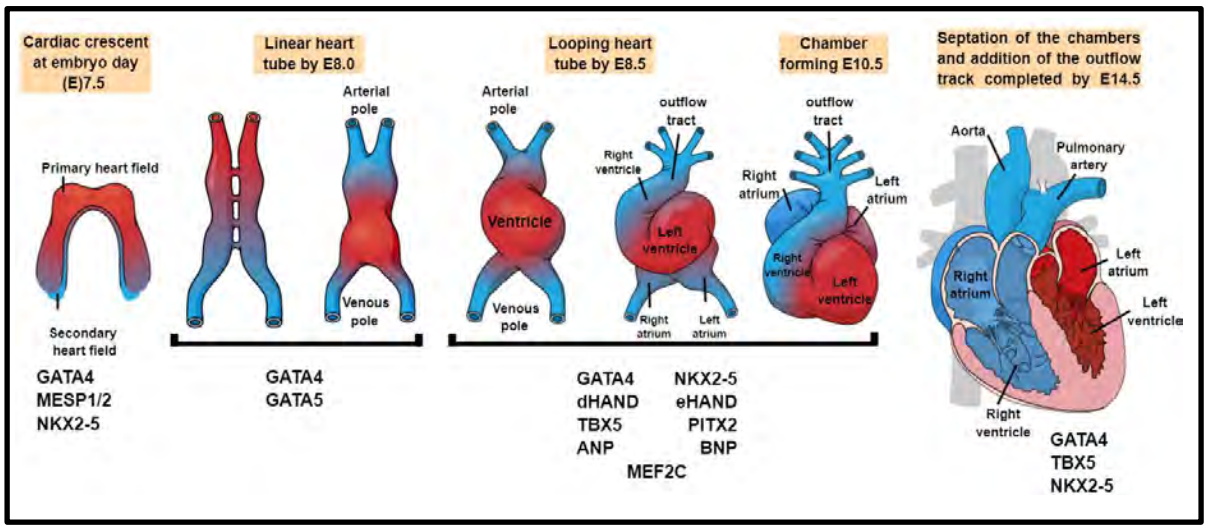
According to Chapuli and his colleagues (2010), cardiogenesis is defined as the growth of new heart tissue from cardiac precursor cells. Bartunek and his team demonstrated that the concept of cardiogenesis has recently gained importance due to extensive research on the stem cell segment and its potential in the repair and treatment of failing heart symptoms that occur in relation to cardiovascular precursors (Bartunek et al., 2009).

The different sources of heart cell precursors identified in the embryo are cardiac neural crest cells (CNCCs), the proepicardium (PE), and cardiogenic mesoderm cells (CMCs). In the mammalian embryo, there is a migration of the cardiac precursor cells specified by the expression of cardiac-specific TFs to the anterior primitive streak at the start of gastrulation (Nakajima, 2010), and undergo epithelial to mesenchymal transition (EMT). They then ingress through the anterior primitive streak, forming the

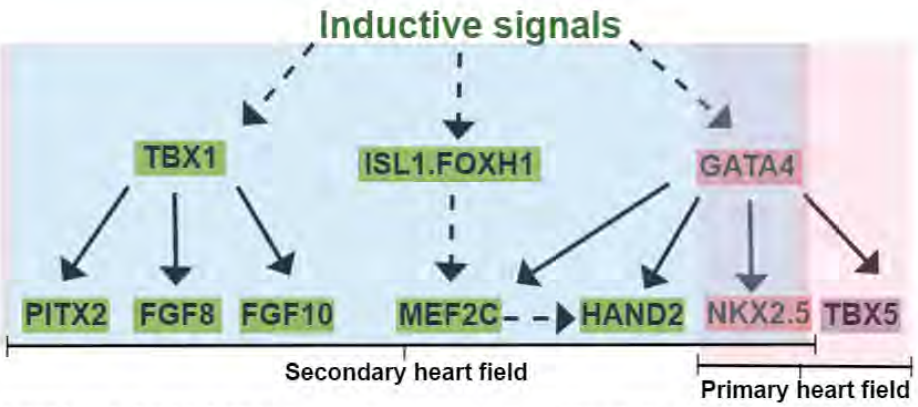
cardiac mesoderm. The cardiac precursor cells migrate laterally and anteriorly until they are located beneath the head folds, forming a cardiac crescent at embryo day (E) 7.5 as shown in Figure 2.2 A (Buckingham et al., 2005; Aanhaanen et al., 2011).

The cardiac precursor cells, which can be characterized by the expression of *NKX2.5*, *GATAa4*, *ISL1,* and *e/dHAND* move medially and ventrally until they fuse with the midline. This creates a linear heart tube by E8.0. At E8.5, the heart tube loops toward the right, with the dorsal portion becoming the inner curvature, and the ventral portion forming the outer curvature. The heart morphology is almost complete by E10.5, with subsequent septation of the chambers, and addition of the outflow track completed by E14.5. During septation, the systemic and pulmonary circulation separate through the assembly of the right ventricle to the pulmonary trunk and the assembly of the aorta to the left ventricle. After septation, the heart continues to grow as many cells follow numerous, complex differentiating pathways to form the diverse and specialized cells of the mature heart. The model in Figure 2.2 shows of the critical stages occurring in heart development from cardiac crescent through maturation, and indicates the predominant TFs expressed at each stage.

(A)



(B)



GATA4 and NKX2.5 (NK2 homeobox 5) are central transcription factors in the primary heart field and the secondary heart field (purple shading). The transcription factor T-box 5 (TBX5) is only expressed in the primary heart field (pink shading). Genes expressed in the secondary heart field are shaded in blue. The ISL1 protein is a marker for cardiac progenitor cells in the secondary heart field. The protein product of TBX1 is a central regulator of the secondary heart field, as it controls the development of the cardiac outflow tract. FGF, fibroblast growth factor; FOXH1, forkhead box H1; MEF2C, myocyte-specific enhancer factor 2C; PITX2, paired-like homeodomain transcription factor 2. Dashed lines indicate that additional components are involved, and solid lines indicate direct-regulation.

Figure 2.2 Mammalian heart development**.** (A). The embryonic days are for heart

development. (B) The signaling pathways involved in the induction of cardiomyocytes. Adapted from Xin et al., 2013 (Xin et al., 2013).

The primitive linear heart tube is composed of endothelial cells, which are separated from the external myocardial layer by a gel-like matrix called cardiac jelly. Cardiac precursor cells that produce the primitive heart tube are primarily found in the lateral splanchnic mesoderm and are called the first heart field (FHF) precursors. The second heart field (SHF) contributes to the primitive tube via the migration of cardiac precursor cells to the venous and arterial poles from the pharyngeal and Medio caudal splanchnic mesoderm (Mjaatvedt et al., 2001; Kelly, 2005). The FHF precursors are the prominent source of the cardiomyocytes in the left ventricle, while the SHF precursors give rise to the entire right ventricle, a portion of the left ventricle, the proximal outflow tract, and a large portion of the atrial myocardium (Buckingham et al., 2005; Moretti et al., 2006).

Gene expression controlling cardiomyogenesis is controlled temporally and spatially by a myriad of signaling networks (Kimmel et al., 2010). Cardiac induction is initiated by many signaling pathways; most prominently, Notch, Wnt, bone morphogenetic protein (BMP), and sonic hedgehog (SHH) (Kimmel et al., 2010). These signaling pathways control expression of TFs which are important in controlling expression of genes that are crucial for patterning and growth of the heart and skeletal muscle-specific genes.

TFs essential for heart development include GATA TFs, NKX2.5, MEF2c, TBX5, and the natriuretic peptides (Stefanovic et al., 2015). The process of cardiomyogenesis in the embryonic heart is also affected by cell proliferation and recruitment of supplementary cells in the arterial and venous poles of the heart tube, resulting in the development of

interactions between cardiomyocytes, epicardial and endocardial cells, as well as cardiac neural crest cells (CCNCs) (Brade et al., 2013).

2.2 Zebrafish as a Model for Heart Development

Zebrafish is a small freshwater fish native to northern India and adjacent countries. It is a common aquarium fish worldwide (Hill et al., 2005). With sequencing of the zebrafish genome steadily progressing and the massive increase in articles being published using zebrafish as an animal model, it has become a well-established model in the scientific community. One of the main reasons for its popularity is the relative ease to maintain large systems with hundreds of animals. Zebrafish exhibit many advantages over more conventional vertebrate models such as the mouse or rat, including its size, husbandry, and rapid embryonic development. The embryos of zebrafish usually are only 1.5 mm in diameter, which helps to minimize the costs of testing, since only small amounts of dosing solutions, chemicals, and disposable labware are required to treat and maintain the embryos. Zebrafish are capable of optimally producing 200–300 eggs per pair of adult fish. The high fecundity of this species, when bred in proper ratios, allows for the collection of a large number of embryos, helping to minimize the inconsistencies that can stem from small sample sizes (Hill et al., 2005). Sexually mature males and females can be distinguished by their body shape, as males are slender while females are more rounded, and their coloration, in which males display a yellowish tint and females have a silvery greyish-blue hue (Figure 2.3.A).

The zebrafish embryo is transparent, providing an unobstructed view of the developing

embryo for 2–4 days after fertilization. This also makes fluorescently tagged gene products much easier to detect (Dahm et al., 2006). The ability to visually identify malformations in the developing embryo is of great benefit (Hill et al., 2005). The zebrafish is an ideal model for embryonic microinjection due to its external fertilization, hence easy collection of fertilized embryos (Hill et al., 2005).

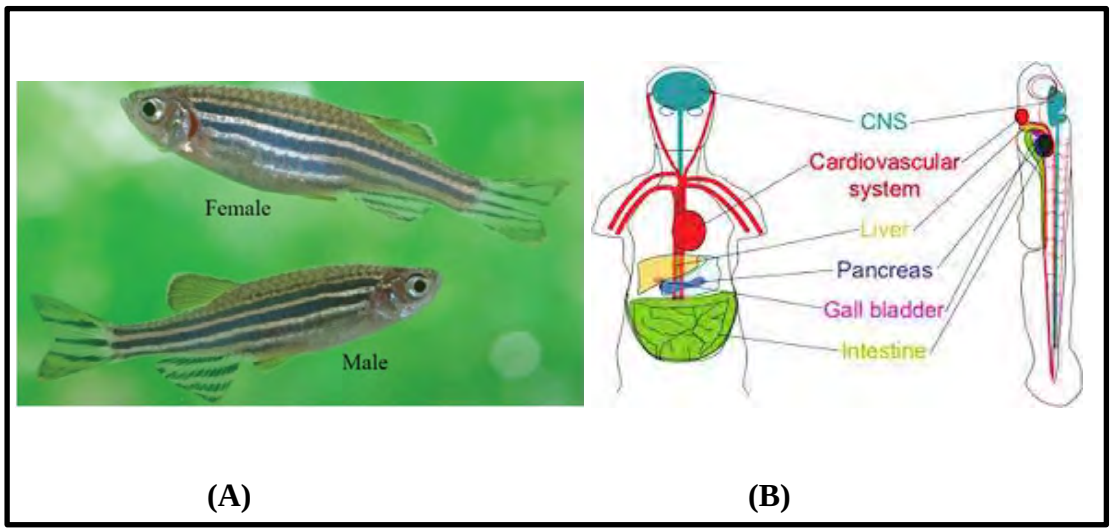


Figure 2.3 Zebrafish as a model animal (A) Adult male and female of the AB strain of zebrafish (B) Some of the conserved organ systems between zebrafish and humans (Teame et al., 2019)

According to research by Lakstygal et al. (2018) the epigenetic mechanisms in zebrafish is primarily related to developmental events that occur at the time of germ cell

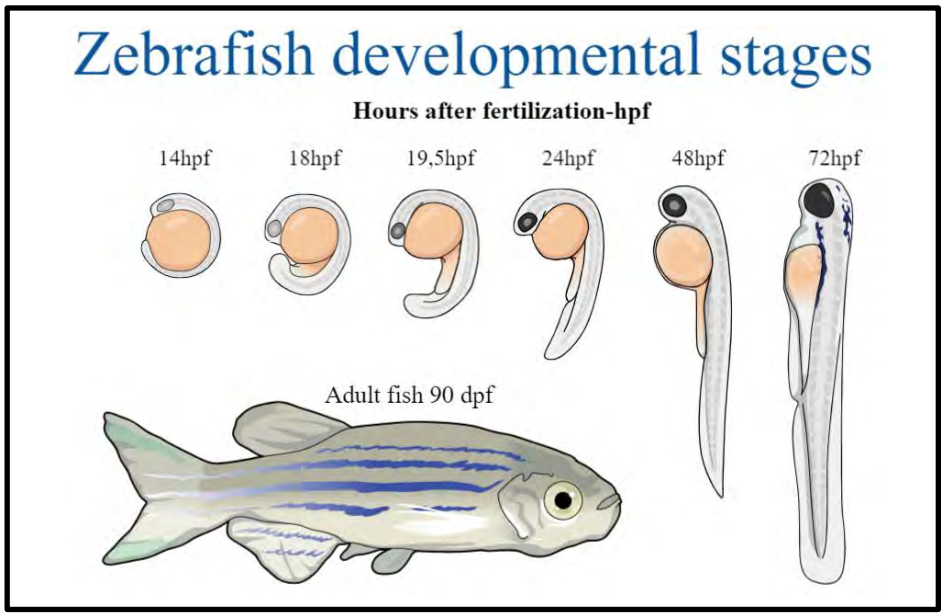
programming. These mechanisms include reversible histone modifications such as acetylation, methylation, phosphorylation (Bannister et al., 2011). DNA methylation in the zebrafish model takes place effectively (Ramsahoye et al., 2000), it is believed to be restricted to cytosine bases located 5′ to guanine (i.e. CpG-sites). The reaction, which is catalyzed by enzymes called DNA methyltranferases (DNMT), adds a methyl groups (-CH3) to cytosine from the donator Sadenosyl methionine (SAM) (Watson et al., 2002; Tang et al., 2007).

With regards to the early phases of life, development is a highly-conserved process in all vertebrates (Hill et al., 2005). Compared to smaller models like *Caenorhabditis elegans* or *Drosophila* as shown in Figure 2.3 B, zebrafish are much more biologically relevant to humans because they are vertebrates. Apart from the placenta, lungs and mammary glands, all other human organs are also found in zebrafish. There is a corresponding zebrafish gene for 71% of human genes. Further, in the context of genes associated with human disease such as endocrinological disorders and hematological disease, neurodegenerative diseases, cardiac arrhythmias, this statistic goes up to 80%.

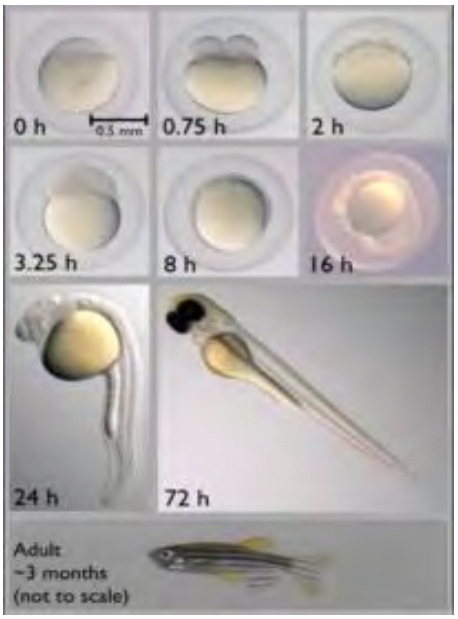
2.2.1 Zebrafish Developmental Stages

During early development, the age of the embryo is defined by the number of hours that have passed since fertilization (hours post-fertilization, or hpf). Periods of early development include zygote, cleavage, blastula, gastrula, segmentation, pharyngula, hatching, and early larva. Figure 2.4 shows the description of normal zebrafish embryo (ZFE) development (Kimmel et al., 1995).

(A)


Zebrafish developmental stages
Hours after fertilization-hpf
14hpf
18hpf
19,5hpf
24hpf
48hpf
72hpf
Adult fish 90 dpf

(B)



Period	h	Description
Zygote	0	The newly fertilized egg through the completion of the first zygotic cell cycle
Cleavage	¾	Cell cycles 2 through 7 occur rapidly and synchronously
Blastula	2¼	Rapid, metasynchronous cell cycles (8, 9) give way to lengthened, asynchronous ones at the midblastula transition; epiboly then begins
Gastrula	5¼	Morphogenetic movements of involution, convergence, and extension form the epiblast, hypoblast, and embryonic axis; through the end of epiboly
Segmentation	10	Somites, pharyngeal arch primordia, and neuromeres develop; primary organogenesis; earliest movements; the tail appears
Pharyngula	24	Phylotypic-stage embryo; body axis straightens from its early curvature about the yolk sac; circulation, pigmentation, and fins begin development
Hatching	48	Completion of rapid morphogenesis of primary organ systems; cartilage development in head and pectoral fin; hatching occurs asynchronously
Early larva	72	Swim bladder inflates; food-seeking and active avoidance behaviors

Figure 2.4 Periods of Zebrafish Early Development. (A) Zebrafish developmental stages. (B) description of zebrafish developmental stages (Kimmel et al., 1995).

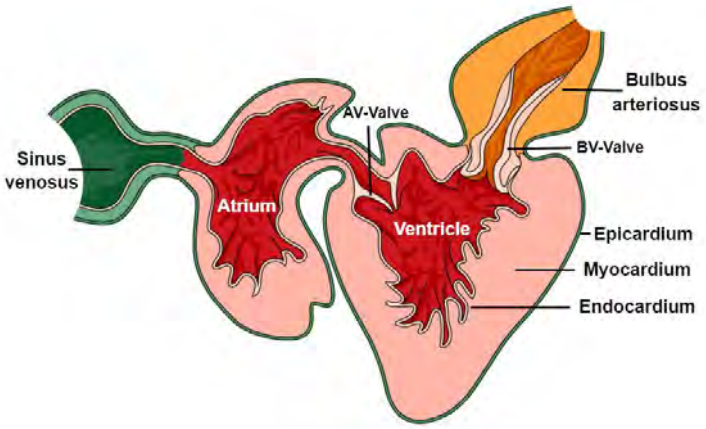
2.2.2 Zebrafish Heart Development and Maturation

There are various anatomical differences between the zebrafish and mammalian hearts. Understanding the key differences can aid in interpreting data obtained when analyzing zebrafish hearts, which would be difficult or impossible in human hearts (Genge et al., 2016).

The adult zebrafish heart consists of a single ventricle and atrium which is contained within a pericardial sac located between the pectoral bone and operculum, anteroventrally to the thoracic cavity (Hu et al., 2001). Blood returns to the heart via hepatic portal veins, anterior cardinal veins and posterior cardinal veins, which enter the sinus venosus. Blood drains into the atrium, which is located on the dorsal side of the ventricle. Blood is then pumped through the ventricle along the bulboventricular orifice and into the bulbus

arteriosus, a pear-shaped appendage located between the ventricle and the ventral aorta (Hu et al., 2001). The ventral aorta is a cylindrical vessel connected to four pairs of afferent branchial arteries which branch off into afferent branchial arterioles inside the gill filaments. The gill filaments are located under the operculum for protection, where oxygenated water flows over the filaments and allows oxygen to diffuse into the blood. Blood then flows into four pairs of efferent branchial arteries that converge to form the dorsal aorta as shown in Figure 2.5.

(A)



(B)

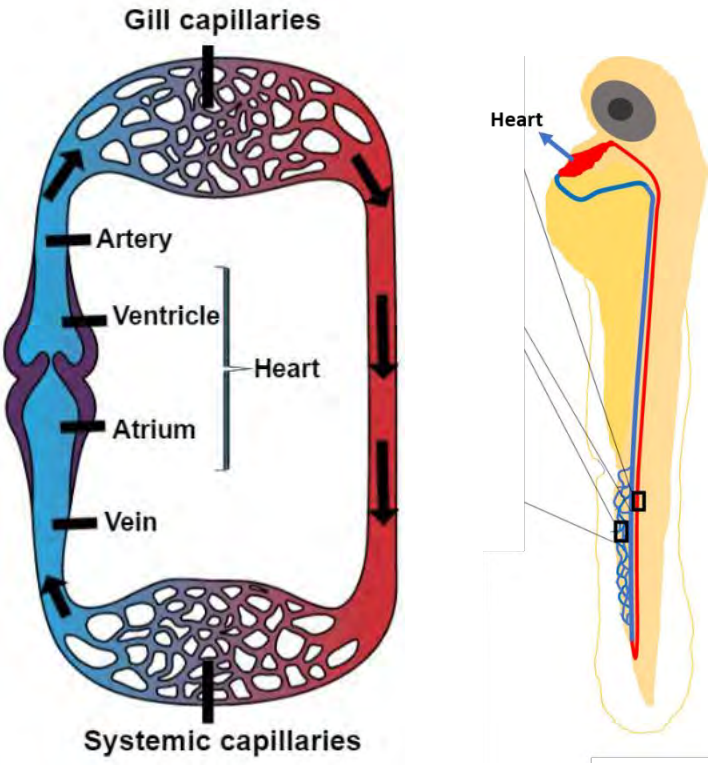


Figure 2.5 Zebrafish Circulatory System. (A) Two chambers of the zebrafish heart. (B) The closed-loop circulatory system, common in all fish. Blue denotes deoxygenated blood and red oxygenated blood.

Notable discoveries made using the zebrafish have helped to further describe the cellular and molecular mechanisms activated during vertebrate heart development (Bakkers, 2011; Brown et al., 2016; Knight et al., 2016). As in mammals, migration of the cardiac precursor cells occurs after gastrulation, forming a single heart tube in the embryonic zebrafish (Stainier, 2001), as depicted in Figure 2.6. The heart tube loops toward the left and differentiates to form a defined ventricle and atrium with an atrioventricular valve from endocardial cushions. The ventricle and atrium are composed of myocardium and endocardium and have myosin heavy chain isoforms that are specific to each chamber (Schoenebeck et al., 2007). Although the genetic determinants leading to formation of the heart tube are similar between mammals and zebrafish (Stainier, 2001), septation does not occur, as it does in mammals and amphibians. Genes involved in mammalian heart development also play prominent roles in zebrafish heart development (Bakkers, 2011).

Mesodermal angioblasts translocate, forming a primitive vascular loop at the midline and later becoming the cardinal vein and aorta. Angiogenic sprouting occurs from the axial aorta and cardinal vein, forming vasculature throughout the ZFES (Lawson et al., 2002). The aortic arches remain symmetrical and attach to the gills and lateral aortae, which eventually merge to form one dorsal aorta. Similar observations have been made in embryonic mammals prior to aortic arch extension, regression, and remodeling. The same critical genes are involved in vasculogenesis in zebrafish and mammals, such as sonic hedgehog, NOTCH, and vascular endothelial growth factor (VEGF) (Lawson et al., 2002).

Cardiac contraction is first observed at roughly 20 hpf, with the circulation of blood occurring around 24 hpf. The conduction system in hearts of adult zebrafish appear to be specialized with two trabeculae bands conducting atrioventricular contraction (Sedmera et al., 2003). Separate atrial and ventricular depolarization waves have been reported in embryos from 5 days post fertilization (Forouhar et al., 2004). The mechanism and onset of trabeculation in zebrafish hearts are thought to be very comparable to those of mammals (Schwartz et al., 1991). As chamber development progresses, compaction and thickening of the myocardial walls occur, along with the formation and thickening of trabeculae (Schwartz et al., 1991; Liu et al., 2012; Zakaria et al., 2018). Main morphological changes in zebrafish heart development along with genes governing heart development process are summarized in Figure 2.6.

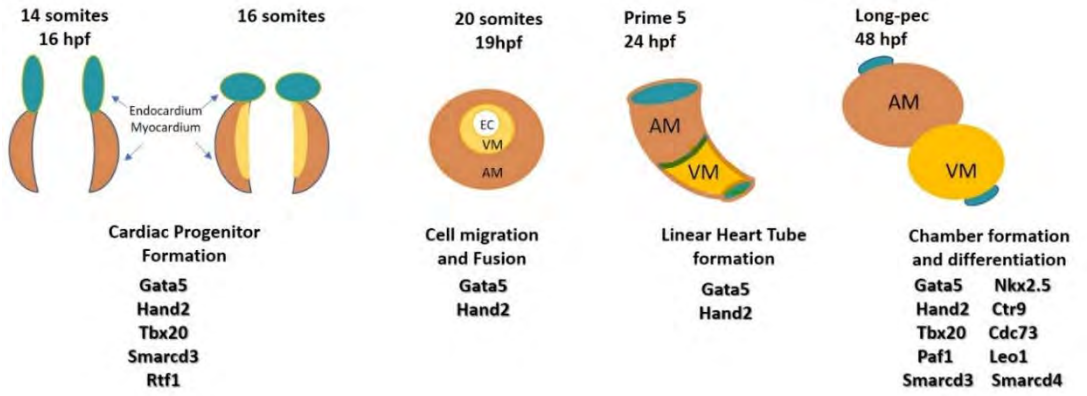


Figure 2.6 Stages of zebrafish heart development: when the embryo at 14 somites stage, the cardiac precursors emerged from the anterior lateral plate mesoderm. The resulting cardiac precursors then move and merge at the midline forming a cone-like structure when the embryo at 20 somites stage. After 1 day of development, the tube-like structure heart starts to beat and support circulation to the embryo body. After 2 days of development, well-demarcated cardiac chambers, as well as looping, has been completed (Zakaria et al., 2018).

After initial cardiac development, cardiac growth continues linearly with the length of the zebrafish (Bertaso et al., 2013). Cardiac development in zebrafish is a consequence of cardiomyocyte hyperplasia via the proliferation of existing cardiomyocytes. This is in contrast to the process in mammals, where hypertrophy and binucleation cause postnatal cardiac growth (Li et al., 1996; Soonpaa et al., 1996). According to Bouveret and his team, the heart is made up of cardiac muscles and is known as the most modified muscular vessel that is regulated with the help of gene regulatory networks (GRNs). The GRNs are controlled by TFs that generally present in the form of subnetworks, known as kernels in the body. These kernels are classified into different subparts, which include NKX2-5, GATA5, CDC73, and TBX5/20, as well as others. The kernel TFs are largely responsible for developing and defining organ-specific functions (Bouveret et al., 2015; Lu et al., 2016).

2.3 Epigenetics and Post-Translational Modifications

Over the last few decades, the human proteome has been shown to be far more complicated than the human genome. Although it is estimated that only 20,000 to 25,000 genes are encoded by the human genome (International Human Genome Sequencing, 2004), the human proteome is comprised of over 100,000 proteins (Jensen, 2004). These data indicate that a single gene in the human genome encodes an average of at least four to five different proteins.

The complexity of the human proteome is a result of multiple mechanisms that increase the number of proteins encoded by a single gene. These include alternative splicing, transcriptional initiation at alternative promoters, and genomic recombination (Ayoubi et al., 1996), These are besides the epigenetic events accountable for hereditary changes and

changes in gene expression. Epigenetics, which means "above" or "on top of" genes, refers to external chromatin alterations that turn genes "on" or "off." Although the modifications do not alter DNA sequence, they significantly affect the cells' gene expression and the transcriptional regulatory pathway (Mazzio et al., 2012).

Appreciable progress has been reported in recent history in the diagnosis, medicine and treatment of cardiovascular disease. The addition of epigenetics to the study of cardiovascular medicine has revealed a significant number of modifications that affect the overall development and progression of cardiovascular diseases (Castro et al., 2003). However, there have been no significant reports of epigenetic modifications of the genome in cardiovascular diseases, whereas in the case of cancer, histone deacetylase inhibitors, histone methylation inhibitors, and DNA methylation inhibitors have been successful (Shailesh et al., 2018).

Post-translational modifications (PTMs) play a vital role in the modulation of protein activity and function, half-life, interactions, and cellular localization. Since a single PTM can alter a protein's function, it stands to reason that various combinations of modifications can impart increasingly distinct functionalities to protein subsets (O'Malley et al., 2008; Nussinov et al., 2012). This concept is known as the "protein modification code".

The language of the PTM code is complex and involves cooperative, order-dependent, antagonistic, and mutually exclusive modifications (Nussinov et al., 2012). Combinatorial PTMs result from the activities of distinct signaling pathways and enable the modified protein to function as a signal integrator. PTMs in proteins are highly

dynamic processes that occur in response to a wide range of stimuli, thereby representing an adaptive response to the environment. They allow a cell to operate with a fewer number of genes and allow for rapid responses to physiological or pathological changes through the addition or removal of modifications.

As chemical modifications of proteins, PTMs are carried out by several enzymes during or after protein biosynthesis. Enzymes represent approximately 5% of the human proteome and are able to catalyze over 200 different types of PTMs (Jensen, 2006). Kinases, phosphatases, and methyltransferases are among the best-known enzymes that can add or remove functional groups to the side chains of amino acids.

Protein PTM is the covalent addition of chemical functional groups to protein-incorporated amino acids. Protein modifications vary in their permanence and may be present at low stoichiometry (Afjehi-Sadat et al., 2013). As of 2016, there are over 450 known types of PTMs in the universal protein database UniProt (Venne et al., 2014). The variability of PTMs' chemistry and physical properties is immense and includes differences in modification charge, polarity, hydrophobicity, volume, and shape (Nussinov et al., 2012). PTM may alter protein conformation and influence protein-substrate, protein-protein, or protein-nucleic acid interactions.

Protein methylation is one of the most frequently occurring PTMs. It is estimated that 1–2% of prokaryotic as well as eukaryotic genomes encode methyltransferases. The methylation of protein-incorporated amino acids at histidine, lysine, arginine, glutamine, asparagine, glutamate, D-aspartate/Lisoaspartate, cysteine, and both N-terminal and C-terminal residues (Afjehi-Sadat et al., 2013; Clarke, 2013) can regulate multiple cellular

processes, such as messenger RNA (mRNA) translation, splicing, and DNA damage. Furthermore, protein methylation acts as a central player in epigenetic regulation of gene expression, as the balance between histone methylation and demethylation regulates the availability of DNA for transcription (Cheung et al., 2005).

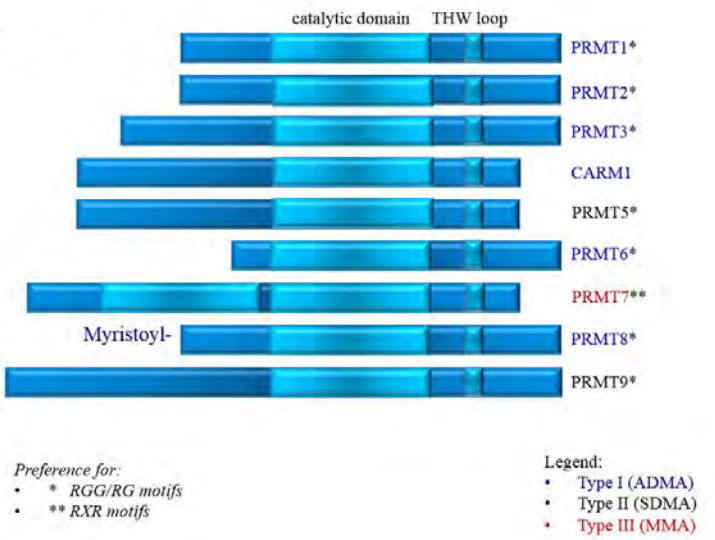
Protein methylation is carried out by specific enzymes called methyltransferases, which transfer the methyl group to the proteins, mainly from the primary donor group S-adenosylmethionine (SAM) to lysine or arginine residues. These two types of methylation are catalyzed by different types of enzymes: lysine methyltransferases and protein arginine methyltransferases, respectively. A single or multiple methyl groups can be added to target proteins (Clarke, 1993). Protein methylation eliminates potential hydrogen bond donors from the arginine side chain to nitrogen or oxygen of specific amino-acid side chains (N- and O-methylation respectively). Methylarginine-containing residues alter binding interfaces and have been shown to both impede and promote protein-substrate interactions, depending on the context (Boisvert et al., 2005; Arkov et al., 2006; Krause et al., 2007; Yang et al., 2013).

Nine PRMTs (PRMT1-9) have been identified in mammals. All of the PRMTs carry a highly conserved methyltransferase-catalytic domain, which is known to oligomerize into a ring-like structure (Weiss et al., 2000; Zhang et al., 2003), as well as a threonine-histidine-tryptophan (THW) loop, recognized to relate with the methyl-accepting substrate arginine and mediate substrate specificity (Jain et al., 2016) as shown in Figure 2.7 A. However, PRMTs differ regarding the cellular localization, presence of additional protein domains, and tissue expression. Despite the fact that PRMTs share the same catalytic domain, it has been demonstrated that there is no significant redundancy within

the activity of PRMTs, as mouse knockouts for different PRMTs generally result in different phenotypes (Bedford et al., 2009).

PRMTs are divided into three types. Type I PRMTs form ω-NG, NG-asymmetric dimethylarginine (ADMA), through the addition of two methyl groups to the same terminal nitrogen of arginine. This category contains PRMT1, PRMT3, PRMT4 (also known as CARM1), PRMT6, and PRMT8 (Figure 2.7B). Type II PRMTs mainly catalyze ω-NG, NG symmetric dimethylarginine (SDMA), through the addition of a second methyl group to the second nitrogen of arginine. Members of this category are PRMT5 and PRMT9 (Bedford, 2007). Finally, type III PRMTs enzymes catalyze the ω-NG-monomethyl arginine (MMA) reaction, and to date, only PRMT7 has been established as a member of this category (Atkinson et al., 1967; Bedford et al., 2009; Zurita-Lopez et al., 2012).

(A)



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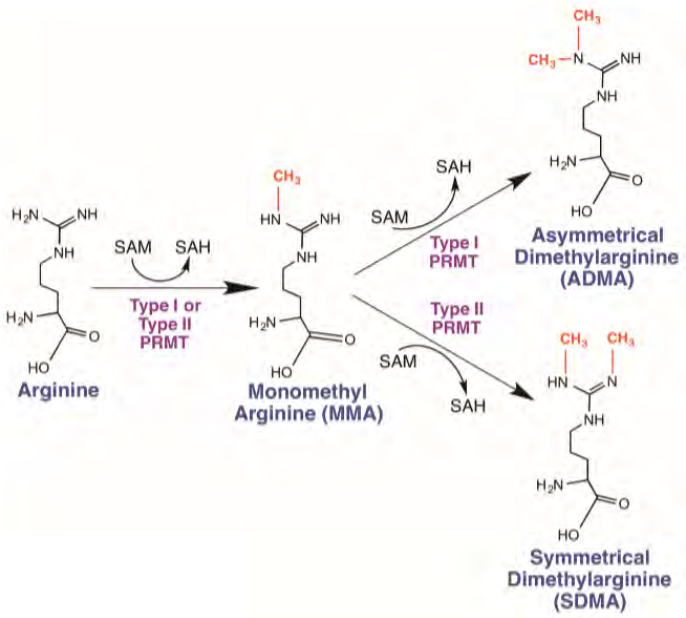


Figure 2.7 Members of the protein arginine methyltransferases family. (A) The nine members of the protein arginine methyltransferases family. (B) The three types of protein arginine methyltransferases and how they catalyze formation of MMA, ADMA, and SDMA. (Auclair et al., 2013)

2.3.1 Role of Protein Arginine Methyltransferase in Skeletal Muscle

Current research on of skeletal muscle PRMTs has mainly focused on PRMT1, PRMT4, PRMT5, and PRMT7. Relative to the other PRMTs, *PRMT4* mRNA is expressed to the highest degree in skeletal muscle, followed by *PRMT5* and *PRMT1* (Harris et al., 2014). Relative protein expression and enzyme activity levels between PRMTs have not yet been determined in skeletal muscle. PRMT1, PRMT4, and PRMT5 contribute to the regulation of muscle development. During myoblast fusion, PRMT1 exhibits the highest level of methyltransferase activity (Richard et al., 2005), while PRMT4 is required to express genes necessary for the later stages of skeletal muscle differentiation (Boisvert et al., 2004; Schroder et al., 2004). Inhibition of *PRMT4* has been shown to abrogate expression of transcription factors essential for initiating differentiation, such as myogenin and myocyte enhancer factor-2C (*MEF2C*) (Boisvert et al., 2004; Hassa et al., 2008).

In vitro studies using muscle cells, as well as several other *in vitro* and *in vivo* studies on non-muscle tissues, have shown that PRMT1, PRMT4 and PRMT5 by way of their specific methyltransferase activities can cotrol expression of molecules important for muscle remodelling. For example, p53 activity is significantly altered when it is methylated by PRMT1, PRMT4, or PRMT5 (An et al., 2004; Scoumanne et al., 2009). RIP140 transcriptional corepressor activity is suppressed by PRMT1 methylation at any one of three specific arginine residues including R240, R650, or R948 (Mostaqul Huq et al., 2006). PRMT1 has been shown to potentiate PGC-1α coactivator activity by methylating arginine residues in the C-terminal Glu-rich E region of PGC-1α, which plays a vital role in the coactivator function of the enzyme (Teyssier et al., 2005).

PRMT5 is shown to methylate three arginine (Arg 333, Arg 335 and Arg 337) in p53, and accordingly adapt its DNA binding activity. This, in turn instigates a change in the p53- expression program. PRMT5 also inhibits E2F1activity via methylation of R111 and R113, this leads the getting bound by Tudor domain protein p100-TSN, suppressing its apoptotic activity and enhancing its growth stimulating roles (Arkov et al., 2006; Bedford et al., 2009; Tee et al., 2010; Yang et al., 2013; Shailesh et al., 2018). Collectively, these studies demonstrat that PRMT1, PRMT4, and PRMT5 can affect muscle remodelling via their methyltransferase activities as shown in Figure 2.8.

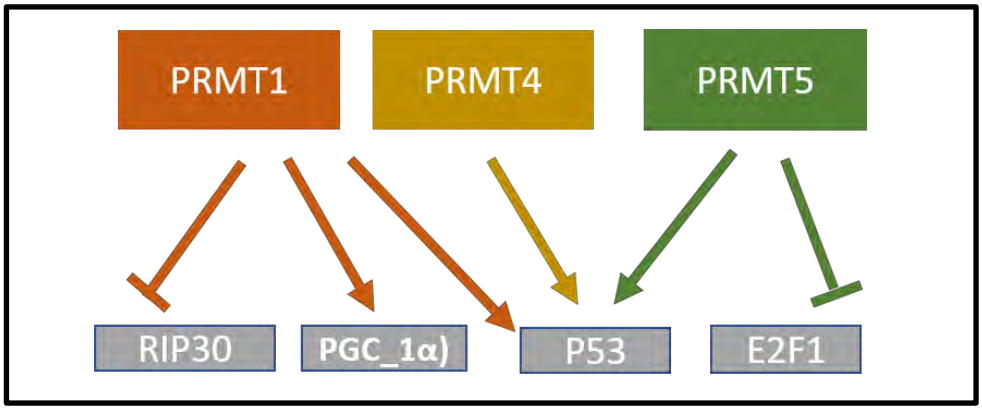


Figure 2.8. Putative PRMT targets

PRMT5 is required for early gene expression, suggesting that distinct PRMTs are preferentially active at different times throughout myogenesis (Schroder et al., 2004; Hassa et al., 2008; Karkhanis et al., 2011). PRMT5 has also been reported to regulate myogenin, *MEF2C*, *MYOD*, and myogenic regulatory factor 5 (*MYF5*) expression (Marx et al., 2000; Dacwag et al., 2007). In addition, PRMT4 and PRMT5 can be employed to the promoter of the myogenin gene to enhance its transcriptional activation during cell differentiation (Dacwag et al., 2007; Wei et al., 2013). Notably, these studies describing the expression and function of PRMTs in muscle development were all performed *in vitro*, utilizing rodent muscle cell culture models. Recent investigations demonstrate that PRMT4 and PRMT5 are also crucial for muscle regeneration *in vivo* (Batut et al., 2011). Following an injury to skeletal muscle, PRMT4 methylates *PAX7* and is required for the induction of *MYF5* transcription during regeneration (Baltimore, 2011). Zhang and colleagues showed that PRMT5 is required for proliferation and differentiation of muscle stem cell (MuSC) during recovery from cardiotoxin induced muscle damage (Zhang et al., 2015). Taken together, these studies highlight the potential role of PRMT1, PRMT4, and PRMT5 in the regulation of skeletal muscle plasticity.

2.3.2 Protein Arginine Methyltransferase 5 (PRMT5)

According to Shaileshet al. (2018), PRMT5 is essential in cellular processes at various cell cycle stages in a tissue-specific manner as illustrated in Figure 2.9. Apart from methylating histones, PRMT5 can also methylate other important transcription factors (Table 2.1), which highlights its key role in cellular regulation. (Shailesh et al., 2018).

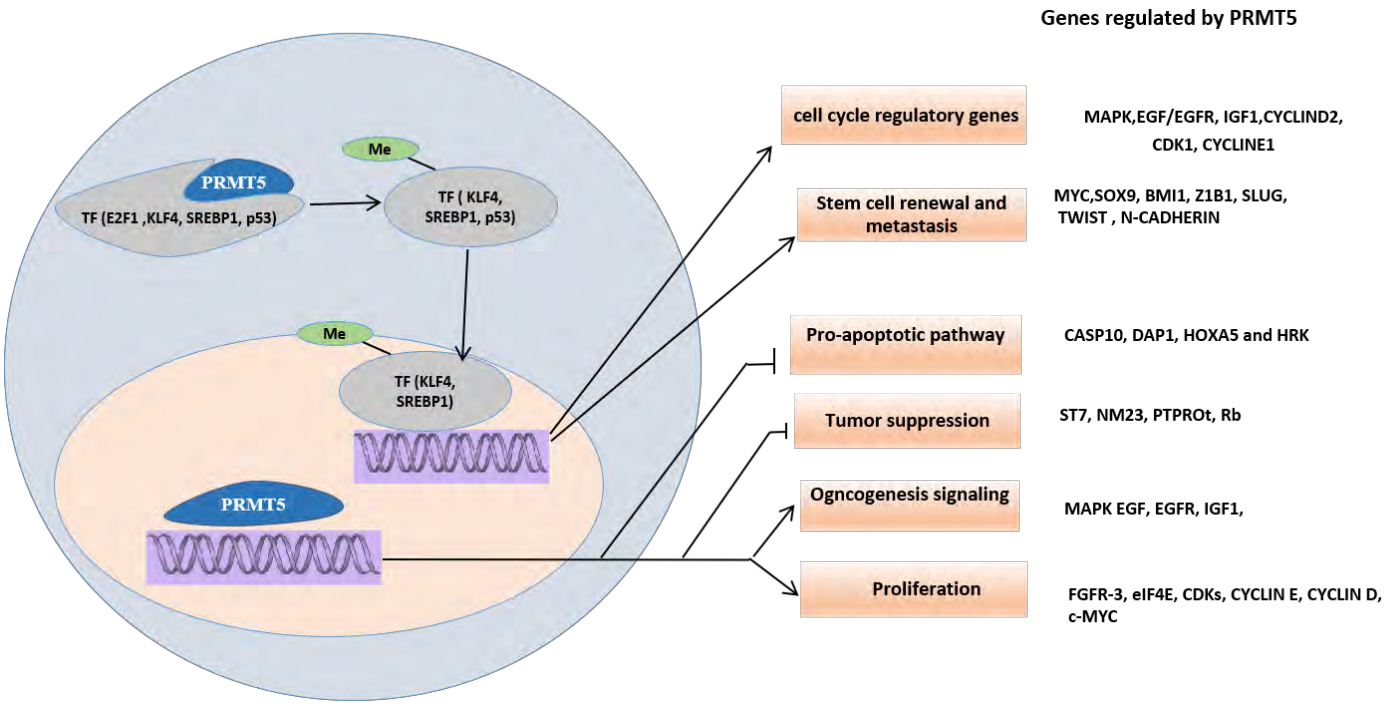


Figure 2.9 Schematic model of PRMT5-regulated cellular processes. Cytosolic PRMT5 induces arginine methylation of various transcription factors that translocate into the nucleus and regulate expression of their respective target genes. Nuclear PRMT5 is also directly recruited to the promoter regions of specific target genes to enhance cellular proliferation and oncogenesis (Shailesh et al., 2018)

PRMT5 is the predominant type II methyltransferase producing MMA and SDMA in mammals (Pal et al., 2007). PRMT5 was first identified in humans via a two-hybrid yeast screen for Janus kinase binding protein, and was named Jak2 binding protein 1 (JBP1) (Pollack et al., 1999). According to Tee and his team, PRMT5 is ubiquitously expressed in mammalian cells. It also allows the early detection and proper functioning of embryonic systems. It is an essential enzyme, since *PRMT5* null mice (*PRMT5* -/-) display early lethality between 3.5 and 6.5 embryonic days (Tee et al., 2010).

Table 2.1: PRMT5 methylated proteins (Shailesh et al., 2018)

PRMT5 substrate	Biological function	Reference
Histones (H3R8, H4R3, H3R2), SPT5, FCP1, MBD2, KAP1, N-MYC PAX3, RNA polymerase II	Regulation of transcription	(Kwak et al., 2003; Pal et al., 2004; Amente et al., 2005; Tan et al., 2006; Pesiridis et al., 2009; Tsai et al., 2013; di Caprio et al., 2015; Park et al., 2015; Wu et al., 2015; Zhao et al., 2016)
SmD3, CF 1(m)68	Post transcriptional regulation	(Pesiridis et al., 2009; Martin et al., 2010)
FGF-2, RPS10, G3BP1	Regulation of Translation	(Bruns et al., 2009; Ren et al., 2010; Tsai et al., 2016)
p53, ASK1, PDCD4, CRN5,	Regulation of apoptosis	(Jansson et al., 2008; Fay et al., 2014; Rastetter et al., 2015; Chen et al., 2016)
EGFR, RAF, E2F-1, FEN1, Androgen receptor, srGAP2	Regulation of cell proliferation, migration, differentiation and survival	(Guo et al., 2010; Guo et al., 2010; Andreu-Perez et al., 2011; Hsu et al., 2011; Zheng et al., 2013; Mounir et al., 2016)
NF-κB p65, HOXA9	Immune response regulation	(Harris et al., 2016)
MBP, GM130	Cellular integrity	(Branscombe et al., 2001; Zhou et al., 2010; Bandyopadhyay et al., 2012)
Piwi proteins, Rad9	Genome stability	(Vagin et al., 2009; He et al., 2011)
SHP, TDH, SREBP1	Metabolic regulation	(Kanamaluru et al., 2011; Han et al., 2013; Liu et al., 2016)

Much of the current literature on PRMT5 describes PRMT5 as a factor that negatively regulates transcription through histones methylation, which affect transcriptional outcome by influencing higher-order chromatin structure, chromatin accessibility, and TF recruitment (Lee et al., 2005; Krause et al., 2007). Indeed, the general assumption that PRMT5 functions as a transcriptional repressor is not cut and dry, as there are examples where it functions as a coactivator.

PRMT5 is capable of symmetrically methylating various residues on histones H2A, H3, and H4 histones *in vivo*, generating the repressive marks H3(Me_2)R8, and H4(Me_2)R3, and activation marks H2A(Me_2)R3, and H3(Me_2)R2. (Pal et al., 2003; Pal et al., 2004; Migliori et al., 2012; Karkhanis et al., 2016).

PRMT5 methylation marks at H3(Me_2)R2 and H3(Me_2)R8 have been associated with transcriptional repression as well as activation (Bedford et al., 2009; Migliori et al., 2012). For example, symmetric methylation of H3R2 by PRMT5 upregulates expression of the *forkhead box protein 1* (*FOXP1*) transcription factor, which is known to be associated with normal and cancer stem cell function (Chiang et al., 2017). Moreover, H3(Me_2)R8 found at the myogenin promoter is dimethylated in muscle cells, this methylation facilitates the formation of a transcription complex involving the Brg1 ATPase and the myogenic transcription factor, MyoD (Dacwag et al., 2007).

PRMT5 selectively methylates cytosolic histone H2A(Me_2)R3 to repress differentiation genes in ES cells, but not H4R3(Tee et al., 2010). The distinction between roles for H2AR3 and H4R3 methylation by PRMT5 suggests that each histone tail and targeted arginine has a unique function (Bandyopadhyay et al., 2012).

PRMT5 forms two dimers in a head-to-tail arrangement that is typical to all PRMTs. Directly binding to MEP50, PRMT5 greatly enhances its histone methyltransferase ability, primarily through an increased affinity for protein substrate. The PRMT5–MEP50, or methylosome complex, has a higher level of methyltransferase activity compared to PRMT5 alone (Antonysamy et al., 2012). The methylosome is formed as a hetero-octameric complex composed of four PRMT5 proteins and four MEP50 proteins (Ho et al., 2013).

The activity and localization of PRMT5 are regulated in multiple ways, including the association with different binding partners. PRMT5 binds to pICln or the Rio domain-containing protein (RioK1) in a mutually exclusive manner in order to modulate choice of substrate. Recruitment of RioK1 by PRMT5 favors methylation of the spliceosomal machinery components (Guderian et al., 2011). Another interactor is a Coordinator of PRMT5 (COPR5), which is associated with histones in the nucleus and recruits PRMT5 to nucleosomes (Lacroix et al., 2008). COPR5 acts as a chromatin adaptor for PRMT5 and promotes histone H4R3 methylation at specific loci, including *CCNE1*, a crucial regulator of cell proliferation (Lacroix et al., 2008). These examples allow us to conclude that interacting partners of PRMT5 are very important in regulating its activity.

In conclusion, the epigenetic factor PRMT5 is believed to play vital roles in a wide range of cellular processes, such as transcription, control of cell cycle, and cell differentiation. However, the mechanisms by which PRMT5 has its epigenetic effect on heart development are not fully explained.

CHAPTER 3: MATERIALS AND METHODS

3.1 Optimal zebrafish care/ husbandry

Zebrafish were kept in re-circulating stand-alone aquarium racks from AQUA NEERING ZD560 (San Diego, California, USA) in the Biomedical Research Center at Qatar University. Room temperature was kept at 26°C and the water temperature around 28°C. The room was on a diurnal light schedule, allowing 14 h of light (7:30 am to 9:30 pm) and 10 h of darkness (9:30 pm to 7:30 am). To ensure high quility embroys, adult wild-type zebrafish of the AB strain were raised and kept under standard laboratory conditions as described in (Westerfield, 2000).

AB is a standard (wild type) line that is very well characterized and commonly used in research. while the zebrafish were bred in aquaria breeding tanks filled with saline reconstituted (Instant Ocean) RO water (60 mg/liter), normal habitat was achieved by controlling the circulated water and temperatures. The tanks were bleached, scrubbed, and rinsed very thoroughly between breeding to prevent any contamination, growth, or infection in the tanks. To enhance maximal egg production, the fish were fed two times daily, in the morning and afternoon with brine shrimp (hatched from frozen cysts BS90 purchased from Brineshrimpdirect). All food was prepared fresh and kept at 4°C when not in use. The night prior to injection, male and female zebrafish were separated via a mesh barrier in a mating basket, the next morning (as the lights turned on), the barrier was removed, and the zebrafish were left to mate in shallow water undisturbed for ~ 20 mins. The embryos were collected in Embryo medium (E3M), freshly prepared E3M according to Westerfield (Westerfield, 2000) was used to maintain the zebrafish

embryos (ZFEs). All ZFEs were staged and fixed at specific hour post fertilization as described by (Kimmel et al., 1995).

In order to block PRMT5 enzyme expression successfully, the knockdown morpholinos (MO) injection must be performed at 1-4-cell stage (approximately 1-2 hpf). This means that the embryos must be collected immediately after breeding to have adequate time to inject a sufficient quantity of embryos for the experiment. Initially, the embryos harvested from the breeding tank were examined, and opaque embryos (unviable) were separated from the clear (viable) embryos and discarded.

3.2 Morpholino design for PRMT5.

Morpholino (MO) is frequently used to determine a phenotype or probe gene function by identifying the role played by various gene products through a loss of function testing. MO have the advantage of biological stability over DNA oligos because MO is not susceptible to enzymatic degradation. For optimal effectiveness, MOs are injected into embryos at the 1-4 cell stage. The length of knockdown effects are variable, and the MO is believed to eventually lose its effect due to dilution (Heasman, 2002).

The MO itself typically consists of short-chain (around 25 nucleotides**).** MO is capable of knocking down gene expression by splice blocking or translation blocking. The different mechanism of action for the two types of MO means that a different method of testing is required to verify the efficacy of the knockdown. Translation blocking MOs bind to targeted RNA and block translation of the mRNA at the translation start site. The MOs do not directly degrade their target mRNA; instead, they bind to it and inhibit its translation until it can be naturally degraded, in this case, western blotting is the most

reliable method to validate silencing as the protein of interest should not be expressed even though the messenger RNA is present. The splice-blocking MO inhibit the splicing of the primary transcript to the mature mRNA for the gene, so RT-qPCR would be the most reliable test (Gene Tool manufacturer's protocol).

MO sequences can be adapted from the literature. MO-modified antisense oligonucleotides sequences for the zebrafish *PRMT5* gene were previously published (Batut et al., 2011). MO sequences that were used in this study were summarized below in Table 3.1. All of these MOs were purchased from Gene Tools, Philomath, OR, USA.

Table 3.1: List of morpholino oligonucleotides

Genotyping	Company	Nucleotide position	Sequence (5'-->3')
PRMT5	Gene tools	3-27	5′-GACGCCATCGTTAGGAGACGAGATG-3′
HAND2	Gene tools	483 – 507	5'-CCTCCAACTAAACTCATGGCGACAG-3'
Control (scrambled)	Gene tools	-------	5′-CCTCTTACCTCAGTTACAATTTATA-3′

Alternatively, these oligos can be designed online by Gene-tools. Gene-tools offers a free and fast online design service, which can be accessed through the following link: https://oligodesign.gene-tools.com/. By providing information about the genes of interest, such as sequences or accession numbers, a custom MO can readily be designed. The following specific steps summarize how I designed MO against *PRMT5* in zebrafish:

1. First, I searched for details of the gene of interest from the NCBI database. For *PRMT5*, in zebrafish: https://www.ncbi.nlm.nih.gov/gene/368664

2. GenBank mRNA transcript ID from NCBI https://www.ncbi.nlm.nih.gov/nuccore/NM_001007183.2

3. mRNA sequence was found at the following link: https://www.ncbi.nlm.nih.gov/nuccore/NM_001007183.2?report=fasta

4. As a negative control, either a mismatched or scrambled MO (similar sequences with random base pair changes) can be injected. This would help attest to the specificity of the phenotype(s) observed with specific MO injections and help minimize the risk that the observed effects are due to an artefact of the injection procedure. MO sequences that were used in this study were summarized in Table 3.1.

3.3 Preparation of morpholino injection

3.3.1 Stock preparation

Upon ordering, MOs are delivered in a vial typical package size: 300 nanomoles (about 2.5 mg or 75 OD units for 25-mer). The best solvent for MO stocks is ddH_2O. Diethyl pyrocarbonate (DEPC) can damage MOs and may be toxic to embryos. Hence, DEPC-free ddH_2O needs to be used. While Gene Tools recommends 1 mM stock solutions (approximately 8 ng/nl), this can be too low, especially if the morpholino requires a high dose, or is being mixed with other MOs. Therefore, it is ideal to make various concentrations of MO stock solutions (2 or 3 mM). Once resuspended, Gene Tools

recommends keeping MOs at room temperature in airtight containers to prevent evaporation or to keep them frozen at -20°C.

The MO stock solutions were diluted with RO using the Gene Tools guideline as shown in Table 3.2. It was reconstituted by adding 0.15 ml of sterilized RO water to the 300 (2.5 mg) nanomole lyophilized oligonucleotide to produce a 2.0 mM solution. Because there is lack of information in literature about the adverse effect of *PMRT5* and *HAND2* MO concentration on embryos survival (Batut et al., 2011), different concentrations from these MOs were prepared to achieve the optimal injectable concentration that cause minimum mortality and deformity. Therefore, at the time of injection, these MOs were diluted to a final concentration either of 3.2 ng/nl, 6.4 ng/ nl, or 8.0 ng/nl. The diluent contains 0.5 % phenol red (Sigma P4633, UK) in E3M solution. The 60X E3M stock solution recipe consists of 5.0 mM NaCl, 0.17 mM KCl, 0.16 mM $MgSO_4$-$7H_2O$, 0.4 mM $CaCl_2$-$2H_2O$ in 1 L MilliQ water ddH_2O with a final pH of 7.6. No details of the impact of this MO on zebrafish embryos other than the specific concentration used were reported (Batut et al., 2011), so I had to optimize the dosage in order to determine the effects of the morpholino. The 0.4 mM (3.2 ng/ nl) concentration was prepared 10 µl at a time using 2 µl of stock *PRMT5* MO, 2 µl of phenol red, and 6 µl of E3M solution. The 0.8 mM (6.4 ng/ nl) concentration was prepared 10 µl at a time using 3 µl of stock *PRMT5* MO, 2 µl of phenol red, and 5 µl of E3M solution. The 1 mM (8 ng/ nl concentration was prepared 10 µl at a time using 4 µl of stock *PRMT5* MO, 2 µl of phenol red, and 4 µl of E3M solution. The 0.4 mM (3.2 ng/ nl) concentration was prepared 10 µl at a time using 2 µl of stock *HAND2* MO, 2 µl of phenol red, and 6 µl of E3M solution. The injection solution was stored at room temperature and prior to

use it was warmed in a thermal cycler (Eppendorf Mastercycler) to 65°C and vortexed to ensure that any precipitated MO was re-dissolved.

Table 3.2 Gene tools recommended dilutions for Morpholino Oligonucleotides.

The concentration of lyophilized oligonucleotide	**Sterilized RO water ddH_2O**	**Concentration of stock solution**
300 nanomoles	300 µl	1.0 mM
300 nanomoles	150 µl	2.0 mM
300 nanomoles	75 µl	4.0 mM
100 nanomoles	100 µl	1.0 mM

3.3.2 Preparation for Injection

3.3.2.1 Pulling needle using Narishige PC-100 puller

The microinjection procedure was done using a Picoliter Injector (Harvard Apparatus cat. # PLI-90A), and a Narishige micromanipulator (MN151). The needles used in the microinjector were pulled on Narishige PC-100 Micropipette Puller using 1.0 mm glass capillaries with filament (World Precision Instruments, Sarasota, FL: cat. no TW100F-4). The needles were pulled using the following settings: Pull=70, Heat=68, Time=200, and Velocity=80**.** After the needles were pulled, a scalpel (Baxter SP surgical blade) was used to cut the tip and then the needle was sharpened by Sutter Instruments BV10 capillary beveller (Sutter Instruments Product cat. # BV10).

3.3..2.2 Calibrating the needle to estimate the amount of injection.

Next, the needle was loaded into the microinjector; the needle was filled to the tip with mineral oil (Fisher Scientific cat. # 0121-1, USA) that drives the microinjector to prevent any bubbles in the system that would negatively impact the microinjector performance. Briefly, morpholinos were mixed with mRNA/morpholino solution before loading it into the needle**,** so that particles that could clog the needle are fully dissolved. Then 5 µl of the appropriate injection solution was pipetted onto a sample slide, and negative pressure was used in the microinjection apparatus to draw the solution into the tip of the needle.

3.3..2.3 Picoliter Injector setup protocol

Harvard Apparatus PLI-90A picolitre injector (Harvard Apparatus cat. # PLI-90A) was used for zebrafish injections. The injector was adjusted using the following specifications:

P-balance: Must be 0. It should be slightly negative to prevent yolk flow back into the needle, which would result in dilution of the MO solution in the needle. Conversely, if the back pressure is too high, the MO will constantly flow out of the needle even without exerting any pressure on the pedal, resulting in variability and inconsistencies in the dose injected and the phenotypes observed.

P-inject: Pressure should be between 20 and 25 psi. This can be toggled to give the right injection volume. The injection pressure can vary from 10 to 30 psi.

Injection time: It should be adjusted to ~300 milliseconds. In order to get the optimal injection volume, the time of injection needs to be adjusted first.

3.4 Sequential steps of MO injection

In preparation for the test embryos to be stored in the incubator, two Petri dishes of N-phenylthiourea (1x PTU which is E3M+PTU) water were placed in the incubator shortly before the embryos were collected from the zebrafish. PTU water is used to suppress pigmentation to make embryos transparent and facilitates taking time-lapse videos. To streamline the microinjection technique, the embryos to be injected were aligned in a single column in the 2% agarose injection chamber with the vegetal pole facing towards the needle tip. Using a transfer pipette, I transferred the collected embryos to the furrows. All excess PTU water around embryos was removed to prevent from floating of embryos (Figure 3.1B). 2% agarose injection chamber was prepared as follows: 0.6 g agarose in 30 ml E3 egg water. The agarose was poured into a petri dish, an injection mold (Adaptive Science Tools (cat. # TU-1) was placed on the agarose. Once cooled, furrows were formed for placing and stabilizing embryos Figure 3.1A.

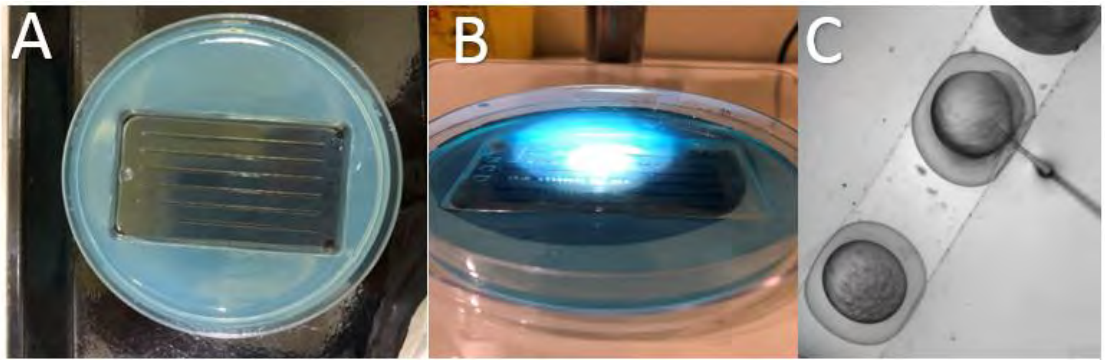


Figure 3.1 Steps for injection of MO solution into the yolk. (A) Injection chamber. (B) Using a transfer pipette, transfer the collected embryos to the furrows. (C) Insert the needle through the chorion, inject near the cell/yolk boundary of the embryo.

3.5 Injection of MO solution into the yolk

The microinjection procedure was performed on embryos approximately 60-90 mins post fertilizeation. The MO solution was injected near the cell/yolk boundary of the embryos at the 1-4 cell stage (1-2 hour post-fertilization) (Figure 3.1C) to ensure the distribution of the MO evenly to daughter cells. For the injection procedure, the tip of the needle was gently manipulated into the vegetal pole of the embryo using the joystick attached to the micromanipulator, and approximately 1 nl of injection solution was dispensed. To ensure that each embryo was receiving the correct amount of MO, the microinjector handle was turned 1 unit of the smallest labelled measure, this volume of the injected bead was visually estimated to be $1/5^{th}$ of the embryo volume (Rosen et al., 2009). The needle was withdrawn slowly from the embryo and care was taken to do as little damage to the embryo as possible. Next, subsequent eggs were injected in the same manner until 100 embryo embryos were injected. This resulted in 0.4 mM (3.2 ng/ nl), 0.8 mM (6.4 ng/ µl) and 1 mM (8 ng/ µl) of MO being injected into the embryo. The injected embryos were then rinsed off of the 2% injection chamber with PTU water. A Petri dish of PTU water was placed in the incubator at 28°C til 3-days post fertilization (dpf), with daily replenishment of PTU water. Phenotypic assessment was done using a Zeiss Stereo Discovery V8 Microscope.

The used needle was discarded, and a clean needle was inserted into the pipette holder following the same loading procedure used before. The needle was again filled at the tip with mineral oil, and the control injection solution was drawn into the needle. The same procedure was followed with the control scrambled MO injections into another set of embryos, and they too were placed in the incubator, in a separate labelled Petri

dish. For each experiment, uninjected embryos (collected from the same breeding group) were kept as a control to be monitored at the same time points as the injected embryos for comparison. Finally, an injection of 250 pg of human *PRMT5* mRNA, which was prepared as mentioned in section 3.7 using an *in vitro* T7 driven transcription kit, was performed to confirm the rescue of phenotype induced by *PRMT5* knockdown. The success of PRMT5 knockdown was assessed using Western blotting.

3.6 Embryo monitoring after injection

The embryos were examined under a Zeiss stereo discovery V8 Microscope equipped with Hamamatsu Orca Flash high-speed camera and a workstation equipped with HCImage software for opacity developmental endpoints at 24, 48, 72 hpf. Necrotic embryos were removed after each checkpoint to prevent contamination of the remaining live embryos. If any developmental abnormalities were present, the defective embryos were photographed along with the control scrambled MO injected embryos which were injected, at the same time.

3.6.1 Morphological assessment

The effects of injection with different concentrations of *PRMT5* MO on ZFE morphology were assessed at multiple time points, using a Zeiss SteREO Discovery V8 microscope to monitor developmental stages, mortality, hatching, spontaneous movement, response to touch, presence of deformities and heart rate.

3.6.2 Mortality and hatching percentage

The mortality percentage was determined by counting the number of dead ZFEs per group at 24, 48, and 72 hpf divided by the total number of injected embryos X 100.

Dead embryos were removed at each monitoring time point. Assessment of potential neuro/muscular defect at 24-hpf by tail flicking assay (burst/min) was measured by Danio Scope software. Hatching percentage was determined by counting the number of hatched ZFEs per group at 48 and 72 hpf divided by the total number of injected embryos X 100.

3.6.3 Assessment of cardiac function:

3.6.3.1 Live imaging of zebrafish

Zebrafish heart composed of two chambers, one ventricle and one atrium (Shin et al., 2010; Yalcin et al., 2017). Several heart function/hemodynamics parameters can be measured, these parameters include cardiac output (CO), blood velocity, vessel diameter, and heartbeat (Yalcin et al., 2017; Yalcin, 2018; Zakaria et al., 2018).

3.6.3.2 Mounting and imaging zebrafish embryos

A drop of 3% methylcellulose solution (room temperature) was put in a concave well imaging slide. Embryos was Positioned in the well using a suitable plastic dropper Figure 3.2 A, B (overfilling the well may cause fish displacement out of the well). The 3% methylcellulose drop was gently mixed with the E3M to stabilize the hatched embryo. For the preparation of 3% methylcellulose solution, 3 g of methylcellulose powder was dissolved in 100 mL PBS, or other mounting media, in a flask. A stirring magnet was placed in the flask containing the mixture and the flask was placed on a magnetic stirring plate. The speed was set to “low” and kept it at 4^{o}C for approximately one day to dissolve all the clumps. Once the methylcellulose was completely dissolved, it was aliquoted into small tubes and stored at −20°C.

The embryo should be positioned on its left, with its right side facing up and anterior point to the left. This would facilitate unambiguous imaging for the ventricle Figure. 3.2 C. Under the microscope, zoom-in on the embryo heart with 100X magnification and recording was started for approximately 5 second. Ventricle boarders should be positioned inside the imaging window. Figure 3.2 D. The useful formats for the movie were either AVI movie format or TIFF (or JPEG) image sequenced format.

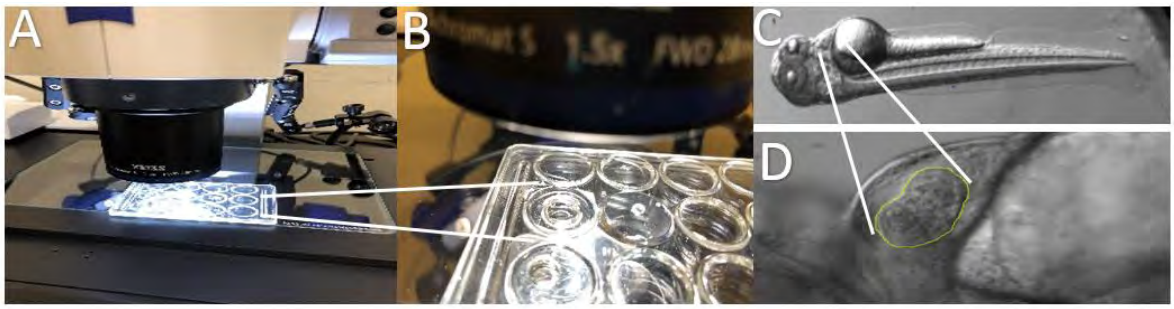


Figure 3.2 Mounting and imaging of zebrafish embryos. (A) The concave well imaging slide with zebrafish embryo under the microscope. (B) Magnified view of the well. Fill the wells ¾ of volume with E3M medium. (C) The positioning of the embryo should be on its left side. (D) Ventricle can be seen clearly in this configuration (zoomed image on left, ventricle borders are highlighted). Anterior is to the left.

3.6.3 Analysis of the blood flow in major blood vessels

There are two major blood vessels in zebrafish: the dorsal aorta (DA) and the posterior cardinal vein (PCV). Blood flow in these vessels can be easily imaged from the trunk of the embryos. By tracking red blood cells (RBC) movements in the DA and PCV, it

is possible to measure the heartbeat, as well as the average and peak flow velocities in these vessels. The same region in the DA and PCV was localized to measure the flow velocity, arterial pulse and vessel diameter as shown in Figure 3.3. There are multiple available algorithms from providers including Danioscope (Noldus Information Technology Inc, USA) which was used. In our experiments, we utilize MicroZebraLab blood flow from Viewpoint (version 3.4.4, Lyon, France).

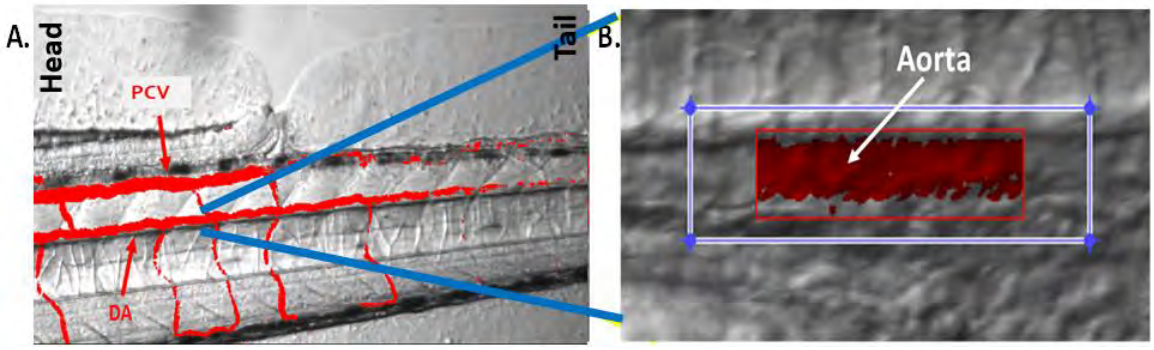


Figure 3.3 Blood flow analysis of zebrafish embryo. (A) Automated detection was initially performed to aid the localization of the arteries. (B) Automatic detection of blood vessels using ZebraLab. DA: dorsal aorta and PCV: Posterior Cardinal Vein.

At 72 hpf, 6 embryos from each experimental group were mounted using 3% methylcellulose and visualized using Zeiss SteREO Discovery V8 Microscope equipped with Hamamatsu Orca Flash high-speed camera and a workstation equipped with HCImage software. For each embryo, a 10-second bright field video of the beating

heart and the tail was recorded at 100 frames per second (fps) and 100 X magnification. This analysis was carried out for injected, and control scrambled MO injected embryos.

Blood velocity measurements were used to estimate the frictional shear stress levels in the cardiovascular system. Shear stress (τ, dynes/cm^2) was calculated using this formula $\tau = \frac{4\mu V_{mean}}{D}$, where μ is the blood viscosity (dynes/cm^2), V is the average blood velocity (μm/s), and D is the vessel diameter (μm). Cardiac output (CO, nl/min, also known as flow rate), was measured using this formula F = V · A, where V is the average blood velocity (μm/s) and D is the vessel diameter (μm) (Yalcin et al., 2017; Yalcin et al., 2018; Zakaria et al., 2018).

3.7 O-Dianisidine staining

O-dianisidine staining was performed to detect haemoglobin in RBCs of whole embryos as previously described (Paffett-Lugassy et al., 2005). The principle for this assay is that the heme in haemoglobin catalyzes oxidation of o-dianisidine in the presence of H_2O_2, producing a dark brown colour in haemoglobin-positive cells. Briefly, At 72 hpf, control scrambled MO, and PRMT5 knockdown embryos (10 embryos in each group) were dechorionated and anaesthetized with 0.02% Tricaine. Then, embryos were stained in the dark for 30 mins with 1 ml staining solution (containing 0.6 mg/ml o-dianisidine (Sigma), 11 mM sodium acetate (pH 4.5), 43% ethanol (v/v) and 0.65% hydrogen peroxide). Staining was stopped by rinsing embryos with 70 % ethanol. Embryos were fixed in 4 % formaldehyde in 1X PBS saline overnight at 4°C, and then embedded into 3.0% (w/v) methylcellulose for imaging. Images were taken using Zeiss Axiocam ERc 5s camera under bright field microscopy

(Stemi 508 Zeiss) at 50X. The intensity and the size of red-coloured areas in the yolk sac of each embryo were measured using ImageJ software. In Image J, the Polygon Selections tool was used to draw a polygon around the red area, A standard two-tailed *t-test* was used to calculate the p-value at a 95% confidence interval.

3.8 Behavior and locomotion assays

Embryos at 3 dpf from both groups, *PRMT5* knockdown and controls scrambled MO-injected were distributed into individual wells of a 96-well flat bottomed plate, 1 embryo per well in E3M, and allowed to acclimatize in the incubator for 1 h prior testing. The multi-well plate was placed in the Danio Track system chamber (Noldus Information Technology, NL, USA) set at 28°C and illuminated with white light for an adaptation period of 20 mins. The EthoVision XT 11.5 software was programmed depending on the protocol needed to record individual locomotor activity over 50 mins with alternating 10 mins dark, 10 mins light phases during the experiment. The arena settings and the detection settings are adjusted, so that optimal tracking is achieved. When the experiment was finished, the larvae were euthanised. Data were exported to Excel and graphed using GraphPad Prism8. The results were compared to controls scrambled MO-injected. Due to circadian rhythms, all locomotion assays were performed from 1:00 pm onwards to ensure steady activity of zebrafish embryos (MacPhail et al., 2009).

3.9 Protein extraction and PAGE (western blot analysis)

Western blot assay was used to verify the success of PRMT5 knockdown. Embryos were collected at specific time points (18, 24, 72 hpf) and dechorionated using Pronase enzyme (Sigma-Aldrich 10165921001, UK) (if the embryos were being tested before hatching). The embryos were then deyolked as previously established (Link et al., 2006) to efficiently remove the interfering major yolk protein, Vitellogenin which is a phospholipo-glycoprotein that functions as a nutritional source for the developing embryo. This method enabled high-resolution 2D gel electrophoresis and improved Western blotting considerably. Next, the embryos were transferred into 1.5 mL microcentrifuge tubes and washed twice using refrigerated E3M solution. Embryos were then collected by centrifugation at low speeds (<3000rpm) as needed, and the supernatant was discarded. To extract proteins from embryos, a solution of 10 ml Radioimmunoprecipitation (RIPA) buffer (Thermo Fisher Scientific 89900, USA) plus 1 tablet of Protease Inhibitor Cocktail (Thermo Fisher Scientific 88668, USA) was added to each tube and the embryos were dissociated with microcentrifuge polypropylene pellet pestles (Thermo Fisher Scientific 3411D62, USA). Samples were incubated on ice for 30 mins, before centrifugation for 15 mins at 13,000 g at 4°C. The supernatant was collected and transferred into a new 0.5 ml microcentrifuge tube that was stored at -80°C. Before loading protein on the gel, it is important to determine the protein concentration, the protein concentration was determined using Pierce Bicinchoninic acid assay (BCA) Protein Assay kit (Thermo Fisher Scientific 23227, USA). BCA was used to calculate the unknown protein concentration of the samples based on their ODs at 280 nm. A volume of Laemmli sample loading buffer (4x)

(Sigma-Aldrich 70607, UK**)** containing reducing agents including SDS, β-mercaptoethanol was added to the sample according to sample volume (1:3 loading buffer to sample ratio). The samples were then incubated at 95°C for 5 mins in a heat block to denature the protein. A 20 µg of each sample and 1 well of protein ladder (SMOBiO -PM2500) were loaded and run on a 10% sodium dodecyl sulphate-Poly acrylamide gel (SDS-PAGE) to separate the proteins. The 10% SDS gel (contained 2.5 mL 40% Acrylamide-Bisacrylamide, 2.5 mL of 1.5M Tris (pH 8.8), 0.1 mL of 10% SDS, 4.85 mL of dH_2O, 0.05 mL of 10% ammonium persulfate, and 0.01 mL of TEMED), was run at 70 V for 90 mins in 1x Running Buffer (composed of 25 mM Tris, 190 mM glycine (pH 8.3) and 20% SDS).

The gel was transferred to a container containing western transfer buffer (composed of 100 ml transfer buffer (10X) containing tris base 25 mM, glycine 192 mM + 200 ml MeOH + 700 ml H_2O) and agitated for approximately 5 mins to rinse the gel. Next, for protein transfer, a piece of 0.45 Polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific- 88518) was cut to the same dimensions as the gel. The membrane was then immersed into 100% methanol and then rinsed in Milli-Q water.

To transferring the gel onto the PVDF membrane, a sandwich was made by layering a filter paper, gel, membrane, then another filter paper. Bubbles were removed using a rolling with light pressure. The sandwich was then placed in western blot cassette. The cassette was placed into the western blotting transfer apparatus (Cleaver Scientific Blotting CVS10D Omni PAGE Mini Electrophoresis System). This device applied a 100V for approximately 3 h at 4°C.

Once the transfer was complete, The PVDF membrane was blocked with 15 mL of 5% nonfat dried milk in TBS-T. The membrane was incubated overnight at 4°C on a roller with 1:500 of the appropriate primary antibody (see Table 3.3). The next day, the membrane was washed 3 times in TBS-T then incubated with the appropriate secondary antibody for 1 h (see Table 3.3). The membrane was then washed 3 times in TBS-T and developed using an Electrochemiluminescence (ECL) kit (Abcam Biochemicals ab65623, UK). The membrane was then developed into a film in the darkroom using a developer. The image was examined, and depending on the intensity of the bands, the exposure was repeated as necessary. The Molecular weight of the protein band was estimated by comparing it to the Molecular weight of the closest protein ladder bands, which are of known size. The Western blot was scanned and saved as a tiff file, and the bands were quantify using ImageJ software.

Table 3.3: List of Antibodies.

Type	Target	Application	Source
Mouse monoclonal Anti-PRMT5	PRMT5	WB, ChIP	Thermofisher PRMT5-21
Rabbit poly clonal GAPDH	GAPDH	WB	abcam ab209856
Anti-H3R8me2s	H3R8me2s	WB, ChIP	Dr Said
Anti-H4R3me2s	H4R3me2s	WB, ChIP	Dr Said
Anti Mouse IgG	Secondary anti-mouse	WB	abnova PAB0096
Anti Rabbit IgG	Secondary anti-rabbit	WB	sigma A0545

3.10 *In vitro* transcription

In vitro transcription was used to generate human *PRMT5* mRNA, that was microinjected into embryos in PRMT5 phenotype rescue experiments. Plasmid pBS(KS+)/Fl-*PRMT5* which generated previously (Pal et al., 2003), was first linearized by EcoRI restriction enzyme (NEB, R0101S, USA) and purified using a DNA purification kit (AllPrep DNA/RNA Mini Kit [cat. no. 80204]). The purified linearized DNA was then used as a template for *in vitro* transcription according to the manuals of mMESSAGE mMACHINE T7/T3/SP6 Transcription Kit (Thermo Fisher, USA) For the generation of mRNA for injection.

3.11 Real time RT-qPCR:

Total RNA was isolated from whole ZFES *PRMT5* knockdown and control scrambled MO (20~30 each), using IBI DNA/RNA/Protein Extraction Kit (IBI Scientific -r IB47702, USA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using the SuperScript™ IV VILO™ Master Mix kit (Thermo Fisher Scientific 11756050, USA) according to the manufacturer's instructions. Quantitative analysis of specific mRNA expression was performed using TaqMan® Fast Advanced Master Mix (Applied Biosystems®, USA) and specific primers and probes that were designed (Applied Biosystems®, USA) against the genes of interest (Table 3.4); these include; *PRMT5*, atrial natriuretic peptide (*ANP/NPPA*), brain natriuretic peptide (*BNP/NPPB*), Myogenic factor 5 (*MYF5*), GATA Binding Protein 5 (*GATA5*), and Cell Division Cycle 73 (*CDC73*). The signal was read using RT-qPCR (QuantStudio™ 6 Flex RT-qPCR System). The relative quantity was calculated based on the $2^{-\Delta C_T}$ method (Rao et al., 2013), and the fold change was calculated in reference to the geomean of a group of housekeeping genes *B2M*,

RPL13A, *EF1α*.

Table 3.4 List of primers for RT-qPCR

Gene Name	Assay ID	Exon Boundary	Assay Location	Amplicon Length
PRMT5	Dr03422718_m1 Catalog # 4351372	4-5	436	66
MYF5	Dr03074150_m1 Catalog#: 4331182	203	564	108
CDC73	Dr03100174_m1 Catalog #: 4351372	2-3	245	65
GATA5	Dr03086725_m1 Catalog#: 4331182	2-3	895	83
ANP– ppa	APGZVJD Catalog#:4331348	2-3	240	63
BNP-nppb	APGZEPV Catalog#:4331348	2-3	230	63
B2M	Dr03432699_m1 Catalog #: 4351372	1-2	168	99
RPL13A	Dr03101114_g1 Catalog#: 4331182	2-3	200	58
ELF1	Dr03118707_m1 Catalog#: 4351372	6-7	937	62

3.12 Mass spectrometry

Protocol for tryptic digestion was used to do the mass spectrometry, the band of PRMT5 protein was cut from the SDS polyacrylamide gel immediately after running the gel using a blade. The gel slice was placed in a MS compatible Eppendorf tube and centrifuged in order to collect the gel piece to the bottom of the tube. Regardless of whether the gel was stained with glutaraldehyde-free silver, TCS, or CCS, it is necessary to destain the gel piece, as the stain will interfere with MS analysis. Approximately, 40 µl of destain solution (100 ml acetic acid, 450 ml methanol, 450 ml H_2O) was added and washed again 5 times. After this, 20 µl of 0.1 M NH_4CO_3 (Ammonium carbonate) which works as a buffering system was added and incubated 2-3 times for 10 mins each. The gel was then washed with 20 µl of 50% acetonitrile one time, then 4-5 times with 20 µl 100% acetonitrile to dehydrate the gel, till it becomes white like paper colour. The gel was air-dried for 15-20 mins, and enzymatic digestion was performed using trypsin solution 10 mg/ µl; (8 µl trypsin of 0.1 ug/ µl + 32 µl of 0.1 M ambic), it was added and incubated on ice for 5-15 mins. After that, it was incubated overnight at 37° C.

The next day, 0.1 % Trifluoroacetic acid (TFA) in water (to improve peak shape and retention times) was added to the sample for 1-3 h while shaking. Sample was eluted with 0.8 µl of acetonitrile containing alpha- cyano-4-hydroxycinnamic acid which serves as a matrix. The peptide then was loaded on ZipTip and run the MALDI-TOF-MS/MS.

3.13 Chromatin immunoprecipitation (ChIP) assay

Single-cell suspension from ZFES and larvae was obtained using 0.25% trypsin-EDTA + Collagenase 100 mg/ml following a well-established protocol (Bresciani et al., 2018). The single-cell suspension was cross-linked with 1% formaldehyde (from the 37% commercially available stock, Fisher) for 10 mins at RT in the shaker (slowly to mix). The cross-linking process was quenched with the addition of glycine diluted to a final concentration of 125 mM for 5 mins at RT on the shaker. Cells were transferred to a falcon tube and spun at 1000rpm for 4 mins to collect the cells. The cell pellet was washed twice with 10 ml of 1X PBS. Each sample was lyzed in 100 µl SDS lysis buffer (100 mM Tris-HCl [pH 8.6], 15 mM NaCl, 60 mM KCl, 1 mM $CaCl_2$, 3 mM $MgCl_2$, and protease inhibitors-aprotinin (Roche), 1 µM PMSF (Sigma), 1 µg/mL Pepstatin, 5 µg/mL Leupeptin.

To shear DNA to chromatin fragments that range in size from 250-500 bp (90-95%). 0.3 unit of MNase (stock is 1 U/µl) was added to each sample and incubated at 37°C for 20 mins. The reaction was stopped immediately by adding stop buffer (100 mM Tris-HCl [pH 8.6], 0.45% SDS, 2.55% Triton-X100, 5 mM EDTA [pH 8.0], and protease inhibitors as above). It was then spun in the cold room at 14000 rpm, for 10 mins. The supernatant was transferred to a new tube, snap frozen in liquid nitrogen, and stored at -80°C for ChIP assay. Approximately, 15 µl of the sample was analyzed by adding proteinase K (20 mg/ml) and 10% SDS and incubated at 37°C overnight. Next day the sample was analyzed on 1% agarose gel to confirm the size of chromatin fragments.

In the meantime, protein Sepharose A beads needed for immunoprecipitation were resuspend (500 PCV Beads) in 10 ml of 1X PBS (cold). The supernatant was removed,

and beads were washed again 5 times, Beads were then resuspended in an equal amount of 1X PBS (cold) so that a 50% slurry was formed. Beads were next blocked with 0.2 mg/ml sonicated single-stranded salmon sperm DNA (8 mg/ml) and with 0.5 mg/ml BSA (10mg/ml) at 4˚C on a rotor overnight.

Chromatin was diluted in immunoprecipitation buffer (IP buffer: 0.1% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, and adjusted to final volume of 500 ml dH_2O) in a ratio 1:10 and the resulting diluted chromatin fraction was incubated with the pre-cleaned/pre-blocked protein Sepharose A beads (Roche) for at least 2 h at 4˚C. Pre-cleaned chromatin fraction was afterwards incubated at 4°C on a rotor, with primary antibodies against PRMT5 mouse IgG was used as control. After 5h beads were added in every sample and were incubated on a rotor, at 4 °C overnight.

The following day, samples were centrifuged at 2,000 rpm for 3 mins at room temperature and washed with mixed micelle buffer (20 mM Tris [pH 8.1], 150 mM Nacl, 5 mM EDTA, 5% w/v Sucrose, 0.2% Triton X-100, and 0.2% SDS), then with buffer 250 (50 mM HEPES [pH 7.5], 0.1 % Na – DOC, 0.2 % Triton X-100, 250 mM NaCl, and NaCl EDTA), and washed with washing buffer (10 mM Tris [pH 8.0], 0.5 % Na – DOC, 0.25 % NP – 40, 150 mM LiCl, and 1 mM EDTA), finally washed with TE buffer [pH 7.6], following centrifugation at 2,000 rpm for 3 mins.

The immunoprecipitated beads/antibody/chromatin samples were then incubated twice for 10 mins in elution buffer each time (50 mM Tris [pH 8.0], 10 mM EDTA, 1% SDS) in a thermomixer at 65°C, centrifuged at 1,400 rpm for 2 mins. The supernatant was collected in new labelled tubes. Finally, samples were reversed-cross linked with the

addition of 5M NaCl and RNase (0.5 µg/µl). DNA purification was performed using Phenol: chloroform extraction and RT-PCR was performed using specific primers and probes that were designed (Applied Biosystems®) against the genes of interest; these include: brain natriuretic peptide (*BNP/NPPB*), Myogenic factor 5 (*MYF5*), GATA Binding Protein 5 (*GATA5*), retinoblastoma-like 2 (p130) *RBL2* as positive control.

Table 3.5 List of primers for ChIP assay RT-qPCR

Gene Name	Assay ID	Assay Location in promotor sequence
MYF5 Promoter	APDJZYF Catalog#:4331348	AATATCCTGTAGAGATCAAAAT
GATA5 Promoter	APGZHN7 Catalog#:4331348	ATTCACAGATCGTGTTTGCACTG
BNP-nppb Promoter	APEPVJD Catalog#:4331348	AAACCTTCACGTATCCTCCAGG
RBL2 promoter	APCE7TR Catalog #: 4331348	TGTTGGGTGCTTTTTATATATGC

3.14 Statistical analysis:

Statistical analysis was performed using Graphpad Prism 8 software. Distribution was determined using Kolmogorov–Smirnov normality test. Parametric data were analysed using one way-ANOVA with Sidak post-hoc test, two-way-ANOVA with Dunnett test and unpaired two-tailed *t*-test. While nonparametric data were analysed using the Kruskal-Wallis test with Dunn's post-hoc test. A p-value of less than 0.05 was considered statistically significant.

CHAPTER 4: RESULTS

4.1 PRMT5 expression fluctuates during development

Study of early embryonic development may reveal important insights into the transcriptional programs controlling key steps, and essential components required for this process. Differential expression of PRMT5 during developmental stages is crucial for muscle development, as previous studies have confirmed the cooperation between muscle regulatory factors (MRFs) including myogenin (Chen et al., 2002; Dacwag et al., 2007). Earlier reports on PRMT5 in mouse embryonic development revealed that high levels of PRMT5 protein in the cytoplasm of mouse embryonic stem (ES) cells are responsible for interaction with STAT3 and maintenance of embryonic stem cell pluripotency, via transcriptional repression of *FGF5*, *GATA6*, *LHX1*, *FOX2, HOXA3*, *HOXA7*, and *HOXD9* (Tee et al., 2010). Moreover, expression of both PRMT4 and PRMT5 are detected in the somites during early somitogenesis and enriched in the pre-somitic mesoderm (PSM) (Holley, 2007; Batut et al., 2011). Further, it has been suggested by multiple reports that PRMT4 as well as PRMT5 are essential for myogenesis (Chen et al., 2002; Dacwag et al., 2007; Dacwag et al., 2013).

To investigate the role of PRMT5 in heart development using zebrafish**,** relative expression of *PRMT5* mRNA during zebrafish embryogenesis was evaluated during developmental stages. Real time RT-PCR analysis revealed that *PRMT5* mRNA was detected at 1 hpf and underwent slight increase between 18-72 hpf (Figure 4.1 C). This was consistent with the PRMT5 protein expression profile evaluated using western blot at the same developmental stages. Our results also showed that PRMT5 was

dynamically expressed during the different developmental stages. We found that PRMT5 expression started early at 6 hours post-fertilization, and that its expression gradually increased until 18 hpf. After this, PRMT5 expression was reduced and kept steady between 24 hpf and 48 hpf. Remarkably, PRMT5 expression at 72 hpf was further reduced, and its mobility shifted to a lower level compared to other developmental stages. To gain further insight into the unique pattern of PRMT5 expression, we used mass spectrometry to analyze the two PRMT5 isoforms, which were detected at 24 and 72 hpf.

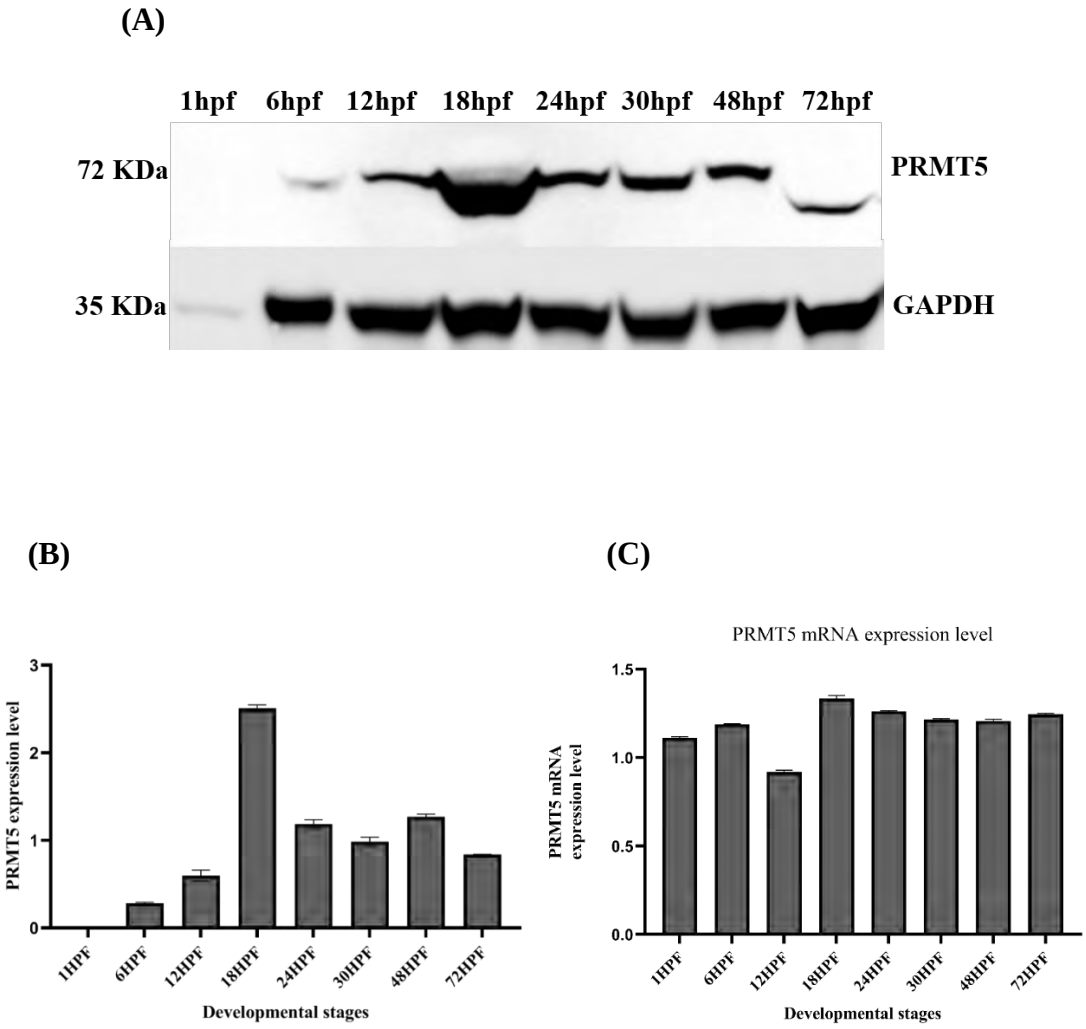


Figure 4.1 PRMT5 expression during zebrafish embryogenesis. (A) Western blot of wild type zebrafish showing bands of protein levels in different developmental stages from 1 hpf -72 hpf. (B) Quantification of Western blot bands intensity for PRMT5 protein expression during zebrafish developmental stages from 1-72 hpf, Analysis was done using Image J, n=3. (C) Relative expression of *PRMT5* mRNA during zebrafish embryogenesis. The relative quantity was calculated based on the $2^{-\Delta CT}$ method, and the fold change was calculated in reference to the Geomean of a group of housekeeping genes (B2M, RPL3, and EF1).

4.2. Expression of different PRMT5 isoforms during zebrafish embryonic development.

In order to understand the shift in size of PRMT5 protein band in western blotting, which was detected at 72 hpf, we carried out a mass spectrophotometry analysis. PRMT5 SDS-PAGE at 24hpf and 72hpf were excised out and trypsinized before they were analyzed using MALDI-TOF-MS/MS.

Using profound searching tool http://prowl.rockefeller.edu/prowl-cgi/profound.exe, we found that a unique peptide sequences appear at 24 hpf but not at 72 hpf (Table 4.3), the changes in PRMT5 protein sequence at 72 hpf was similar to isoform 4 of PRMT5 protein (identifier: O14744-4), Length:466, Mass (Da):53,580 (Figure 4.2B). Sequence alignment between PRMT5 isoform 1 that appears at 24 hpf and PRMT5 isoform 4 that appears at 72hpf (Figure 4.2C), showed that missing peptide sequences in PRMT5 isoform 4 were detected between 106-259 base pair which are the same fragment missing in PRMT5 protein at 72 hpf sample, as shown in the peptide mass map of this stage (Figure 4.2A). This finding indicates expression of different PRMT5 isoforms during zebrafish embryonic development due to alternative splicing.

(A)

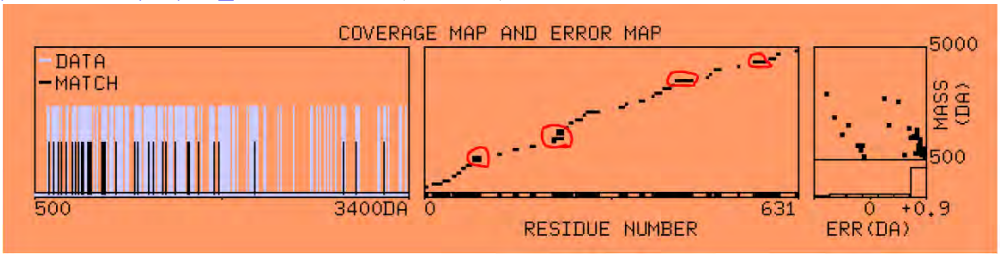
Protein Sample at 24 hpf

Measured peptides: 255

Matched peptides: 36

Min. sequence coverage: 58%

1. gi|118722330|ref|NP_001007184.2 (PRMT5) [Danio rerio]



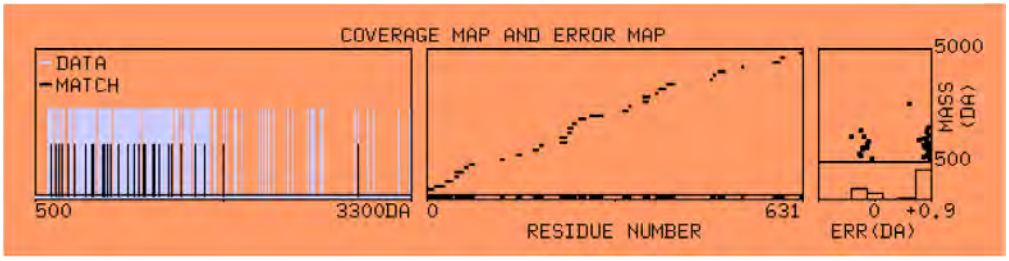
Protein Sample at 72 hpf

Measured peptides: 336

Matched peptides: 32

Min. sequence coverage: 47%

1. gi|118722330|ref|NP_001007184.2 (PRMT5) [Danio rerio]



(B)

PRMT5 Isoform 4 missing base pairs PRMT5 at 72 hpf missing base pairs

(C)

```
PRMT 5 isoform1    1   MAAMAVGGAGGSRVSSGRDLNCVPEIADTLGAVAKQGFDFLCMPVFHPRFKREFIQEPAK   60
PRMT 5 isoform4    1   -----MRG---PN--SG-------TEKGRLVIPEKQGFDFLCMPVFHPRFKREFIQEPAK   43
                           : *    .  **         . *    **************************

PRMT 5 isoform1   61   NRPGPQTRSDLLLSGRDWNTLIVGKLSPWIRPDSKVEKIRRNSEAAMLQELNFGAYLGLP  120
PRMT 5 isoform4   44   NRPGPQTRSDLLLSGRDWNTLIVGKLSPWIRPDSKVEKIRRNSEA--------------   88
                       ******************************************

PRMT 5 isoform1  121   AFLLPLNQEDNTNLARVLTNHIHTGHHSSMFWMRVPLVAPEDLRDDIIENAPTTHTEEYS  180
PRMT 5 isoform4   89   ------------------------------------------------------------   88

PRMT 5 isoform1  181   GEEKTWMWWHNFRTLCDYSKRIAVALEIGADLPSNHVIDRWLGEPIKAAILPTSIFLTNK  240
PRMT 5 isoform4   89   ------------------------------------------------------------   88

PRMT 5 isoform1  241   KGFPVLSKMHQRLIFRLLKLEVQFIITGTNHHSEKEFCSYLQYLEYLSQNRPPPNAYELF  300
PRMT 5 isoform4   89   -------------------LEVQFIITGTNHHSEKEFCSYLQYLEYLSQNRPPPNAYELF  129
                                          ****************************************

PRMT 5 isoform1  301   AKGYEDYLQSPLQPLMDNLESQTYEVFEKDPIKYSQYQQAIYKCLLDRVPEEEKDTNVQV  360
PRMT 5 isoform4  130   AKGYEDYLQSPLQPLMDNLESQTYEVFEKDPIKYSQYQQAIYKCLLDRVPEEEKDTNVQV  189
                       ************************************************************

PRMT 5 isoform1  361   LMVLGAGRGPLVNASLRAAKQADRRIKLYAVEKNPNAVVTLENWQFEEWGSQVTVVSSDM  420
PRMT 5 isoform4  190   LMVLGAGRGPLVNASLRAAKQADRRIKLYAVEKNPNAVVTLENWQFEEWGSQVTVVSSDM  249
                       ************************************************************

PRMT 5 isoform1  421   REWVAPEKADIIVSELLGSFADNELSPECLDGAQHFLKDDGVSIPGEYTSFLAPISSSKL  480
PRMT 5 isoform4  250   REWVAPEKADIIVSELLGSFADNELSPECLDGAQHFLKDDGVSIPGEYTSFLAPISSSKL  309
                       ************************************************************

PRMT 5 isoform1  481   YNEVRACREKDRDPEAQFEMPYVVRLHNFHQLSAPQPCFTFSHPNRDPMIDNNRYCTLEF  540
PRMT 5 isoform4  310   YNEVRACREKDRDPEAQFEMPYVVRLHNFHQLSAPQPCFTFSHPNRDPMIDNNRYCTLEF  369
                       ************************************************************

PRMT 5 isoform1  541   PVEVNTVLHGFAGYFETVLYQDITLSIRPETHSPGMFSWFPILFPIKQPITVREGQTICV  600
PRMT 5 isoform4  370   PVEVNTVLHGFAGYFETVLYQDITLSIRPETHSPGMFSWFPILFPIKQPITVREGQTICV  429
                       ************************************************************

PRMT 5 isoform1  601   RFWRCSNSKKVWYEWAVTAPVCSAIHNPTGRSYTIGL                        637
PRMT 5 isoform4  430   RFWRCSNSKKVWYEWAVTAPVCSAIHNPTGRSYTIGL                        466
                       ***********************************
```

Figure 4.2 Expression of different PRMT5 isoforms during zebrafish embryonic development. (A) Mass spectrophotometry analysis for protein Sample at 24 and 72 hpf. (B) Putative conserved domains of PRMT5 with Unique peptide sequence at 72 hpf compared to 24 hpf zebrafish developmental stages. (C) Sequence alignment of PRMT5 isoform 1 that appears at at 24 hpf and PRMT5 isoform 4 that appears at 72hpf.

Table 4.1 Unique peptide sequences of PRMT5 at 24 hpf **but not at** 72 hpf stage

Peptide sequences of PRMT5 isoform 1 at 24 hpf that are missing in isoform 4 at 72hpf	**Residues Position**	**Computed Mass**
1- LSPWIETDSELTTERR	82-97	1931.958
2- WLGEPIKAAILPTSIFLTNK	217-236	2211.266
3- HSEKDLR	268-274	883.451
4- EWTSPEK	418-424	875.402
5- ADIIVSELLGSFGDNELSPECLDGAQHFLK	425-454	3216.563
6- EVTLSIKPETHSPGMFSWFPILFPLK	556-581	3000.581
Total computed mass		**12960.69**

4.3 PRMT5 MO titration

Stable genetic approaches for genome engineering may provide an ideal experimental system; however, there are currently no transgenic zebrafish models involving cardiomyopathy related mutations or zebrafish PRMT5 gene knockout model. PRMT5 is an essential enzyme and *PRMT5* null mice (*PRMT5* -/-) display early lethality between 3.5 and 6.5 embryonic days (Tee et al., 2010). Therefore, to examine the involvement of PRMT5 in zebrafish embryonic development, we interfered with its translation using antisense morpholino oligonucleotides (MO), which were designed to target the 5' UTR of *PRMT5*. No data in the literature was found about the optimal concentration of MO required for PRMT5 silencing in zebrafish. Therefore, optimization of the MO dosage was necessary.

The MO optimization experiment showed that zebrafish injected with scrambled MO had similar survival percentage as compared to un-injected embryos. This finding rules out

any embryo lethality attributed to damage from the morpholino injection solution. However, the low survival percentage before 12 hpf could be attributed to the injection of unfertilized embryos.

The graph in Figure 4.3 illustrates the average percent of embryos surviving at 24, 48, and 72 hpf for both PRMT5- specific MO and control scrambled MO injected embryos. The 1 mM (8 ng/ µl) and 0.8 mM (6.4 ng/ µl) MO injected embryos showed a significant reduction in survival percentage compared to control scrambled MO injected embryos. This was observed across each measured time point where lethality or malformation was observed. Our results indicated that a high concentration of PRMT5 MO had a significantly lower survival percentage due to off-target effects and toxicity of MO. While 0.4 mM (3.2 ng/ nl) PRMT5 MO injected embryos did not show a significant reduction in survival percentage; however, some similar phenotypes in the experimental group were noted such as delayed in heart development, elongated tub-like structure heart and pericardial edema in comparison to control scrambled MO injected embryos (Figure 4.4).

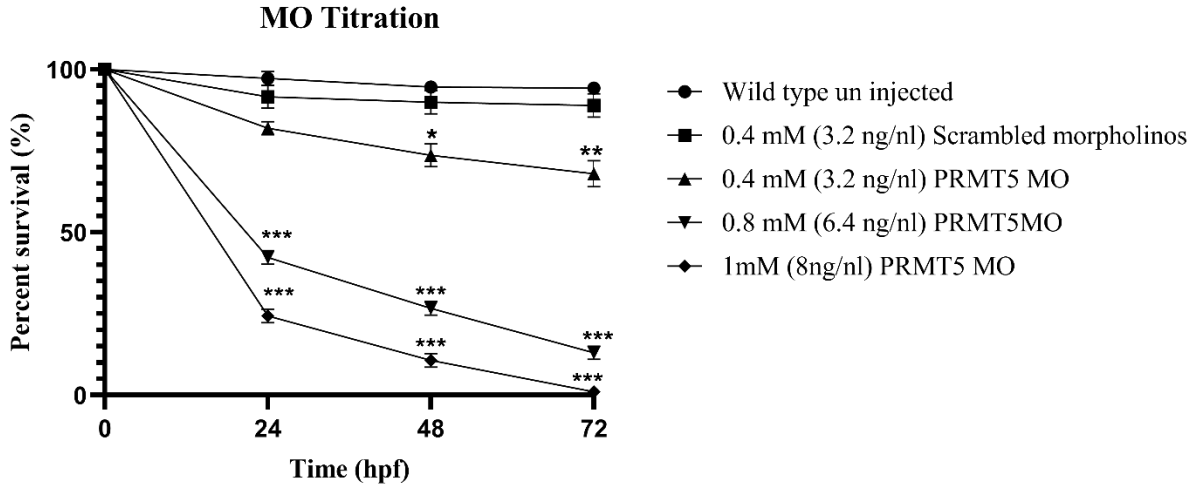


Figure 4.3 PRMT5 MO titration. Embryonic survival for different PRMT5 MO concentrations was converted to a percentage, averaged and plotted. Percent survival of MO injected embryos was calculated at 24, 48, and 72 hpf. All data points represent as mean ± SEM, (100 embryos were used in each group; experiment was performed in triplicate). The analysis was done by two-way-ANOVA with Dunnett test. *$p<0.05$, **$p<0.01$, ***$p < 0.001$, and ****$p<0.0001$.

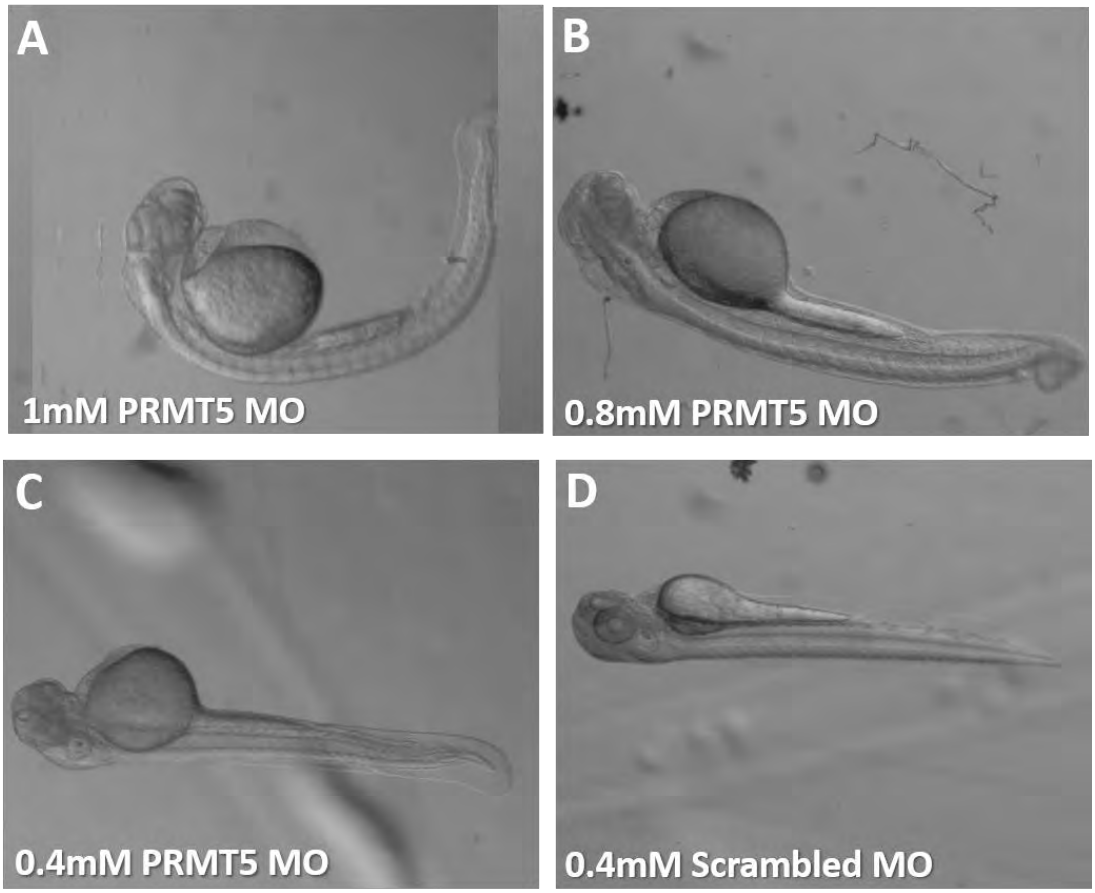


Figure 4.4 Embryonic phenotypes for different PRMT5 MO concentrations.

(A) 1 mM (8 ng/ µl). (B) 0.8 mM (6.4 ng/ µl). (C) 0.4 mM (3.2 ng/ nl). (D) 0.4 mM (3.2 ng/ nl) scrambled MO.

4.4 PRMT5 morphants exhibit distinct and specific phenotypes

PRMT5 knockdown embryos exhibited distinct and specific phenotypes. Phenotypes of the morphants were examined between 24 and 72 hpf under a light Microscope. PRMT5 loss of function gave rise to embryos with a delay in heart development, and an elongated heart shaped like a tube with pericardial edema. As a result, embryos with this defect in heart formation behaved differently in term of cardiac performance as well as locomotion in comparison to scrambled MO (Figure 4.5).

HAND2 is a transcriptional regulator controls cardiac development, whose expression domain demarcates the heart forming region in the ALPM (Schoenebeck et al., 2007). HAND2 has an early impact on cardiac progenitor cells formation, that's why HAND2 knockdown embryos were used as a positive control for heart development defect. Knocking down *PRMT5* was not as severe as knockdown of a heart specific gene (HAND2) where it showed abnormal heart size and structure.

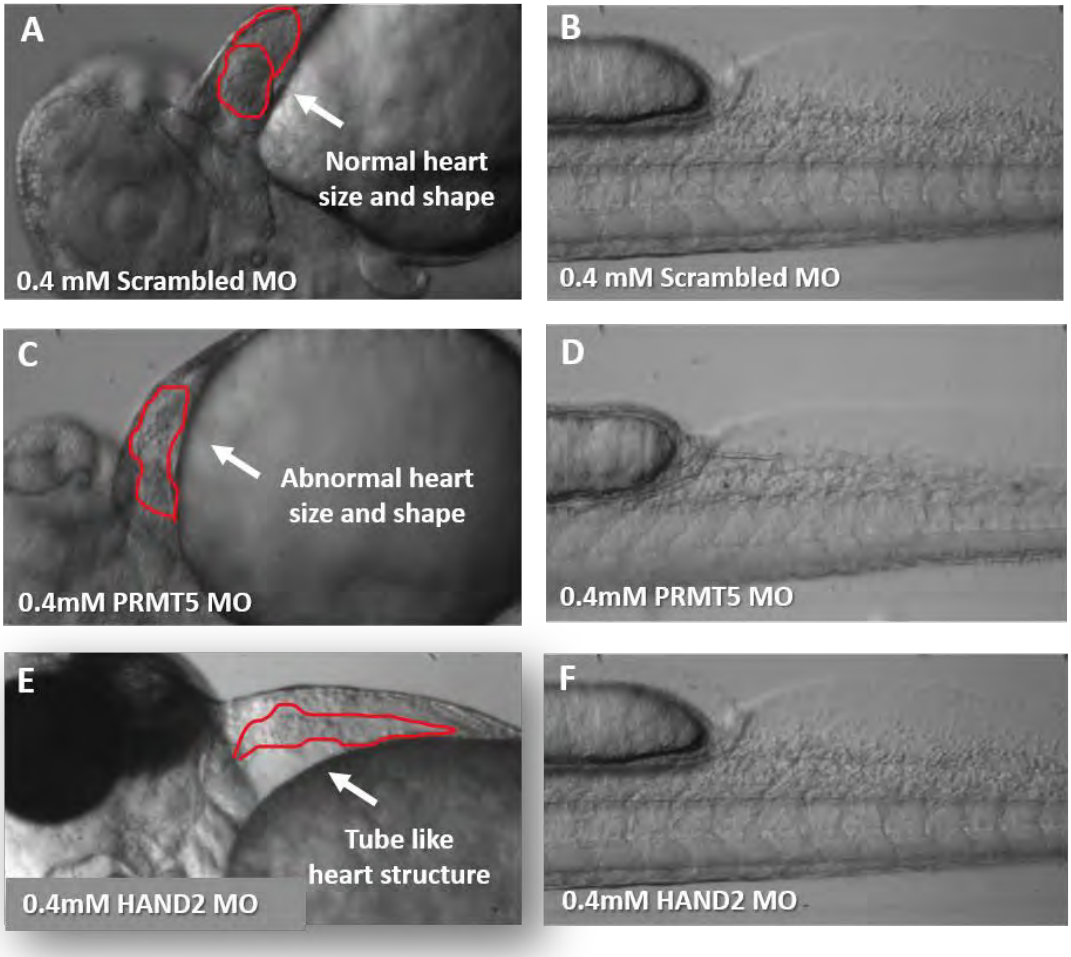


Figure 4.5 PRMT5 morphants exhibit distinct and specific phenotypes. Hearts and tails of 72 hpf embryos were examined under a light microscope (100X magnification). (A, B) Control scrambled MO shows normal heart development without pericardial edema. (C, D) 0.4 mM PRMT5 MO-injected embryos shows abnormal heart size and shape with pericardial edema. (E, F) 0.4 mM HAND2 MO injected embryos as positive control shows tube like heart structure.

4.5 Rescue of cardiac morphant using exogenous human PRMT5 expression

Expression of PRMT5 was strongly reduced after injection of PRMT5 MO as confirmed by western blot and led to abnormal heart development. To confirm that these phenotypes are due to loss of PRMT5 expression, a rescue experiment was conducted using human *PRMT5* mRNA.

Embryos at 1 hpf were injected with scrambled MO alone, PRMT5 MO alone, scrambled MO in combination with *in vitro* synthesized human PRMT5 mRNA, or PRMT5 MO with PRMT5 mRNA. Next, PRMT5 protein expression was measured 24-hour post injection.

A representative Western blot bands for protein samples from PRMT5 knockdown and rescue embryos at 24hpf are shown in Figure 4.6A, Our result showed that PRMT5 protein level expression significantly reduced by 3-folds ($p< 10^{-4}$) in PRMT5 knockdown embryos and was significantly restored by 2.4-folds ($p = 10^{-4}$) when the PRMT5 MO was co-injected with human *PRMT5* mRNA (Figure 4.6B).

Altogether, the results showed successful rescue of PRMT5 expression at protein level when the PRMT5-MO was co-injected with human *PRMT5* mRNA. The impact of PRMT5 rescue on zebrafish heart function, tail flicking, and locomotion phenotypes will be shown in the upcoming sections confirming that phenotypes appeared were due to PRMT5 knockdown and not caused by as MO off-target effects.

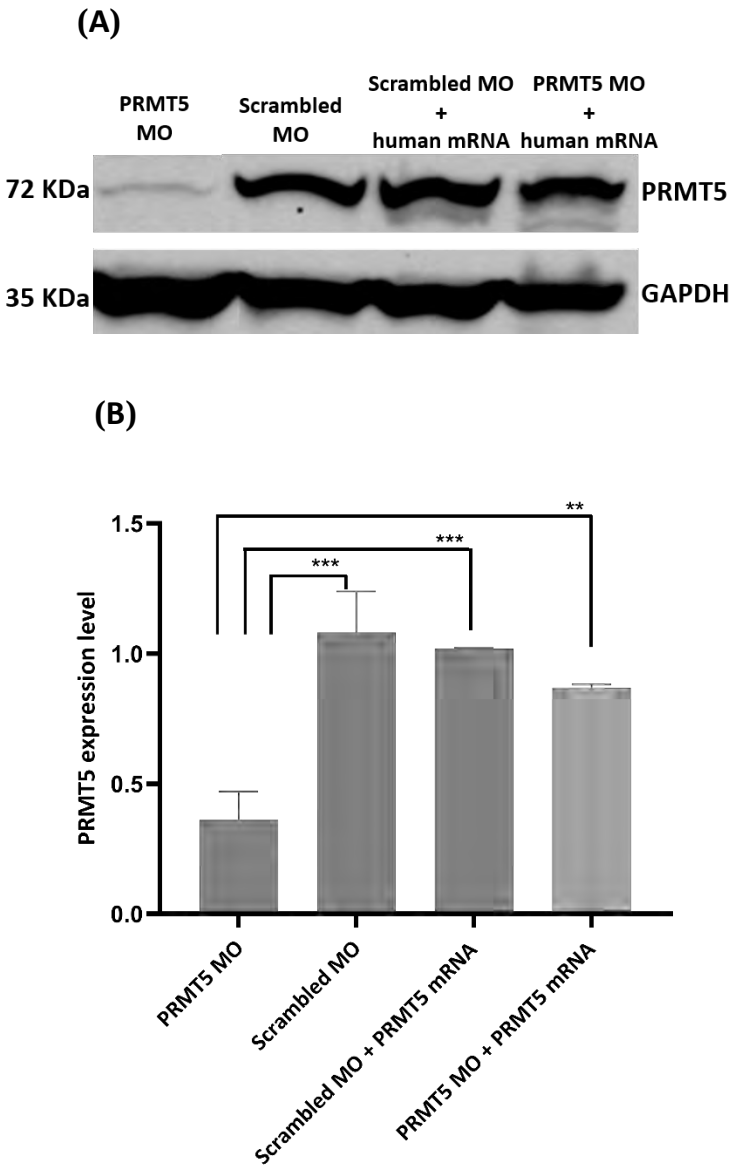


Figure 4.6 PRMT5 knockdown and rescue. (A) Western blot analysis of extracts from the PRMT5 knockdown groups in comparison with control scrambled MO injected group. (B) Quantification of Western blot bands was done using Image J. All data are presented as mean ± SEM (experiment was performed in triplicate). The analysis was done by one-way-ANOVA with Sidak post hoc test. **p<0.05, **p<0.01, ***p < 0.001 and ****p<0.0001.*

4.6 Phenotype Analysis:

4.6.1 PRMT5 is essential for proper myogenesis

To better understand how loss of PRMT5 impacts muscle cell differentiation and more specifically heart development, we analyzed phenotypes that affected muscle cell development including tail flicking, hatching function, locomotion of embryos, heart shape, as well as heart function during the different stages of embryonic development.

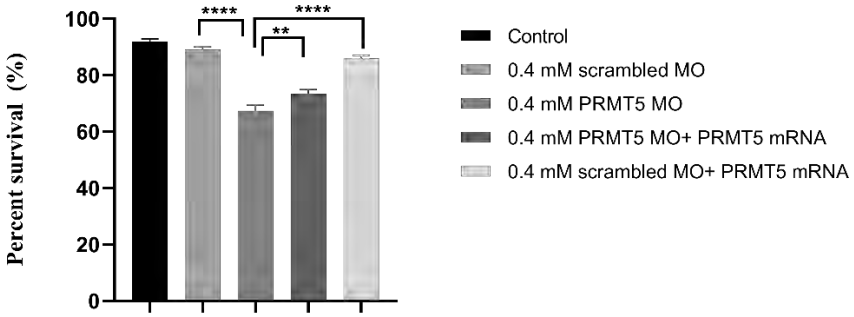
Survival and phenotype of zebrafish embryos expression were checked at specific time points for each injection experiment. As shown in Figure 4.7A, the percent survival of zebrafish embryos at 24 h post-fertilization remained high in all experimental groups except for the PRMT5 knockdown group, where a 20% decrease was observed ($p < 10^{-4}$). Survival of PRMT5 knockdown embryos increased by 6% ($p = 10^{-3}$) when human *PRMT5* mRNA was coinjected. More significantly, PRMT5 Knockdown severely impacted the hatching ability of zebrafish embryos. The hatching percentage was reduced by 4-fold ($p < 10^{-4}$) at 48 hpf (Figure 4.7B). In contrast, hatching of PRMT5 knockdown embryos was significantly increased by 3-fold ($p < 10^{-4}$) in the presence of human *PRMT5* mRNA.

Furthermore, PRMT5 knockdown decreased locomotion and tail flicking by 4.7-fold ($p< 10^{-4}$) at 24 hpf, indicating potential defect in skeletal muscle myogenesis (Figure 4.7C). This deficiency was partially rescued by 3.5-fold (p value < 0.0001) when human *PRMT5* mRNA was co-injected with PRMT5 MO.

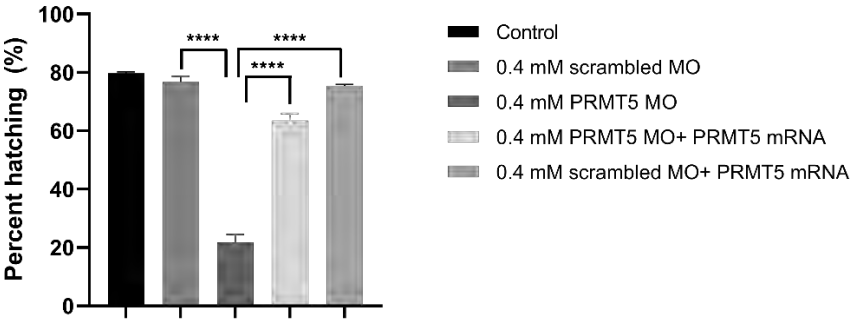
These findings showed that PRMT5 knockdown was associated with delay or inhibition of embryos hatching and locomotion/tail coiling, indicating that there is a defect in

muscle contraction and potential defect in skeletal muscle. This result agrees with earlier reports on PRMT5, which implicated it is required for early gene expression, and its preferentially activity is important at different times throughout myogenesis.

(A)



(B)



(C)

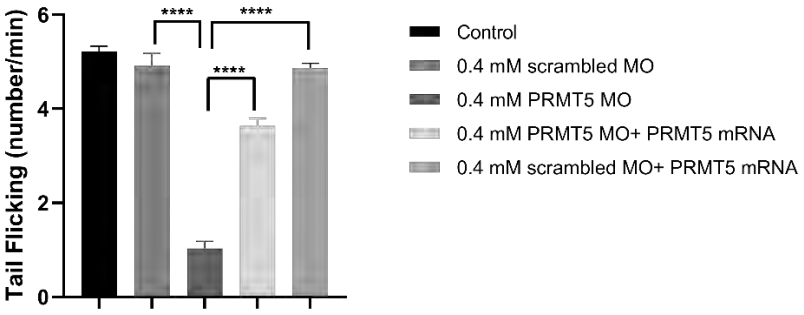


Figure 4.7 PRMT5 is essential for proper myogenesis. (A) Percent survival of control embryos and experimental group. (B) The effect of PRMT5 knockdown on experimental group hatching percentage. (C) Assessment of the potential effect of PRMT5 on muscular system on the experimental group at 24 hpf. All data are presented as mean ± SEM (50 embryos were used in each group; experiment was performed in triplicate). One-way ANOVA with Sidak post hoc test was used to compare the differences between groups. *$p<0.05$, **$p<0.01$, ***$p < 0.001$ and ****$p<0.0001$.

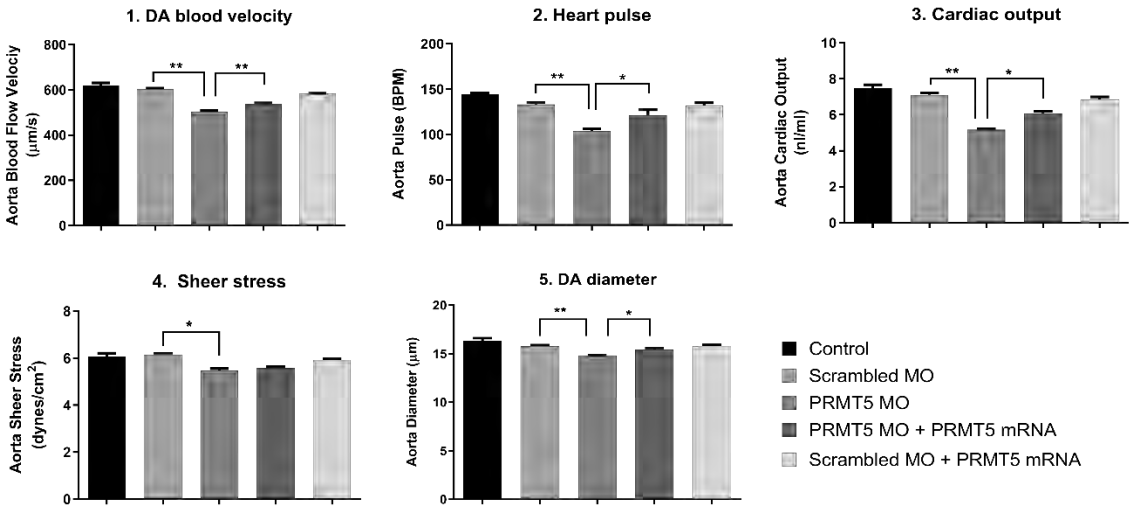
4.6.2 PRMT5 is essential for proper cardiomyocyte development.

Pathomorphological changes in the zebrafish heart are similar to those of the human heart (Giardoglou et al., 2019). These abnormal changes result in reduced cardiac output in humans, and it would be interesting to see if changes induced by PRMT5 knockdown can cause reduced cardiac output in zebrafish. For assessment of heart function, most calculated physiological parameters are heart rate, blood velocity and cardiac output. In our investigation, we have characterized the changes in the zebrafish heart shape, cardiac output, and blood flow in control and PRMT5 knockdown animals. Blood flow velocity was measured by tracking down moving RBCs, which were used to determine shear stress levels; an important mechanobiological factor on endothelial cells.

Cardiac output and blood flow analyses showed that PRMT5 knockdown triggered a delay in heart development in comparison to wild type embryos. This alteration resulted in a 1.2-fold ($p < 10^{-4}$) decrease in dorsal aorta blood velocity (Figure 4.8 A1), 1.2-fold ($p < 10^{-4}$) drop in heart pulse (Figure 4.8 A2), and 1.4-fold ($p < 10^{-4}$) reduction in cardiac output (Figure 4.8 A3). In agreement with the negative impact of PRMT5 knockdown on cardiac function, sheer stress was also reduced significantly by 1.1-fold ($p< 10^{-4}$) (Figure 4.8 A4) in comparison to the control scrambled MO injected embryos. Most of the heart function failure was rescued when PRMT5 MO was co-injected with human PRMT5 mRNA, which resulted in a .9-fold ($p < 10^{-3}$) increase in dorsal aorta blood velocity (Figure 4.8 A1), 1.1-fold ($p < 10^{-2}$) increase in heart pulse (Figure 4.8 A2), and 1.1-fold ($p < 10^{-3}$) restore in cardiac output (Figure 4.8 A3). These results are in agreement with cardiac output and blood flow analyses results for PCV (Figure 4.8 B).

Collectively, our data suggested that PRMT5 might play critical roles in cardiomyogenesis during zebrafish development. In upcoming sections (4.9 and 4.10) we will examine the expression of master regulators for muscle development and the role played by PRMT5 to control their expression during heart development.

(A)



(B)

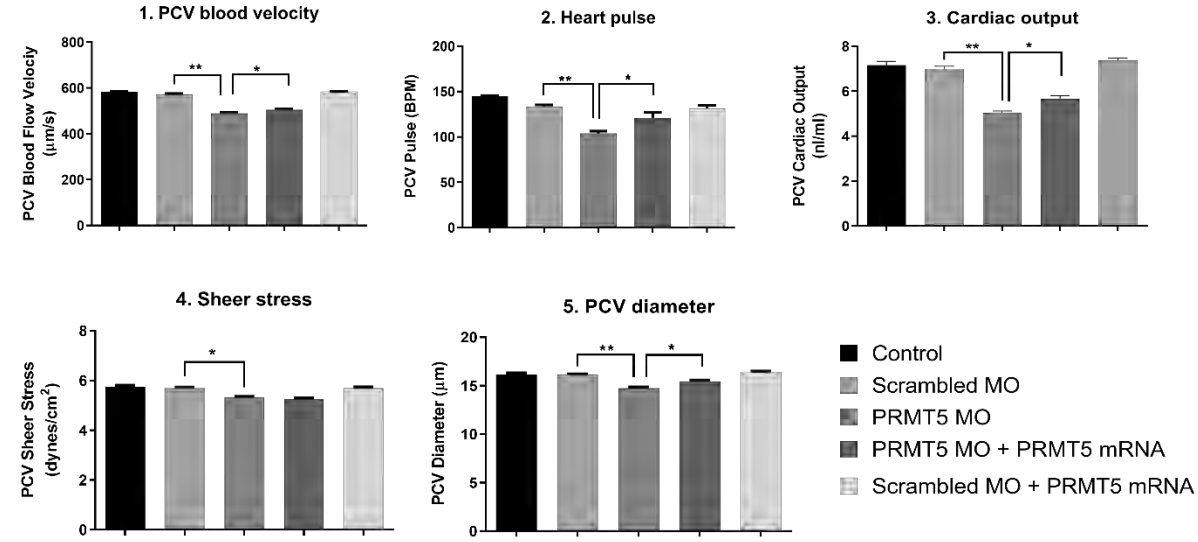


Figure 4.8 Assessment of cardiac function (A) Dorsal aorta blood flow analysis, (B) Posterior Cardinal Vein (PCV) blood flow analysis. 1-4 cell stage zebrafish embryos were injected as groups with scrambled MO, PRMT5 MO, PRMT5 MO + PRMT5 mRNA rescue, and scrambled MO + PRMT5 mRNA rescue. un-injected embryos were used as control. All data are presented as mean ± SEM (6 embryos were used in each group; experiment was performed in triplicate). The analysis was done by one-way-ANOVA with Sidak post hoc test. *$p<0.05$, **$p<0.01$, ***$p < 0.001$ and ****$p<0.0001$.

4.7 PRMT5 knockdown reduced RBCs production

PRMT5 plays an essential role in blood progenitor cell specification (Greenblatt et al., 2016). Using the *o*-dianisidine staining, we tested whether PRMT5 knockdown could have an adverse effect on erythropoiesis in zebrafish. Microscopic images of the embryos stained with o-dianisidine staining shows reduced number of hemoglobin positive RBCs in PRMT5 knockdown embryos in comparison to scrambled-MO (Figure 4.9 A). Quantification of the number of erythrocytes in PRMT5 knockdown embryos using Image J software, revealed that there was a 2.1-fold ($p < 10^{-4}$) decrease in the haemoglobin peroxidase activity compared to control scrambled MO injected embryos (Figure 4.9). This result suggests that PRMT5 knockdown negatively affect hematopoiesis.

(A)

(B)

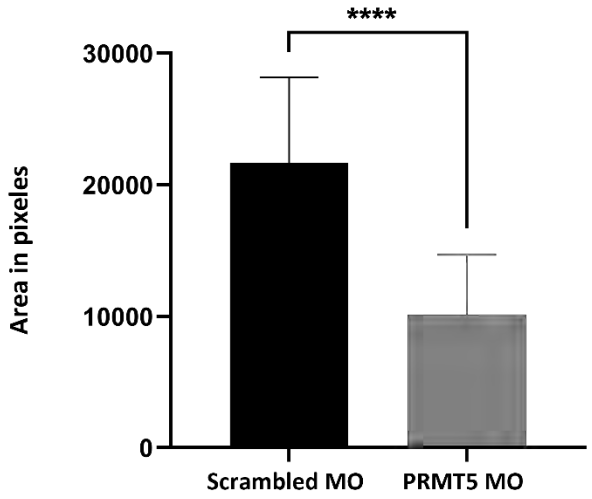


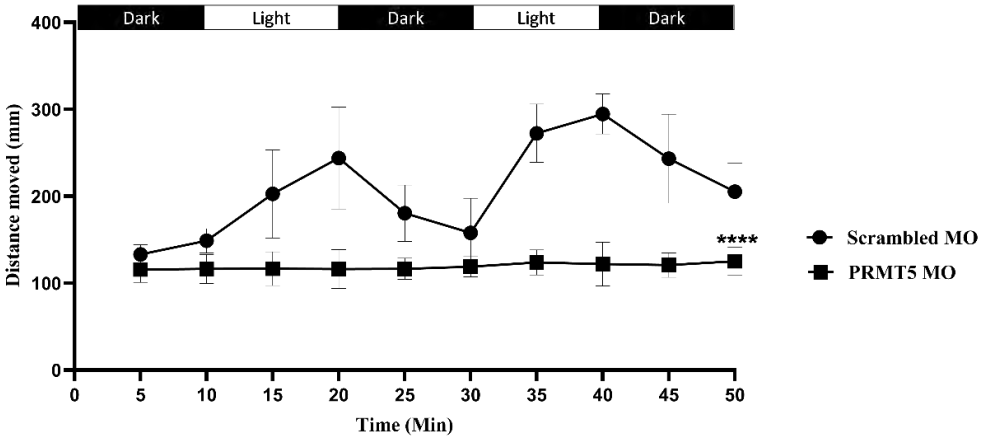
Figure 4.9 Distribution of hemoglobin positive RBCs by O-dianisidine staining at 72-hpf. (A) Microscopic images of the embryos stained with o-dianisidine to assess the hemoglobin peroxidase activity. (B) Quantification of the number of erythrocytes in the areas stained by o-dianisidine, analysis was done using Image J software. All data are represented as mean ± SEM. (20 embryos were used in each group; experiment was performed in triplicate). The analysis was done by unpaired two-tailed t-test. **p<0.05, **p<0.01, ***p < 0.001 and ****p<0.0001*.

4.8 PRMT5 knockdown negatively impacts zebrafish locomotion

Spontaneous movement by zebrafish embryos occurs early during development, and indicates normal primary motor neuron innervation of skeletal muscle. To investigate the impact of PRMT5 knockdown on motor neuron activity of zebrafish embryos, we measured the distance travelled by embryos when PRMT5 expression has been reduced compared to scrambled MO injected embryos. The locomotion assay revealed that PRMT5 knockdown embryos showed an abnormal locomotion in the total and average distance travelled (mm) per h under dark/light phases in comparison to control scrambled MO injected embryos. PRMT5 knockdown significantly decreased the average distance moved (mm) per h by 2 -2.9-fold ($p < 10^{-4}$) during dark/light phases (Figure 4.10A). Furthermore, the total distance moved by PRMT5 knockdown embryos at 72 hpf was reduced by 1.7-fold ($p < 10^{-4}$) in comparison to control scrambled MO injected embryos. (Figure 4.10B).

Notable, the locomotion assay results showed that silencing of PRMT5 has a significant impact on the muscles, indicating that PRMT5 might have a potential role in skeletal muscle myogenesis during zebrafish development.

(A)



(B)

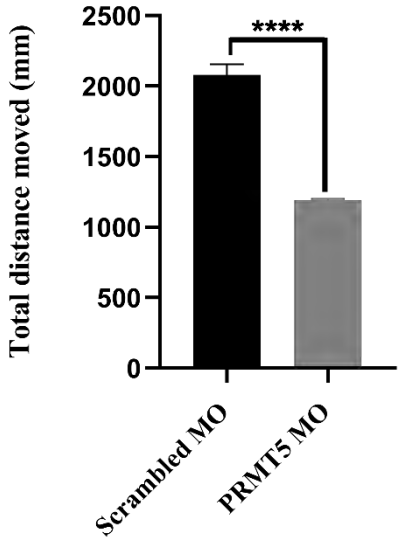


Figure 4.10 Locomotion of PRMT5 knockdown embryos compared to scrambled MO injected embryos. (A) Graph showing the average distance (mm) travelled per h under dark/light phases by control scrambled MO and PRMT5 knockdown embryos. Data of locomotion test are represented as mean ± SEM. (20 embryos were used in each group; experiment was performed in triplicate). The analysis was done by two-way-ANOVA test. *$p<0.05$, **$p<0.01$, ***$p < 0.001$ and ****$p<0.0001$. (B) Total distance (mm) moved per minute by control scrambled MO, PRMT5 knockdown embryos at 72 hpf. Total distance moved data are represented as mean ± SEM. The analysis was done by unpaired two-tailed t-test. *$p<0.05$, **$p<0.01$, ***$p < 0.001$, and ****$p<0.0001$ (20 embryos were used in each group; experiment was performed in triplicate).

4.9 PRMT5 regulates myogenic factors and affects cardiac failure markers expression.

PRMT5 is widely expressed in different human tissues. Importantly, high expression of PRMT5 is observed in the heart. This led us to examine the role of PRMT5 as a master regulator for heart muscle development. Expression of cardiac-specific TFs was significantly reduced due to the absence of PRMT5 compared to control scrambled MO injected embryos (Figure 4.11). For instance, at 24hpf GATA5, MYF5 and CDC73 expression was reduced by 4.7-fold ($p < 10^{-4}$), 2.1-fold ($p < 10^{-4}$), and 2.8-fold ($p < 10^{-4}$), respectively. *ANP/NPPA* mRNA expression was reduced by 1.8-fold ($p < 10^{-4}$), and *BNP/NPPB* mRNA expression was reduced by 1.5-fold ($p = 10^{-4}$). Moreover, real time RT-PCR at 48hpf revealed similar trend in mRNA expression for the examined target genes. While at 72hpf, GATA5, MYF5 and CDC73 expression was reduced by 3.4-fold ($p < 10^{-4}$), 1.6-fold ($p < 10^{-4}$), 2.5-fold ($p < 10^{-4}$) respectively. In contrast, *ANP/NPPA* mRNA expression was unaffected, and *BNP/NPPB* mRNA expression was increased by 1.6- fold ($p < 10^{-4}$). These results confirm that PRMT5 has an essential role during development and its expression is required for regulation of the cardiac muscle transcription factors.

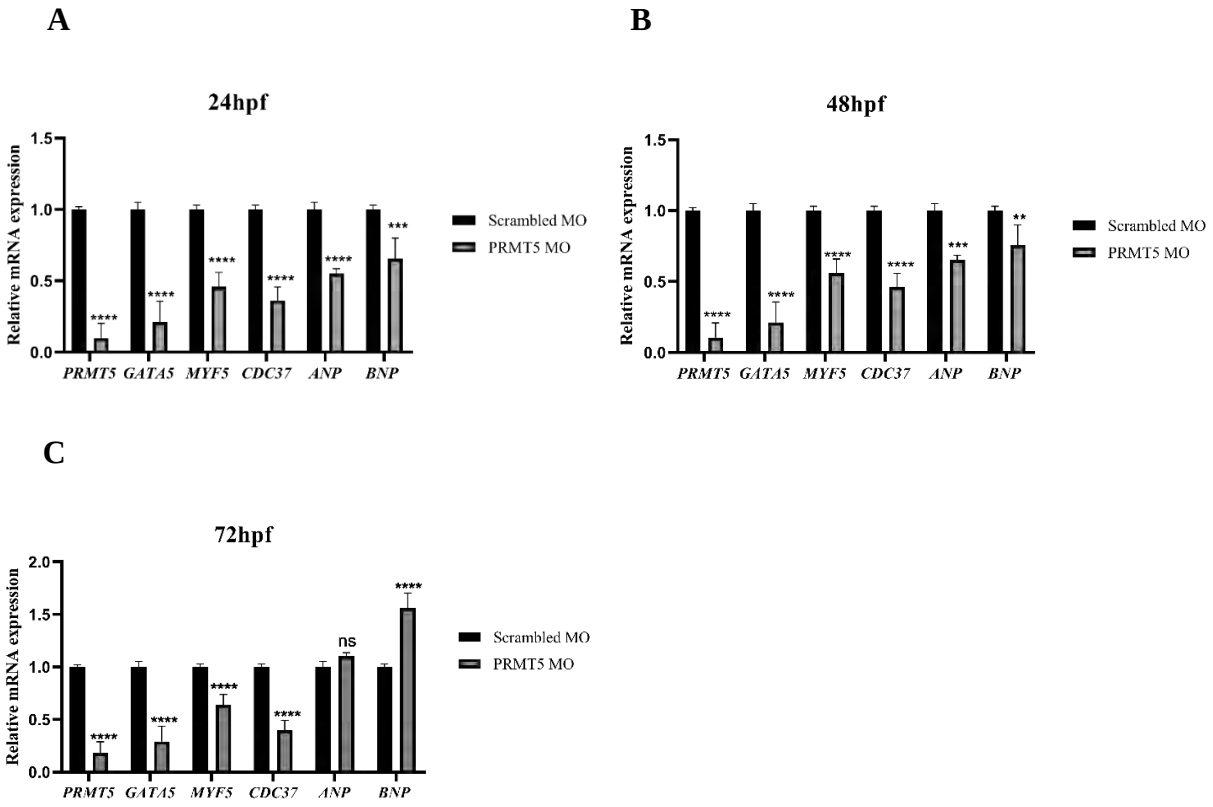


Figure 4.11 PRMT5 regulates myogenic factors and affects expression of cardiac failure markers. Quantitative analysis of mRNA expression of the indicated genes at 24 hpf (A), 48 hpf (B), and 724hpf (C). Analysis of mRNA expression was performed using Taqman RT-qPCR using specific primers and probes designed against PRMT5, GATA5, MYF5, CDC73, *ANP/NPPA*, and *BNP/NPPB*, The exact amount was calculated based on the $2^{-\Delta CT}$ method, and the fold change was calculated in reference to the geomean of a group of housekeeping genes, *B2M*, *RPL13A*, and *EF1α*. All data are presented as mean ± SEM (experiments were carried 3 times in triplicate). The analysis was done by two-way-ANOVA with Sidak post hoc test. *p<0.05, **p<0.01, ***p< 0.001 and ****p<0.0001.

4.10 PRMT5 recruitment to the MYF5 and GATA5 promoter regions

In light of the mRNA expression profiles we observed upon PRMT5 knockdown, we reasoned that PRMT5 might be involved in epigenetic regulation of the examined targeted genes. To check if there was a positive correlation between PRMT5 knockdown and decreased expression of cardiac muscle transcription factors GATA5 and MYF5, ChIP assays were conducted at 3 time points 24,48, and 72 hpf.

Our analysis showed that there was significant enrichment of PRMT5 at the RBL2 promoter (13.5-fold) ($p < 10^{-4}$) during the examined developmental stages, RBL2 was used as a positive control that known to be PRMT5 substrate. Interestingly, significant enrichment of PRMT5 was seen at the GATA5 (11.3-fold) ($p < 10^{-4}$), and the MYF5 (12.5-fold) ($p < 10^{-4}$) promoters at 24hpf. The same result was at 48hpf where significant enrichment of PRMT5 at the GATA5 (11.9-fold) ($p <10^{-4}$), the MYF5 (12.9-fold) ($p < 10^{-4}$) promoters. Furthermore, at 72hpf there was significant enrichment of PRMT5 at the GATA5 (9.8-fold) ($p < 10^{-4}$), the MYF5 (11.5-fold) ($p < 10^{-4}$) promoters. In contrast, no significant enrichment of PRMT5 at the *BNP/NPPB* promoter was seen during any of the examined stages, indicating that PRMT5 affect the expression of *BNP/NPPB* indirectly, not via promoter sequence. Results from CHIP assays reveal that PRMT5 directly recruited to the promoters of specific transcription factors GATA5 and MYF5; which are most commonly cardiac-specific transcription factors studied in the literature and regulate multiple aspects of differentiation and heart muscle development in zebrafish, These findings indicate that PRMT5 activity is required during early stages of cardiomyogenesis to control the expression of key cardiac transcription factors, which are crucial for normal heart development.

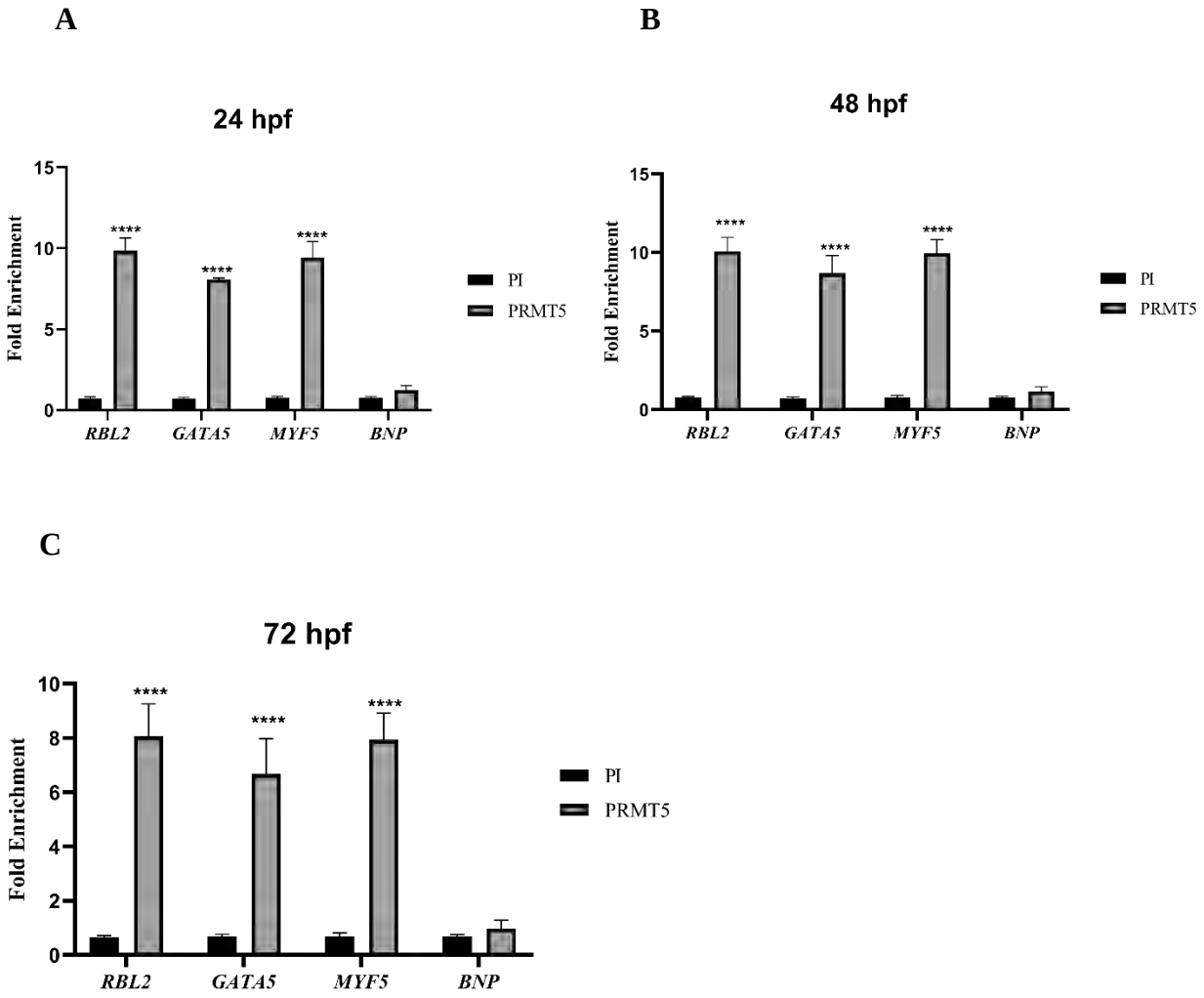


Figure 4.12 Recruitment of PRMT5 at the GATA5 and MYF5 promoters. ChIP assays were performed using cross-linked chromatin from zebrafish embryos using pre-immune (PI) and anti-PRMT5 antibodies. RT-PCR was performed using specific primers and probes designed against *RBL2* as positive control, *GATA5, MYF5*and (*BNP/NPPB*). Fold enrichment with each antibody was calculated relative to the PI sample. ChIP assays showed significant enrichment of PRMT5 at the MYF5 and GATA5 promoter at 24hpf(A), 48hpf(B) and 72hpf(C). All data are represented as mean ± SEM (ChIP assays were carried out twice in triplicate). The analysis was done by two-way-ANOVA with Sidak post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

CHAPTER 5: DISCUSSION

Over the past few years, research on cardiac development focused on uncovering the genetic mechanism that controls cardiac differentiation, and clarifying the embryonic origin of the different cardiac lineages. The study of the physiological mechanisms behind cardiac function has proven instrumental in understanding heart disease and the interplay between different cardiac cells. It has recently attracted great interest due to the potential applications of regenerative medicine to heart failure, which has become one of the major causes of morbidity, especially in developed countries. To gain a better understanding of heart development, it is necessary to study the underlying gene expression and its regulation, by focusing on the developmental gene programs, which are also activated during adult heart regeneration. Multiple TFs work in combination during cardiac development. Moreover, most of the TFs are also utilized in the adult heart in response to disease stimuli, which lead to hypertrophic enlargement and/or dilated cardiomyopathy, as part of the "fetal gene program".

Morphogenesis of the heart is an essential process for life in complex animals. Many pathways and genes are involved in this process, which can allow developmental defects to occur (Lu et al., 2016). These developmental defects can cause congenital heart defects (CHD) (Pierpont et al., 2007; Feng et al., 2014). Researchers are identifying the genes responsible for these defects, as well as the epigenetics or environmental conditions that affect the function or expression of those genes. Two areas of research under exploration are the control of epigenetic modifications (such as acetylation, ubiquitination, and methylation) and discovering novel molecular targets, which may alter heart biology and significantly enhance the efficiency of existing

treatments.

Our focus was on the role of PRMT5 in the essential transcriptional modulators of heart development and the identification of the major players in the early phases of cardiomyogenesis, which include GATA5, MYF5, CDC73. The present study showed, for the first time, that PRMT5 safeguards normal heart development. Further, our investigations confirmed that in comparison to wild type, *PRMT5* knockdown embryos exhibited a delay in heart development accompanied by a reduction in the number of red blood cells and an overall decrease in blood flow and cardiac output. Moreover, when the PRMT5 protein levels were reduced, expression of cardiac transcription factors was reduced. Further analysis by ChIP assays revealed that PRMT5 directly targets the promoter sequences of *GATA5* and *MYF5* genes, which are required for terminal differentiation and development of heart muscle. This finding suggests that PRMT5 governs expression of some cardiac transcription factors, which are crucial for normal heart development and proper cardiomyocyte development.

PRMT5 **is dynamically expressed during zebrafish development**

In this study, characterization of PRMT5 in zebrafish showed that it is ubiquitously expressed throughout the developing embryos, with high expression levels detected during heart and skeletal muscle development (1hpf-72hpf). *PRMT5* mRNA was detected in 1hpf embryos, despite the fact that zygotic *PRMT5* is not yet expressed (Batut et al., 2011). This highlights the crucial role played by PRMT5 during the early stages of embryogenesis.

The level of PRMT5 mRNA was not consistent with level of PRMT5 protein expression during the examined developmental stages, where mRNA levels were higher than protein levels at 1hpf and 6hpf. There are multiple factors that could explain this, the relationship between rate of transcription and mRNA stability or to translation efficiency vs. protein stability is one factor. Some splice variants are not translated, or protein degradation may contribute more for the reverse result. Moreover, *PRMT5* mRNA was detected in1hpf embryos, despite the fact that zygotic *PRMT5* is not yet expressed, suggesting that *PRMT5* maternal gene (oocyte *PRMT5* mRNA) might get transported to the developing embryos (Batut et al., 2011).

Gene expression evaluation by RT-PCR for characterizing the expression of PRMT5 at mRNA level is essential to confirm the expression for PRMT5 at the protein level. The main obstacle I faced during mRNA quantitation analysis was to calculate the relative quantity and the fold change based on the $2^{-\Delta CT}$ method, by selecting the optimum housekeeping gene for RT-qPCR in zebrafish during different developmental stages (Figure 4.1C). To overcome this issue, all ΔCT values were calculated in reference to the geomean of a group of housekeeping genes *B2M*, *RPL13A*, *EF1α* (Vandesompele et al., 2002).

PRMT5 MO titration

Generally, MO solutions must be carefully diluted to establish the lowest concentration needed to induce a specific phenotype. Embryonic lethality for each of the MOs should also be determined. At higher concentrations (above 4-10 ng/nl), MOs tend to elicit non-specific effects, such as brain or general cell death (Bedell et al., 2011). For the

PRMT5 gene, we tested several concentrations to identify the optimal amount of MO needed to knockdown expression without compromising normal development of the zebrafish.

Mortality of PRMT5 MO injected embryos was detected around 24 hpf; this could stem from several causes. One possibility is the inability of the knocked down animals to maintain homeostasis, due to lack of PRMT5. Moreover, the heart start beating around 24 hpf and becomes fully developed by 48 hpf, so the decrease in survival around this time point might be due to the absence of PRMT5, because the heart was still not well developed in PRMT5 knockdown embryos. Another possible reason for decreased survival at later time points (48 and 72 hpf) is that PRMT5 MO induces apoptosis by activation of p53 which leads to inhibition of cell cycle progression (Bill et al., 2009). The occurrence of the increased lethality at this time point supports the belief that PRMT5 plays an active role in zebrafish heart development.

PRMT5 morphants exhibit distinct and specific phenotypes.

After successfully optimizing the condition for MO injection, we further examined whether PRMT5 knockdown affected heart function. Cardiac defects in PRMT5 knockdown zebrafish embryos generally manifested as pericardial edema and faulty circulation at an early stage (Miura et al., 2011). For functional analysis, I began with studying heart rate, as it is an important factor for assessment of pathological heart function in zebrafish (De Luca et al., 2014). PRMT5 MO-injected embryos exhibited a significant decrease in heart rate, confirming that PRMT5 is important for heart function.

Rescue of cardiac morphant using exogenous human PRMT5 mRNA

Successful rescue of PRMT5 expression was confirmed at both structural and functional levels, by assessing the protein level as well as the embryo's phenotype, as shown in western blot and cardiac function analyses, respectively. The results in Figure 4.6 show that PRMT5 expression was significantly diminished at the protein level in the knockdown sample, and that expression was significantly rescued when the PRMT5 MO was co-injected with human *PRMT5* mRNA. This result indicated that PRMT5 knockdown is the cause of the induced phenotype.

Phenotype analysis:

PRMT5 is essential for proper myogenesis

Previous studies have characterized PRMT5 as an important factor for proper myogenesis in zebrafish (Batut et al., 2011). In the present study, PRMT5 depletion was associated with delay or inhibition of embryos hatching and locomotion/tail coiling, suggesting that there is a defect in muscle contraction (Figure 4.7). Moreover, the locomotion assay revealed that PRMT5 knockdown embryos showed abnormal locomotion behavior (Figure 4.10).

This result is in agreement with earlier reports on PRMT5, which implicated it in the regulation of muscle fiber formation via transcriptional control of early myogenic genes, *MYOD*, *MYF5* and myogenin (Hasty et al., 1993; Nabeshima et al., 1993; Knapp et al., 2006). Collectively, these findings suggested that PRMT5 is an important factor for proper myogenesis in zebrafish, and led us to examine the expression of master regulators for muscle development in response to PRMT5 knockdown.

PRMT5 is essential for proper cardyomyogenesis

Cardiovascular disease is remarkably age-related, and relative changes in methylation have been shown not only at the genome-wide level, but also at the proteome level. Considering that expression of PRMT5 in the heart is substantially reduced in aged rats, further investigation of its function will give us new insights that will advance the development of a therapeutic approach to cardiovascular disease prevention.

From a different perspective, cardiac hypertrophy is grouped among the pathological conditions that significantly cause heart failure (Hunter et al., 1999). There is a significant amount of research evidence to support the fact that cardiac TFs including GATA family TF, nuclear factor of activated cells (NFAT) and myocyte enhancer factor 2 (MEF2) all play crucial roles in the reactivation of fetal growth programs that control hypertrophic events (Passier et al., 2000; Liang et al., 2002; Braz et al., 2003). According to Charron et al (2001), GATA4 has been linked to cardiac hypertrophy. Given the important role played by GATA4 in the heart, its transcriptional activity is controlled and regulated through diverse post-translational mechanisms. Previous research studies have concluded that PRMT5 significantly controls the methylation of GATA4 in hypertrophic cardiomyocytes (Chen et al., 2014). PRMT5 also tends to be overexpressed in a way that inhibits GATA4 acetylation. On the other hand, its knockdown caused induced GATA4 activation and cardiomyocyte hypertrophy.

In zebrafish studies, it was reported that *GATA5*-mutant fish exhibited heart defects (Takeuchi et al., 2011). Interaction between BRG1, an ATPase within SWI/SNF chromatin remodeling complex, and *GATA5* was detected and shown to be critical for

mouse and zebrafish cardiomyogenesis (Takeuchi et al., 2011). Components of the BRG1/BRM associated factor (BAF) complex are significantly upregulated in patients with hypertrophic cardiomyopathy (Hang et al., 2010; Cui et al., 2015; Lu et al., 2016). The previously mentioned relationships between GATA5 and BAF components including BRG1 which can be found associated with PRMT5 provokes further investigations for the role of PRMT5 that may open new insights toward better understanding of cardiomyogenesis (Pal et al., 2003; Pal et al., 2004).

After examination of the requirement for PRMT5 in the adipose differentiation in different animal models as well as the skeletal muscle, an emerging similarity is that many PRMT5 dependent genes in these two systems require PRMT5 to be present at the target gene loci for Brg1 chromatic remodeling enzyme to bind to these promoter sequences (Dacwag et al., 2007; LeBlanc et al., 2012). The same results in the two differentiation systems showed that the facilitation of the ATP-dependent remodeling enzyme function is a major stage in tissue-specific gene activation. A more hypothetical version of this model proposed that arginine methylation by various PRMTs may work independently or cooperatively to enhance Brg1 binding and chromatic remodeling (LeBlanc et al., 2012). However, more research studies are required in this section to confirm the important role played by PRMT5 in cardiac biology. The ability to identify appropriate physiological substrates of PRMT5 under different biological conditions will be important in illuminating its value in regulating cardiac function.

PRMT5 knockdown triggers reduced RBC production

The present study tested whether PRMT5 knockdown could have an adverse effect on erythropoiesis in zebrafish. A significant decrease in the amount of hemoglobin (measured in the yolk area) was recorded between control scrambled MO and PRMT5 knockdown embryos (Figure 4.9), suggesting that PRMT5 knockdown interferes with erythropoiesis.

Limited studies investigated the impact of PRMT5 on RBC production. However, previous studies reported that PRMT5 plays an essential role in blood progenitor cell specification (Greenblatt et al., 2016). Liu and colleagues (2015) reported that lack of PRMT5 expression could result in impaired B and T cell differentiation and development (Liu et al., 2015). Other studies suggested that PRMT5 knockdown abolishes TNF-α-dependent methylation of HOXA9. Lack of HOXA9 methylation may interfere with HOXA9's crucial functions in hematopoietic stem cell expansion (Richters, 2017). suggesting that PRMT5 important for hematopoietic cell production.

PRMT5 regulates cardiac-specific transcription factors and affects expression of cardiac failure markers

Our findings suggest that RMT5 knockdown results in reduced expression of *GATA5, MYF5, CDC73, ANP/NPPA*, and *BNP/NPPB*. Further analysis by ChIP assays revealed that PRMT5 directly targets the promoter sequences of the *GATA5, MYF5,* which are both required for terminal differentiation and development of heart muscle. Moreover, significant increase in *BNP/NPPB* expression at 72 hpf compared to 24 and 48 hpf was detected among the PRMT5 knockdown zebrafish embryos. The increase in

BNP/NPPB levels at 72hpf might be to slow down the progress of morphological and functional changes in the zebrafish heart such as reduced heart rate, shape and blood velocity.

These findings are aligned with a previously published study which reported that *ANP/NPPA* and *BNP/NPPB* were absent in healthy adult hearts and upregulated under hypertrophic stimuli (Gardner, 2003). Other studies showed that *ANP/NPPA* knocked out mice have hypertrophy and hypertension under both resting and pressured conditions as compared to wild-type control mice (Wang et al., 2003; Mori et al., 2004). Another study showed *BNP/NPPB* deletion in mice resulted in interstitial ventricular fibrosis (Tamura et al., 2000). Several researchers documented that TF GATA4 upregulates expressions of *ANP/NPPA* and *BNP/NPPB* (Gardner, 2003; Richards, 2007). This may explain that reduced levels of *ANP/NPPA* and *BNP/NPPB* at 24 and 48 hpf in our experiment may be attributed to lack of GATA5 in response to PRMT5 knockdown.

On the other hand, Kuhn et al. (2004) reported upregulated levels of NPR-C among cases of congestive heart failure. NPR-C reduces the effectiveness of *ANP/NPPA* and *BNP/NPPB* (Kuhn et al., 2004). The above literature suggests that natriuretic peptides (*ANP/NPPA* and *BNP/NPPB)* may slow down the progress of hypertrophy but may not prevent it.

Changes in natriuretic peptide expression suggest that some of the mechanisms of pathogenesis are similar to those in humans and other mammals. However, there are some genes that could be reactivated in the heart due to pathological changes.

Nevertheless, the main role of re-expression of cardiac TFs is to promote cardiomyocyte hypertrophy. These changes in zebrafish hearts appear to share molecular similarities with the pathology in mammalian hearts, where *BNP/NPPB* expression was significantly increased, which is a genetic marker of many cardiac pathologies in human and animal models.

This body of data implies that natriuretic peptide signaling is important for the prevention of pathological cardiac remodeling. This suggests that natriuretic peptides could be upregulated in zebrafish hearts at 72 hpf, in an attempt to decrease the extent of pathological remodeling. This implies that increased natriuretic peptide signaling is a secondary response to pathological changes. Importantly, the natriuretic signaling pathway may be activated in order to reduce the severity of heart damage in PRMT5 knockdown embryos.

Furthermore, reactivation of cardiac TFs, such as GATA4 and MEF2C, is often associated with cardiac hypertrophy (Oka et al., 2007; Barry et al., 2008). Surprisingly, upon PRMT5 knockdown and associated heart failure, our data showed that the expression of these TFs was not elevated in zebrafish at all stages. This mean that the expression of GATA5 and MYF5 was not reactivated in response to heart defects in zebrafish. A possible explanation of this is that the absence of PRMT5, which might activate these genes via methylation on their promoter, or by the possibility that the zebrafish heart responds differently to injury-associated stresses (becoming hypertrophied) than the mammalian heart, and thus, cardiac TFs are not re-expressed in the same way.

The most commonly studied cardiac-specific transcription factors include GATA5 and MYF5, both of which have been found to regulate cardiogenesis in zebrafish. Kuo et al (1997) observed that loss of GATA4 in affected mice. Similarly, the loss of GATA5 function in zebrafish *faust* mutants was found to disrupt migration of the endoderm to the midline (Reiter et al., 1999; Reiter et al., 2001). In support of the core role played by the endoderm in migration of cardiac cells to the midline (Kuo et al., 1997; Narita et al., 1997), *faust* mutants were found to show cardia bifida (Reiter et al., 1999). Besides this non-tissue phenotype, embryos of zebrafish that have GATA5 deficiency tend to exhibit a reduced number of cardiac precursors supporting the cell-intrinsic role played by the GATA factors in the synthesis of cardiomyocytes (Reiter et al., 1999). Consistent with this perspective, the GATA5 overexpression widens the extent to which domains of cardiac genes are expressed, including expression of *NKX2.5* in the ALPM (Reiter et al., 1999). Also, zebrafish embryos that lack both GATA5 and GATA6 tend to exhibit a complete absence of *NKX2.5*-positive precursor cells – suggesting a redundant need for GATA factors that may be used for cardiac fate specification (Holtzinger et al., 2007). Recently published literatures have reported interaction of GATA factors and other important cardiomyogenic signaling pathways (Novikov et al., 2013; Zakaria et al., 2018).

In the process of somitogenesis in zebrafish, MYF5 is usually the first to be expressed among the other members of the MRF family. When there is MYF5 knockdown, adverse effects such as malformation of somites and brain defects are likely indicating the presence of impairments in the trunk and head. The presence of such phenotypes supports the need for researchers to develop a wider and in-depth understanding of the

regulation of MYF5 (Chen et al., 2007). Several factors have been regarded as upstream regulators of MYF5. These factor are not limited to extracellular signals, such as fibroblast growth factor (FGF), wingless (WNT), and SHH (Groves et al., 2005; Li et al., 2006). WNT proteins (synthesized by the dorsal neural tube and surface ectoderm) as well as SHH (synthesized by the notochord and floor plate of the neural tube) have been shown to play vital roles in maintaining MYF5 expression in mice (Chen et al., 2005), Xenopus (Shi et al., 2002), and zebrafish (Coutelle et al., 2001). These information supports the fact that the complex nature of the temporal and spatial pattern of the expression of MYF5 gene is largely controlled by multiple upstream regulatory modules (Chen et al., 2007).

Conclusions

This work represents a systematic *in vivo* analysis of important regulatory factors of the cardiac transcription network, comprising DNA-binding transcription factors, as well as epigenetic regulation by histone modifications.

The present study demonstrated for the first time that zebrafish exhibited morphological changes in the heart due to the absence of PRMT5. Moreover, PRMT5 appeared to play a unique role in the regulation of key factors, including GATA5 and MYF5, in order to mediate heart development. Overall, our work supported the combinatorial nature of transcriptional regulation, with a high degree of interdependency that is carefully orchestrated to regulate the correct temporal expression of every gene, to establish cellular function.

This work demonstrates that zebrafish could be used as a model to aid in understanding cardiac development. However, many unanswered questions remain, which might have facilitated a more detailed understanding of cardiac development and led to a therapeutic window of opportunity.

Future directions

In the future, development of individual PRMT5 transgenic models would be ideal for determining the molecular mechanism of disease development. Also, the cutting edge conditional knockdown or CRISPR/Cas-based method offer a promising future for genome engineering in zebrafish (Gaj et al., 2013; Hwang et al., 2013).

Also, it would be useful to evaluate expression of fetal genes by RT-qPCR, to support our data for characterization of zebrafish heart failure. For example, altered expression of indicator genes, such as α-myosin heavy chain (*MYH6*) and β-myosin heavy chain (*MYH7*), were previously observed in mouse heart failure models, so there would be value added in performing similar studies with the zebrafish model (Perrino et al., 2006; Barrick et al., 2007). It would also be beneficial to analyze possible structural differences in the actin filaments of zebrafish embryos containing wild-type versus PRMT5-variant proteins. Detailed myofibril structure could be determined by immunofluorescence and confocal microscopy, or electron microscopy (Glenn et al., 2012).

Although I have reported changes in the heart function and structure at 72 hpf in zebrafish, due to the absence of PRMT5, it would have been better to confirm the significant effect of PRMT5 knockdown on the output of adult zebrafish hearts by using echocardiography, not only in embryos but also in adult zebrafish. This analysis would further validate the use of zebrafish as a model of human cardiac ageing, if the decline in cardiac output is similar to that reported in humans (Vigorito et al., 2014).

In addition, morphological changes in the heart would result in a net reduction of

cardiomyocytes. However, knowing the absolute number of cardiomyocytes in the zebrafish heart at different ages would prove conclusively that there is a reduction in the number of cardiomyocytes, due to reduced cellular differentiation. This could have been achieved by using flow cytometry, which has been successfully carried out on whole mouse hearts elsewhere (Song et al., 2012; Malliaras et al., 2013). Data gathered from these types of experiments could unequivocally prove whether there is a change in cardiomyocyte numbers in zebrafish.

Limitations

During this journey, I would have liked to perform more experiments; although beyond the scope of this thesis, they could have added more to the data garnered. However, this was not possible, due to lack of time and funding, and limits of IACUC approval.

Although all experiments were thoroughly planned and performed as carefully as possible, there were limitations, which were based on the measurements of certain parameters of the zebrafish heart, such as fibrosis, wall thickness, and other heart morphological changes. A scoring system for the level of fibrosis, rather than simply whether it is present or not, might be more appropriate. This could have been achieved by developing a points system based on the severity of fibrosis, or by quantifying the percentage of myocardium that was fibrotic. Also, measuring the thickness of the ventricular walls in zebrafish was very difficult, due to changes in thickness at different positions. This could have been facilitated by echocardiography, but that was not possible because of technical difficulties and the high cost of the specialized equipment that would have been required.

From another perspective, it is difficult to compare the lifespan of humans with that of different animals, particularly zebrafish, which have a much shorter lifespan (Gerhard et al., 2002).

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