ADAMTS9 IS NECESSARY FOR THE SEXUAL DEVELOPMENT OF FEMALE ZEBRAFISH

by
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The ovary is a unique organ as it must cyclically alter its structure to accommodate the growth, development, and release of oocytes. The processes of oogenesis and ovulation require structural modifications made to the extracellular matrix (ECM) enabling cell migration, differentiation, and survival. ADAMTS9 (A disintegrin and metalloprotease with thrombospondin type-1 motif 9) is a metalloprotease that digests ECM proteoglycans, versican and aggrecan. Adamts9 was found to be expressed highly in preovulatory follicular cells of zebrafish, humans and macaques. Increase of adamts9 expression was also found in preovulatory follicular cells treated with progestin and/or gonadotropin. PGRKO zebrafish have significantly reduced expression of adamts9, reduced adamts9 expression in response to progestin treatments in preovulatory follicles and has an anovulatory phenotype- indicating adamts9 may play a key role during zebrafish ovulation. Aberrant migration of the primordial germ cells has been reported in the knockouts of Adamts9 ortholog in C. elegans and Drosophila. Adamts9 was found to be expressed widely in all tissue types and relatively high in the mouse genital tubercle. Unfortunately, Adamts9 knockout mice die before gonad differentiation. So, the function of ADAMTS9 in the vertebrate ovary has not been determined.

To identify the function of Adamts9 in zebrafish ovaries we generated Adamts9 global knockout (adamts9/+ ) zebrafish using CRISPR/Cas9 and characterized the effects of knockouts. From
1149 fish generated from crossing of \textit{adamts9}^{+/−}, we found significantly fewer \textit{adamts9}^{−/−} fish (4\%) than predicted by mendelian ratios (25\%). The surviving mutant fish had a significant male bias (87\%). The females that were found (2\%) had small, ovaries with few stage III and IV oocytes compared to WT counterparts of a similar age and body length. No female-like fish were observed to release eggs and no ovulated ova were observed in histological sections.

Astoundingly, the remaining mutants (11\%) did not appear to have normal testis or ovaries, but instead had an empty, ovarian membrane-bound compartment that filled the abdominal cavity. On the periphery of these ovary-like compartments, seminiferous tubules and various spermatocytes including normal developed sperms were observed. To our knowledge, this is the first report of an established \textit{Adamts9} knockout vertebrate line and the first description of how sex determination and gonadal structure are affected, highlighting the importance of \textit{Adamts9} function in the development of gonads and the value of zebrafish as a model organism.
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By

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I would like to dedicate this work to my husband and late grandmother who have always encouraged me to pursue my passion and to never stop learning, without them none of this would be possible.
I would like to extend my gratitude to my committee members for their valuable advice, it has been an incredible experience to develop professionally with you all. To Dr. Yong Zhu, the value of the opportunity that you have extended to me cannot be overstated. Thank you for helping me realize my potential and how to achieve goals that I have never imagined. Thanks to all the undergraduates who have helped with this project, specifically Mr. Zachary Adam Roach, Ms. Mckenzie Byrnes, and Ms. Lane Ryan, for their help with conducting survival experiments and histological analysis. Finally, I would like to thank my family for their constant love and support. Thank you!
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Introduction

Zebrafish are a widely used model organisms for genetic and developmental studies because they develop rapidly, have a short maturation time, are relatively inexpensive to upkeep and ease of genetic modifications, but it is unclear how ovarian fate is determined and maintained. Unlike mammals who rely on chromosomal composition and the dimorphic expression of sex determining region of the Y chromosome (Sry), zebrafish laboratory strains (AB and Tubingen) are missing a definitive sex-determining locus and rely on multiple genes to determine sexual fate (for Review see Liew and Orbán 2014). Although the chromosomal “master-switch” is lost or reorganized in domesticated zebrafish, evidence shows that downstream genes known to regulate sexual development in mammals are conserved, like transcription factors SRY high mobility-group box related gene 9 (Sox9), Wilms tumour (Wt1), Forkhead box L2 (Foxl2), dosage-dependent region on X (Dax1) genes, as well as hormones like anti-Mullerian hormone (Amh) and estrogenic receptors and aromatase (Cyp19a1a) (von Hofsten and Olsson 2005; Sreenivasan et al. 2014; Sun et al. 2013; Chen et al. 2017). These genes, their downstream targets, and the number of primordial germ cells (PGCs) which inhabit the primordial gonad play a critical role in the development and maintenance of ovarian follicles in zebrafish. Genetic knockouts of PGCs in zebrafish result in the formation of male somatic tissue, although it did not contain any germ cells (Siegfried and Nüsslein-Volhard 2008). By studying genes evolutionarily conserved in the determination and maintenance of sex, we can begin to understand the underlying molecular interactions required to pattern and maintain the ovary, which will give researchers new approaches to address diseases like polycystic ovarian syndrome (PCOS), (POF) and infertility.
Sex Determination

Formation of the Bipotential Gonad

All vertebrates first form a bipotential gonad before adopting a male or female fate during embryogenesis (Polanco and Koopman 2007). The differentiation and migration of primordial germ cells (PGCs) to the presumptive gonad is essential for the function of the organ (Clelland and Peng 2009; Piprek et al. 2018; Raz 2002; Pearson et al. 2016). In mammals, PGCs are specified and segregated early in gastrulation, in mice this happens around 6 days post coitum (dpc) (Lawson et al. 1999). Three days later (around 10dpc), the gonadal primordium starts to develop from bilateral thickenings of the coelomic epithelium (Kusaka et al. 2010) and by 11.5 dpc proliferating PGCs have settled into the genital ridge (Harikae, Miura, Kanai 2013). Zebrafish primordial germ cells (PGCs) are also set aside early during gastrulation (<24hpf) and migrate to the presumptive gonad located along coelomic wall (Hartung, Forbes, Marlow 2014). By 14 days post fertilization (dpf) zebrafish germ cells are actively proliferating (Takahashi 1977), and by 35dpf most germ cells have become meiotically active primordial oocytes (Dranow, Tucker, Draper 2013).

Zebrafish are a gonochoristic species (two distinct sexes) of teleost that utilize transitory hermaphroditism, where all juveniles first form undifferentiated ovaries containing primordial oocytes before undergoing apoptosis to develop as testes, or developing as ovaries (Takahashi 1977). In the early testis, primordial oocytes undergo a highly dynamic, poorly understood process which leaves cavities that become filled with clusters of cells reported to be testicular germ cells (Orban, Sreenivasan, Olsson 2009). The number of PGCs and primordial oocytes play an active role in determining zebrafish sex as embryos and larvae that have reduced numbers of germ cells (Rodriguez-Mari et al. 2010, Tzung et al. 2015), or none (Siegfried and Nüsslein-Volhard 2008), result in male biased populations.
Differentiation of the Ovary

The molecular interactions involved in sex determination are complex and vary depending on species, but evidence suggests that evolutionarily conserved antagonistic mechanism exists between SOX9 expression, promoting testis formation and spermatogenesis (Sun et al. 2013; T. G. Deliagina et al. 2002), and ovarian development determined by Wnt/β-catenin, FOXL2, and estrogen aromatase (cyp19a1a) (von Hofsten and Olsson 2005; Sreenivasan et al. 2014; Chen et al. 2017). In XX mammals, β-catenin represses endogenous SOX9 signaling and facilitates ovarian follicular cell organization in the absence of Sry (Deliagina et al. 2002), promoting factors like WNT4 (wingless type MMTV integration site family, member 4), and FOXL2 (ForkheadboxL2). Ovarian fate in zebrafish is promoted by an oocyte-derived signal, as depletion of oocytes in adult ovaries results in the regression of ovarian tissue and production of sperm (Rodriguez-Mari et al. 2010; Dranow, Tucker, Draper 2013). However, this oocyte-derived signal and the downstream genes responsible for the rearrangement and differentiation of gonadal tissue has not been characterized.

Sex Reversal in Adult Vertebrates

Sex reversal in mammals usually refers to the phenomenon where the phenotype of an individual doesn't correspond to their genotype (XX or XY), which is the result of gaining or losing function of the Sry gene during embryonic development (Warr and Greenfield 2012). Once an adult, the ovary and testis are thought to be terminally differentiated, but a 1999 knockout of the estrogen receptor ESR1/2 (J. F. Couse et al. 1999), and a 2009 report on conditional knockouts of transcription factor L2 (FOXL2) with ESR1 in mice (Uhlenhaut et al. 2009), suggests that sexual identity must be actively maintained throughout life. The XX
knockout mice would initially develop ovarian tissue as adolescents, but between 8 weeks and 2.5 to 7 months mutant ovarian follicular cells (Granulosa and Theca) appear to transdifferentiate into testicular-like support cells (Sertoli and Leydig), as well as reorganization of ovarian stroma into structures that resemble spermatogenic tubules (Uhlenhaut et al. 2009; Couse et al. 1999).

While it is known that hormonal signaling induces proliferation, organization, and death of cells in the ovary during oogenesis, ovulation, and formation of the corpus luteum, the function of genes that alter the extracellular matrix (ECM) during this time remain uncharacterized. Molecular signaling that alters the ECM mediates cell adhesion, therefore regulating gonad morphogenesis and embryogenesis. Cleavage of cell-surface and ECM molecules by matrix metalloproteases (MMPs), or ADAMTS (A disintegrin and metalloprotease with thrombospondin type-1 motifs) is essential for embryonic development, organ morphogenesis, bone remodeling, nerve growth, angiogenesis, and ovulation (Handbook of proteolytic enzymes, 2012). Expression, trafficking and turnover of proteases that alter the ECM are heavily regulated by: growth factors like TGF-β, cytokines like TNF-alpha, hormones, endogenous inhibitors, precursor zymogens, as well as cell-cell and cell-ECM interactions (Handbook of proteolytic enzymes, 2012). Aberrant expression or misregulation of these proteases results in many common diseases and disorders like cancer, arthritis, ulcers, fibrosis, infertility, and many others (Handbook of proteolytic enzymes, 2012).

**Extracellular Matrix (ECM) as an Inductive Scaffold**

The extracellular matrix (ECM) is a highly dynamic, acellular framework comprised of bioactive molecules and secreted proteins that provides tissue-specific structure to organs (Brown and Badylak 2014). Cells not only contribute to the content of the ECM but can also
receive signals from the ECM through mechanical and chemical means, which is essential for cells to respond to their environment, and key for embryonic development and homeostasis (Theocharis et al. 2016).

Secreted proteases that alter the ECM in response to hormonal signaling have been analyzed in the developing gonad and cells of mice, humans, and macaque (Piprek et al. 2018; Rosewell et al. 2015; Peluffo et al. 2011]. Matrix metalloproteases (MMPs) and members of the ADAMTS (A disintegrin and Metalloprotease with Thrombospondin Type1 motifs) family were found to be induced in the ovarian follicles (Piprek et al. 2018; Rosewell et al. 2015; Peluffo et al. 2011). Among all studied metalloproteases upregulated in distinct cell-types, ADAMTS (A disintegrin and metalloprotease with thrombospondin type-1 motifs), like MMPs, have nonredundant function during sexual differentiation (Piprek et al. 2018). In mice, ADAMTS were selectively expressed both in XX/XY stromal and support derived cells (Piprek et al. 2018). Out of all the family members expressed, ADAMTS1 and ADAMTS9 were more ubiquitous than others, suggesting they may play a role in the development of the bipotential gonad (Piprek et al. 2018). Studies on the expression of matrix metalloproteases in the gonad during differentiation is lacking. Only a study from our lab on the expression of proteases in the follicular cell layer at the terminal stage before ovulation has been reported recently (Liu et al 2018).

**ADAMTS9 in Fertility**

Adamts9 is a cell-autonomous antiangiogenic metalloprotease that is widely expressed throughout mouse development (Jungers et al. 2005). It is released into the extracellular space when activated by furin-cleavage (Koo et al. 2006), where the metalloproteinase motifs digest large, hydrophilic ECM proteins like versican and aggrecan (Kern et al. 2010; McCulloch et al. 2009; Demircan et al. 2013), generating bio-active fragments that are required for collective cell migration (Jungers et al. 2005), differentiation (Nandadasa et al. 2015), and cell death
(McCulloch et al. 2009) throughout murine embryogenesis. More so than any other ADAMTs protein, Adamts9 domains are highly conserved from its ortholog in C. elegans to mammals (Somerville et al. 2003, Clark et al. 2000). Its function is required for collective cell migration of PGC’s in C.elegans and Drosophila (Blelloch and Kimble 1999; Ismat, Cheshire, Andrew 2013) and it is highly expressed in the mouse genital tubercle (Jungers et al. 2005). Due to embryonic lethality in knockout mice models (Enomoto et al. 2010), the function of this protein during gonad development, differentiation, and maintenance is unknown. However, Adamts9 expression is induced by LH and hCG in mature follicles in humans and monkeys during early ovulation (Rosewell et al. 2015, Peluffo et al. 2011). Interestingly, zebrafish retain this characteristic adamts9 follicular cell expression directly preceding ovulation, but this pattern is significantly downregulated in the follicular cells of anovulatory PGRKO zebrafish (Liu et al 2018).

To determine the function of Adamts9 in the zebrafish ovary, we generated global knockouts using CRISPR/Cas9, then characterized the effect on fertility.
Hypothesis

Due to evolutionarily conserved expression of Adamts9 during the differentiation of the gonad (Bleloch and Kimble 1999; Ismat et al. 2013; Jungers et al. 2005) and expression in adult ovaries (Bakkers 2011; Peluffo et al. 2011; Piprek et al. 2018; Liu et al. 2018), we hypothesize that adamts9 plays a nonredundant role during gonadal development and ovulation in zebrafish.

Specific Aims

- **Aim 1: Generate adamts9⁻/⁻ zebrafish model**
  - Design CRISPR/cas9 guide RNA.
  - Identify frameshift mutations
  - Establish mutant lines.

- **Aim 2: Characterization of adamts9⁻/⁻ mutants**
  - Analyze survival rate, genotype and sex ratios of adamts9(+/⁻) in cross fry.
  - Analyze histology of gonad
Materials and Methods

Designing gRNA

CRISPR (Clustered Regular Interspaced Short Palindromic Repeats)/Cas9 is a primitive immune system originally found in bacteria, where small pieces of single stranded viral genetic material are recognized, reverse transcribed and stored by the bacteria inside a specific DNA locus (Mojica, F., et al., 2016). When previously infected bacteria meet the same kind of virus again, the stored pieces of viral DNA are transcribed and used as guides to bring nuclease Cas9 to the complementary viral sequence. When the guide binds sufficiently, Cas9 creates a double stranded break in the viral DNA, destroying it. We use this technology in zebrafish to generate mutations that help us understand the mechanisms underlying hormonal signaling and pathways. Instead of targeting viral DNA sequence, guides are designed to be complementary to an exon within the gene of interest. An exon close to the transcription start site needs to be targeted in order to generate a frameshift mutation that results in a nonfunctional truncated protein. All gRNA target sites were within an exon of Adamts9 (Table 1), spanning a palindromic restriction enzyme site, upstream of a three-base pair motif that is crucial for Cas9 to recognize before the gRNA can bind and a double-stranded cut is made. The three-base pair motif that is crucial for Cas9 to recognize before gRNA can bind is called the PAM (Protospacer Adjacent Motif), because it sits between the interspaced palindromic repeats from which the CRISPR/Cas9 system was developed. The PAM is any nucleotide followed by two guanine residues and is represented by the nucleotide sequence “NGG”. After Cas9 finds the PAM motif, the gRNA can bind and Cas9 makes a double-stranded break that prompts intracellular repair mechanisms to fix DNA (Mojica, F., et al., 2016). These repairs often result in the insertion or deletion of base pairs, and potentially generate premature stop codons that yield frameshift
mutations. Guides were compared (using BLASTN) against the whole genome, and sequences that had low homology to other sites were chosen in order to reduce off-target cleavage.

**Linearization**

T7 gRNA (46759, Addgene) vector codon optimized for zebrafish was cloned using a modified protocol from (Jao et al.2013, Ran et al., 2013). Briefly, 16.4 uL of the vector (202 ng/uL) was linearized by sequential digestion, first with 0.8uL of BglII (10ug/uL) and Sall (10ug/uL), overnight at 37˚C (20uL total volume, 2uL 10x OPTIZYME buffer 3). Digestion products were purified using Qiagen spin columns and DNA was eluted with 20uL of sterilized distilled water (SDW). The second digestion was done with 3.2 uL of BsmBI (10ug/uL), and 2uL of DTT (20mM), and the total volume was brought to 40uL with SDW. The reaction was allowed to run overnight at 37˚C and linearization was validated by gel electrophoresis. Successful samples were inactivated at 80˚C for 20 min, column purified, and linearized plasmid concentrations were quantified using Nanodrop 2000.

**Ligating Oligos**

Oligo sequences for desired gRNA forward and reverse sequences were ordered from Sigma (Table 1) and suspended in sterilized water to a final concentration of 100 µM. Oligos (sgRNA forward 1uL, sgRNA reverse 1uL) were phosphorylated using T4 polynucleotide kinase (PNK,1 uL) and reaction were done using a thermocycler with the following settings: 37˚C for 30 min; 95˚C for 5 min; ramp down to 25˚C at -0.08 °C/second.
After phosphorylation, annealed oligos were diluted (1:200) in order to be ligated into the linearized pT7-gRNA vector. Briefly, Diluted oligos (1uL), 2X rapid ligation buffer (2.5 uL, Promega), linearized vector (1uL), and T4 ligase (0.5uL) were allowed to react at room temperature for 30 minutes before bacterial transformation.

**Bacterial Cloning**

**Transformation**

Homemade competent *E. coli* cells were used for all bacterial cloning experiments. Ligation mixture (3 µl) was added to 50 µl competent cells (50 µL), which were incubated on ice for 30 minutes, heat shocked at 42°C for exactly 90 seconds, then placed on ice for 2 minutes. Luria Broth (250 µL) was added to bacteria and incubated at 37°C, 180 rpm for one hour. Then, bacterial cells were spread at an agar plate and incubated overnight at 37°C.

**Miniprep**

Miniprep (QIAprep Spin Miniprep Kit, Qiagen, USA) was used to purify plasmids according to modified manufacturer’s protocol. Briefly, colonies from freshly grown selective ampicillin plates were inoculated in 7mL of LB in sterile conditions. Samples were cultured for 18-24 hrs at 270 rpm at 37°C. After incubation bacterial cells were harvested by centrifugation (3000 x g for 10 min at 4°C), supernatant removed, and pellet was resuspended in buffer P1 (250 µL) and P2 lysis buffer (250 µL) and thoroughly mixed by inversion. Buffer N3 was added to samples (350 µL) which were immediately mixed by inversion to avoid localized precipitation. In a table top microcentrifuge, samples were spun for 10 min at 13,000 rpm (17,900 x g) to form
a compact pellet containing large protein, leaving nucleic acids in the supernatant. Next, 800 µL of the supernatant containing the plasmid was pipetted on a QIAprep 2.0 spin column, centrifuged for 30-60s (9,000 x g), and flow through discarded. Columns were washed by adding 500 µL of PB buffer and centrifuged again, discarding the flow through. Then, samples were washed with 750 µL of PE buffer by centrifuging for 30-60s (9,000 x g), and the flow through was discarded. An additional centrifugation was completed to remove residual wash buffer. Purified plasmid was eluted by placing 30 µL of sterilized water on the center of each spin column which was allowed to penetrate the silicone membrane for 1 minute, then centrifuged for 1 min (9,000 x g). Plasmid concentrations were determined using Nanodrop 2000.

Animal Husbandry

Wildtype (wt) *Danio rerio* (zebrafish), propagated in our lab are a Tübingen strain originally obtained from the Zebrafish International Resource Center, and are housed in automatically controlled rearing systems for zebrafish (Aquatic Habitats Z-Hab Duo systems, Florida, USA). Fish were bred in polycarbonate breeding tanks and reared in multiple 700mL liter holding tanks with water temperature around 28°C, salinity conductivity from 500-1200µS, and pH around 7.2. The photoperiod was set to 14hrs of light (9:00 AM- 11:00 PM) and 10 hours of dark (11:00 PM- 9:00 AM). Fish were fed commercial food with high protein content (Otohime B2, Reed Mariculture, CA, USA) until satiation and supplemented with freshly hatched artemia twice daily (Brine Shrimp Direct, UT, USA). East Carolina University’s Institutional Animal Care and Use Committee (IACUC) has approved all experimental protocols.
Spawning and Fertility

To determine spawning and fertility, a male and female were in labeled polycarbonate breeding tanks on a work-bench, separate from the automatic rearing system. Every afternoon they were fed as previously described. An hour before the lights turn off, their water was changed, and an insert was placed inside of the tank that would allow eggs through to the bottom, preventing adults from eating the released eggs. The morning of next day (11AM), the water was changed. The collected eggs were placed in an incubator (28°C) and grown for an average of 10 days before being put on the automatic system (fry were fed ground commercial food starting 3dpf, freshly hatched artemia 7dpf). If paired fish didn't spawn after one week, they were placed with another partner that has spawned previously. The partner was replaced with another of the same sex, before repeating the process with the opposite sex. Fish were considered infertile when no spawning was observed after being paired with six partners (3 males and 3 females). To determine the average number of eggs released per day, fish were bred daily for two weeks to ensure that they were reproductively healthy before their eggs were counted for the study.

Generating Mutant Lines

Microinjection

Approximately 100 ng/µl of sgRNA and 150 ng/µl of Cas9 mRNA were co-injected into 400 single-cell embryos within 30 minutes after spawning. Microinjection was driven by compressed N2 gas under control of PV820 Pneumatic PicoPump (World Precision
Instruments, Sarasota, FL), accomplished using a microcapillary pipette attached to a micromanipulator, under a Leica MZ6 microscope (Leica, Germany).

**Mutation Screening**

Thirty embryos (F0) per microinjected cohort were collected for estimating efficiencies of gRNAs at 48 hours post fertilization (hpf) to find. Genomic DNA was extracted from embryos using 200 µl of 50 mM NaOH at 99°C. The supernatant (containing DNA) was obtained by centrifugation, which was used directly in PCR. PCR primers (Sigma), designed around the guide were used to amplify the target region. PCR conditions: Initial denaturation for 2 min at 94°C; 35 cycles of denaturation at 94°C for 30 second, annealing at 57°C for 30 second, elongation at 68°C for second, then a final step at 68°C for 10 minute. If pooled F0 embryos had undigested bands, the remaining embryos in their clutch were raised to adult and screened using genomic DNA obtained from tail fin clippings dissolved in 40ul of NaOH at 99 °C. F1 embryos were screened as previously described at 48 hpf. Bacterial cloning was used to isolate mutations from heterozygous samples, and they were sequenced at ECU’s genomic DNA sequencing facility. The remaining clutch from the heterozygous F1 embryos with frame-shift mutations were grown to adulthood. Individuals were screened by fin clipping and those heterozygous for the same mutation were crossed. F2 embryos were collected and grown into adulthood where they were screened by fin clipping and RE digestion. F2 and subsequent generations were crossed, grown into adults and screened as previously described.

**Survival Assay**

To determine the survival rate of fry, adult fish were spawned daily for two weeks before their fry were collected for study. Several pairs and multiple clutches from each adult pair were
used for the experiment. Eggs collected from each parent were counted, labeled, and separated into breeding tanks containing an average of 40 eggs per tank. Survival after 24hrs, 7 days, and 14 days was found by tracking the number of dead fish found during the daily feeding and water change. The survival rate was calculated by taking the percentage of surviving fish from the initial egg count.

RNA Extraction

Tissue Collection

The night before dissection, several spawning tanks with a pair of male and female fish for each were set up, male and female fish were separated by a divider, so follicles would be mature and ovulate in females, but the males would not be able to stimulate the release of her eggs. At 9:00AM fish were sacrificed by decapitation. Their brain and gonad were immediately placed in 1.7mL RNase-free microcentrifuge tubes (GeneMate) on ice, containing (200 µL for brain; 500 µL for the ovary) RNAzol (Molecular Research Center, Inc., OH. Catalog: RN 190).

RNA Extraction

Total RNA was extracted using RNAzol and a Qiagen RNeasy kit according to manufacturer’s protocol. Briefly, after sonification and following the first precipitation with water, BAN solution (4-bromoanisole) was added to purify RNA and eliminate genomic DNA. A subsequent precipitation was conducted, and an equal volume of 100% isopropanol was added. After 15 minutes, the samples were spun (12,000g for 10 min). The supernatant was disposed, retaining the pellet, which was washed with 75% ethanol twice by centrifuging (3min at
12,000g). The RNA pellet was dried and then solubilized with 10µL of RNase-free water. The approximate concentration and purity of samples were determined using a Nanodrop 2000 Spectrophotometer.

**First Strand cDNA Synthesis**

Reverse transcription was performed using SuperScript III Reverse Transcriptase following the manufactures instructions (Invitrogen, Carlsbad, CA) using a 10µl reaction volume. Specific sets of primers targeting *adamts9, efla, and actb* were used (Table 2). PCR efficiency and authentic products were confirmed by gel electrophoresis (2% agarose gel, 2µL of EtBr/30mL) and sequencing.

**Histology**

**Preparing Tissue Samples**

Fish were sacrificed using ice water in accordance to IACUC guidelines. Once heart function ceased, they were placed in 10x buffered formalin (Fisher Scientific) for 24-48 hours before measurements were taken and the gonad dissected. The gonad was then placed in formalin for an additional 24 hrs. After gonads were fixed, they were prepared for histological analyses with either paraffin or JB4. These mounting mediums share the dehydration process but use different procedures for infiltration.

**Dehydration**
Samples are rehydrated in deionized water and for 30 minutes. Then, starting with 70% ethanol, are dehydrated at increasing concentrations of ethanol (80%, 90%, 100%) for 30 minutes. The lowest and highest concentrations were repeated an additional 30 minutes. All samples were placed on a tissue rocker (Fisher Scientific) set at medium speed.

**Paraffin Infiltration and Embedding**

Samples were placed in Xylene (StatLab) for 30 minutes on the tissue rocker, then an additional 30 minutes in an incubator (55-60 °C). Paraffin (Fisher Scientific, T565) was added in a 1:1 ratio with xylene, placed in the incubator, and gently shaken every 10 minutes for 30 minutes. Samples were then transferred to fresh, 100% paraffin, shaken every 15 min for 1 hour in the incubator (paraffin was changed after 30 min), before being placed in a block mold, covered with fresh paraffin, and allowed to cool overnight.

**JB-4 Infiltration and Embedding**

Following manufacturer’s instructions from JB4- kit (00226, Polysciences Inc., PA), JB-4 solution A was made to 100mL total volume using exactly 1.25g of Benzoyl peroxide. The dehydrated gonads were placed in 1mL of JB-4 solution A on the tissue rocker for 1hr, with solution changed after 30 min. After an hour in JB-4 solution A, samples were removed from solution and oriented in JB-4 block holders (15899-50, Polysciences Inc., PA). The embedding solution was freshly made using 50 mL of cold JB-4 solution A and 2mL of JB-4 solution B, which was mixed briefly then immediately transferred into the block using a disposable pipette. Blocks were held on a Styrofoam conical tube holder, and samples were allowed to polymerize for 72 hours before sectioning.
Mounting

Paraffin and JB4 embedded samples were cut into 10 µM and 5 µM sections, respectively using microtome (Reichert-Jung 2030) and coated metal blades (95057-834, VWR). Sections were placed on top of water covered glass slides (12-550-41, Fisher Scientific), then set on a heat block (34 °C). Meyer’s Albumin solution was rubbed on slides to help adhere paraffin sections to the untreated glass slides. The sections were dried overnight on the heat block, or until sufficiently baked (6hrs at 40C).

Deparaffinization

Samples were deparaffinized by exposing slides to 100% xylene for 5 min, followed by changing the xylene and repeat exposure for another 5 minutes. Samples were then transferred to 100% ethanol for 10 min, and then 95% for 10 min, each of these steps had samples changed to fresh solution after 5 min. After a bath in 75% ethanol for 5 min and a final bath water for 5 min, the slides are ready for staining.

Hematoxylin and Eosin Staining

H&E staining was done immediately following the last step of deparaffinization. Slides were placed in Hematoxylin (SH26, Fisher Scientific) for exactly 90 seconds, then washed in running water for 4 minutes. Then slides were placed in Eosin (95057-848, VWR) for exactly 1 minute before being placed in increasing concentrations of ethanol for 4 minutes each (95% and 100% EtOH), slides were changed to fresh solutions after 2 minutes. Slides were then kept in xylene until cover glass (12-541-B, Fisher Scientific), was mounted using Permount (SP15-100, Fisher Scientific).
**Immunohistochemistry**

After fixation and sectioning, sections of *adamts<sup>-/-</sup>* and *adamts<sup>+/+</sup>* were used for immunohistochemistry using Vectastain Elite ABC kit (PK-6101, Vector Laboratories, CA). Before incubation with primary antibodies, antigen retrieval was conducted using 0.1% testicular hyaluronidase (H3506, Sigma, USA) in 30mM NaOAc and 125mM NaCl (pH=5.2) for 2 hours at 37°C. Slides were rinsed with distilled water for 5 min before being washed with 1x PBS (10x PBS: NaHPO<sub>4</sub> 10.9g, NaH<sub>2</sub>PO<sub>4</sub> 3.2g, NaCl 90g, dissolved in distilled water and brought pH to 7.4. 1x PBS: 100 mL of 10x and 900 mL of distilled water) three times for 3 minutes each. After washes, slides were treated with the blocking solution (250mL distilled water and 2.5mL of 30% H<sub>2</sub>O<sub>2</sub>) for 30 minutes and were washed with 1x PBS as described previously. Normal goat serum was made according to manufacturer’s instructions and incubated for 30 minutes before applying the primary antibody. Sections were incubated with primary antibodies (Triple point biologics, Inc., OR) RP1(250x, RP1-ADAMTS-9) and RP5 (1000x, RP5-ADAMTS-9) overnight at 4°C. The next day slides were washed with 1x PBS as described earlier. The secondary antibody was made according to manufacturer's instructions and incubated with slides for 30 minutes. While slides were incubating with the secondary antibody, the AB solution was made according to manufacturer which states the solution needs 30 min of rest before being applied. Slides were washed with water and 1x PBS again before applying AB solution and incubating for 30 min. Another wash with 1x PBS was done before developing slides in 250mL, 50mM Tris (pH 7.2) containing 100mg DAB (3,3’-Diaminobenzidine. D12384, Sigma) and 75mL of 30% H<sub>2</sub>O<sub>2</sub>, for exactly 2 minutes. Adjacent slides were stained with H &E for structural comparison.
Statistical Analyses

Students’ t test (unpaired) was used to determine significant differences between two data sets (expected vs experimental percentages of genotypes) and one-way ANOVA followed by Tukey’s Test was used for comparing more than three data sets (GSI%) with GraphPad prism software (GraphPad Software, Inc., San Diego, CA).
Results

Generating mutant lines

gRNA targets

The *adamts9* gene is located on reverse strand of chromosome 11 consisting of 203,257 base pairs (bp) and 30 exons (Busch-Nentwich, et al., 2013). Although teleost has gone through an additional genome mutation, there is only one paralog of *adamts9* in the zebrafish genome (same as mammals). Target regions were designed on exons 2, 3, 5 and 7 (Fig 1A), and PCR primers were designed to flank this region asymmetrically (Fig 1A). It is vital to our experiment that Cas9 gRNA targets span a restriction enzyme site and that primers are not equidistant from that region (Fig 1A). This design makes screening straightforward due to destruction of the recognized RE motif by insertions/deletions the cell makes in the process of repairing double stranded breaks. Homozygous *adamts9*+/− mutants leave a single uncut band after enzymatic digestion while wild type (*wt; adamts*+++) samples retain the site and are cut into distinct sizes, resulting in two visible bands when separated on an agarose gel (Fig 1B). Heterozygous (*adamts9*+/-) samples have three bands since they have both WT and homozygous alleles (Fig 1B). Multiple mutations were generated, but two separate frameshift deletions Δ10 and Δ11 were selected for propagation (1C).

Propagating *adamts9* mutant lines

Two *adamts9* mutant lines with 10 bp deletion and an 11 bp deletion were selected for propagation (Fig 2). Heterozygous F1 adults with the same mutation were crossed, their progeny (F2) were grown into adulthood, and then were screened by fin clipping and RE digestion.
Verification of Knockout

Verification of mutation in mRNA

Fish homozygous for the 10 or 11 bp deletion were generated by in-breeding heterozygous F1 and subsequent generations (Fig 2). The mutations were verified by purifying brain and ovarian RNA, performing RT-PCR amplification, and then DNA sequencing (Fig 3, Table 2). Genomic mutations were found to be conserved in the cDNA sequence of homozygous samples (Fig 3). The cDNA sequence generated from the Δ10 and Δ11 suggests the formation of a truncated protein that maintains the signal-peptide and prodomain repeats (in silico) but is missing everything following amino acid residue 557/555 (Fig 4A), including the active site motif at amino acid 573.

Verification of mutation using IHC

The Adamts9 protein is composed of 1643aa with multiple domains (Fig 4A). Zebrafish Adamts9 has a signal peptide and prodomain, but is suspiciously missing the GON domain which is a unique characteristic of the subfamily consisting of adamts9 and adamts20 genes (Somerville, et al., 2003). It has not been determined if this domain is at a different location within the protein sequence or the significance of the lost domain in zebrafish.

To verify whether the protein was generated and if the metalloprotease domain remained intact, antibodies that recognize different epitopes of the Adamts9 protein were used. The RP1 antibody (human sequence is 41% identical to zebrafish) recognizes the pro-peptide domain, and RP5 (85.7% identical) recognizes the catalytic domain (Fig 4C-D). H&E staining of adjacent slides shows the structure of the oocyte, especially follicular cells in WT (Fig 4B-b) and Adamts9−/− follicles (Fig 4b'). Immunohistochemistry (IHC) using the RP1 antibody revealed that
WT follicles had Adamts9 expressed specifically in the follicular cells (Fig 4C). Some background stain is apparent but is negligible when compared to signal from follicular cells (Fig 4c). Adamts9−/− follicles of similar size and structure to WT (Fig 4c′) have no detectable signal from the follicular cells.

IHC with the RP5 antibody also show that Adamts9 is expressed specifically in the follicular cells, but in a distinct discontinuous pattern in wildtype zebrafish (Fig 4D-d). When looking at the same antibody stain on follicles of similar size from adamts9−/− ovary, the structures are similar (Fig 4b′ and Fig 4d′) but there is no detectable signal from the follicular cells (Fig 4d′). We have not analyzed whether adamts9−/− can go through maturation and ovulation, due to limited number of female mutants.

**Characterizing Knockout**

**Heterozygous crossings yield low percentage of homozygous progeny**

It is expected that when crossing two heterozygous individuals their progeny would be approximately 25% homozygous for the mutation. F2 and subsequent generations of heterozygous fish were in-crossed and screened to find the genotypic ratio. The overall percentage of homozygous mutants was found to be 11% 1-month post fertilization (mpf) and 4% at 3 mpf (Table 3), significantly less than what was expected.

To understand why the number of mutants generated from heterozygous in crosses were so low, we investigated whether there was a difference in the amount of eggs released and monitored their survival for a week. There was no difference between WT in cross or heterozygous in cross clutches with regards to the average number of eggs released/day (Fig
5A) and the survival of those eggs for the first week (Table 4), indicating female (+/-) fish have similar fertility to wt. At 14dpf however, there is a significant decrease in the survival of the heterozygous progeny (Table 4). We have not yet determined the survival rate after 14 dpf, or the mechanism driving the increased death rate of fry at this time.

Most *adamts9*^/-^ fish are male

Due to difficulty finding homozygous fish, the number of embryos per day was quantified between WT and Adamts9 (+/-) pairs (Fig 5A). No significant difference was found between the average number of eggs released daily.

The ratio of each sex was quantified with fish at least 3 months post fertilization (mpf) to identify if there was a sexual bias in the mutant clutches (Fig 5B). Heterozygous and WT fish were found to have similar ratios of male to females (within the same tank was 2:1). Communal tanks house an average of 30 fish per tank, but the homozygous fish were overwhelmingly male (37/45; 80%). Only half of homozygous mutant males (24/37; 65%) were able to fertilize WT eggs (data not shown). Although a few homozygous females (3/45; 7%) have been found, none of them were observed to spawn. Strikingly, more individuals had empty, fluid-filled membranous shells (5/45; 11%) than ovaries (Fig 5B). These fish are termed as intersex because their secondary sexual characteristics outwardly resemble females (e.g. body shape and color), but their gonadal shells were observed to produce mature sperm upon histological analysis. Closer examination of spawning behavior and frequency is needed to determine the nature of subfertile *adamts9*^(-/-)^ males.
**Adamts9−/− males are like WT**

Adult (7 mpf) *adamts9−/−* males are similar to their WT counterparts' body shape despite spinal deformity (Fig 6 columns A and B). Dissected testes have similar morphology (Fig 6A. III and 6B. III). In the WT sample, testis had very distinct cysts (Fig 6A. III), whereas the homozygous cysts were less defined (Fig 6B. III). Closer examination is needed to determine if less-defined cysts are a phenotype of the mutation or an individual trait.

Histological analyses of the testis further reveal that the relative size and amount of the spermatogonia to the density of mature spermatozoa are similar (Fig 6A. IV and B. IV). No obvious difference between WT (Fig 6A. IV) and *adamts9−/−* (Fig 6B. IV) males was observed. There seems to be extra space between cysts within the homozygous testis, but this may be an artifact of the fixation process. More work needs to be done to quantify the amount of mature sperm produced, average lumen size, the number of cells/cyst, and the expression of common markers indicating male gonad formation (e.g. *amh*, *sox9a*, or *dmrt1*).

**Adamts9−/− females have malformed ovaries**

Overall, adult (7mpf) *adamts9−/−* females resemble the body shape of their WT counterparts despite spinal deformity (Fig 6 columns C and D). However, the mutant female ovary (Fig 6D. II) covers less surface area that WT (Fig 6C. II) when comparing their open abdomens. Body color is not taken into consideration because the homozygous fish were fixed prior to photographs, whereas the WT sample was freshly sacrificed.

Dissected WT ovaries show hundreds of visible eggs and very little interstitial tissue (Fig 6C. III). Mutant females have less of these large ova and more interstitial space (Fig 6D. III). Differences
in vasculature was not observed because the mutant gonad was fixed before photographs taken.

Histological analyses of WT ovaries show that hundreds of eggs concurrently develop, with the most eggs in an intermediate stage (II-III), and less in (early) stage I or (late) stage IV (Fig 6C. IV). Homozygous ovaries were smaller overall, had fewer vitellogenic oocytes (some degrading), and relatively high amounts of early stage I oocytes (Fig 6D. IV).

We have not yet completed analyses of the developmental differences between adamts9−/− and WT females. Currently, we are conducting histological analyses of gonad during development from 1-3mpf, counting the number of ova in each developmental stage, or ascertaining whether apoptosis/other degenerative pathways were activated differentially during oocyte development.

**Adamts9−/− intersex gonads lack internal structure**

Intersex fish (Fig 6 E) outwardly resemble WT female fish in color and body shape, retaining slight spinal malformations characteristic of adamts9−/− mutants (Fig 6 E. I). Remarkably, upon removal of the abdominal wall, the body cavity appears to be empty, except for organs that normally surround the gonad like the intestines, stomach and swim bladder, which were morphologically similar to WT despite excess space (Fig 6E. II). Surprisingly, when the mutant was submerged in water to take higher resolution pictures, an inflated ovarian like structure could be observed in the empty space (not shown). There is a clear absence of tissue and cells within the membrane when comparing this structure to adamts9−/− ovaries (Fig 6E. III). Upon histological analyses, cysts with developing mature sperm were observed in most adamts9−/− intersex samples (Fig 6E. IV, Fig 7). However, one critical sample had both early-stage ova and testis-like cysts (Fig 8).
Others have demonstrated the ability of adult female zebrafish to develop into fertile males by partially depleting germ cells in adults (Dranow et al., 2013). It is possible that the empty gonad shell results from a degenerating ovary, but more work analyzing expression of ovarian-determining factors (e.g. FoxL2 and cyp19a1a) during development of bipotential gonad in adamts9<sup>−/−</sup> mutants needs to be done to determine when and how female development is altered.

**Adamts9<sup>−/−</sup> have multiple uncharacterized phenotypes**

When screening adult fish (3-7 mpf), homozygous mutants were visibly smaller than their WT counterparts (data not shown). Mutants were so small that they were grown in low-density tank conditions (>10 fish/tank) for an average of two weeks in order to be large enough for spawning experiments (data not shown). It has not been determined whether the fish have already begun to mature before separating the mutants from the higher-density communal tanks, or how this affects sexual development.

Most adult adamts9 mutants (41/47) had visible spinal deformities in the midbody and/or caudal peduncle (Fig 6D and E) in a left/right direction. There have been WT fish that also have spinal deformities, but their malformations are more dorsal/ventrally (not shown). The identity of affected vertebrae, when the malformation is first visible, and differentially expressed factors have not been determined
Discussion

**Heterozygous crossings yield low percentage of homozygous progeny**

Indicative of congenital disease and early death, $adamts9^{-/}$ zebrafish are found in ratios well below that predicted by mendelian genetics (Table 3). Many $adamts9^{-/}$ zebrafish have lateral spinal deformities in the midbody or caudal peduncle and grow slower compared to wildtype fish (Fig 6 D. I & E. I). Zebrafish are known to continuously grow until around 6 months post fertilization (Singleman and Holtzman 2014). Growth retardation and spinal curvature often indicate congenital cardiac and renal abnormalities in humans (Janicki and Alman 2007). Adams9 is known to be lost in hereditary renal tumors (Clark et al. 2000) and $adamts9^{-/}$ mice have valvular and aortic abnormalities (Kern et al. 2010). The molecular mechanisms and the function of $adamts9$ in adult vertebrates is unknown due to embryonic lethality of homozygous $adamts9^{-/}$ mutations in mice (Jungers et al. 2005). Our study provides the first global knockout model that will permit investigation of $adamts9$ function in adult vertebrates.

Numerous gene association studies have implicated that a gene variant of ADAMTS9 (rs4607103) is associated with insulin resistance and beta cell function in human type II diabetes (Meigs et al. 2008; Tam et al. 2013). Evidence from *C. elegans* reveals that GON-1, an $adamts9$ ortholog, is required for normal insulin signaling in beta cells and peripheral tissue (Yoshina and Mitani 2015). Insulin-like signaling in mutant phenotypes were partially rescued by overexpression of the c-terminal GON domain (Yoshina and Mitani 2015). The GON domain is incorporated in secreted proteins that may indirectly or directly impact insulin signaling of many species (Benz et al. 2016). There are 19 metalloproteases in the vertebrate $adamts$ family, but
only \textit{adamts9} and \textit{adamts20} retain the c-terminal GON domain. (Robert P. T. Somerville et al. 2003). Transcripts of cDNA from the NIH Pubmed gene database suggest that the GON domain is absent in zebrafish \textit{adamts9} (Brunet et al. 2015). Regardless if zebrafish are naturally missing GON domain, functional compensation by related metalloproteases like \textit{adamts20}, could explain why \textit{adamts9}\textsuperscript{-/} zebrafish survive gastrulation. Insufficient insulin signaling, renal and cardiac abnormalities could be responsible for the increased death rate in \textit{adamts9}\textsuperscript{+/} clutches (Table 4).

**Most adamts9 (-/-) fish are male**

The plasticity of sex in teleost fish is well documented (Santos et al., 2017), but the plasticity of the mammalian gonad is less understood. Unlike mammals, zebrafish lab strains do not have chromosomally determined sex locus and instead rely on the relative expression of multiple factors to form testes or ovaries (Liew and Orbán 2014). Although teleost fish and mammals differ on the (master) upstream switch for sex determination, many key transcription factors that drive sexual differentiation like SOX9 and FOXL2 are conserved (Siegfried and Nüsslein-Volhard 2008). Mammalian gonads exhibit plasticity after birth in XX knockouts of FoxL2 and ESR1/2 mice (J. F. Couse et al. 1999; Uhlenhaut et al. 2009). As young adults (3-4 weeks) the female knockout mice had functional ovaries, but between 8 weeks and 3 months ovarian follicular cells (granulosa and theca) reorganized, and the expression profiles and morphology resembled testis-like support cells (sertoli and leydig) (J. F. Couse et al. 1999; Uhlenhaut et al. 2009). Genes activated by the FOXL2 transcription factor and estrogenic signaling may be responsible for activating proteinase expression that organizes the extracellular matrix to provide a viable environment for oocyte development. Molecules that are responsible for the physical remodeling of the extracellular matrix during sex determination in mice include metalloproteases, like ADAMTS and matrix metalloproteases (MMPs), whose
expression are cyclically induced by gonadotropins (Piprek et al. 2018). Adamts9 expression is induced by gonadotropins in human ovary (Rosewell et al. 2015) and zebrafish (Liu, et al., 2017) during the early or late stages of ovulation, respectfully. The expression of adamts9 in both forming and adult gonads suggests that this protein has a critical function in ovulation and sex determination. Currently, it is unclear whether the function of adamts9 during development and in adult tissue are similar, or if there are distinct functions during each time. Adamts9−/− zebrafish, although they have not been observed to ovulate, will be indispensable to determine the genetic pathways adamts9 utilizes in embryonic and adult tissue.

In zebrafish, the number of primordial germ cells (PGCs) are critical for the formation of ovaries (Siegfried and Nüsslein-Volhard 2008). Ablation or loss of PGCs entirely during development produce sterile males with no proliferating germ cells C. elegans (Tzung et al. 2015). But reducing the number of PGCs in adults prompts the differentiation into fertile males (Draper et al., 2007; von Hofsten and Olsson 2005; Webster et al. 2017). Like the phenotype that arises from reduced numbers of PGCs, adamts9−/− clutches are overwhelmingly male (fig 5B). It is possible that the PGCs in adamts9−/− mutants do not migrate or proliferate properly, similar to GON-1 knockouts in (Blelloch and Kimble 1999) and Adam-TS knockouts in Drosophila (Ismat, et al., 2013), which would give rise to male-biased populations in adult zebrafish. However, the mismigration or inability of PGCs to proliferate would not fully explain several females (Fig 5A) and female-like fish with a transparent organ with spermatogenic cysts along the periphery (i.e. intersex, Fig 7). The presence of retained oocytes along with the loss of tissue in intersex fish implies adamts9 may play a role in the regeneration somatic tissue surrounding oocytes.

The use of adult mesenchymal stem cells in regenerative medicine has gained popularity since their discovery (Roushandeh et al., 2017). Many organs that express adamts9 in zebrafish are derived from the mesenchyme, including the gonadal ridges, kidney, liver, bone and heart (Brunet, et al., 2015). These organs and connective tissues may also be afflicted in of adamts9(−
zebrafish. Adult female zebrafish ovaries retain germline stem cells (Wong et al. 2011). The environment where the stem cells reside (i.e. the niche) must be established and maintained in a specific combination to keep producing daughter stem cells (Gattazzo et al., 2014). The stem cell niche is the three-dimensional environment where cells are informed of their fate to promote proliferation and differentiation, which enables tissue to regenerate after injury (Demircan et al., 2013). The loss of tissue structure in adams9−/− intersex fish suggests that the ovarian somatic tissue lacked regenerative capacity upon oocyte degeneration, forming cysts that turn apoptotic. One key sample from our research with captures the thin walled organ with oocytes, somatic cell loss, and the development of sperm-like cysts, suggesting that adams9 (−/−) first develop as female before all oocytes degenerate (Fig 8). Whether adams9(−/−) intersex mutants lose tissue structure due to inability to regenerate somatic tissue is a question that has yet to be investigated.

Conclusion

This is the first vertebrate adams9 knockout to survive until adulthood, providing a model to study the function of adams9 in sex determination and the function of adult gonads. The ortholog of adams9, GON-1, is required for the formation of the C.elegan gonad (Blelloch and Kimble 1999), but its function in the formation and function of gonads in vertebrates has been previously unstudied due to embryonic lethality in mice and drosophila (Ismat et al., 2013). This study reveals the necessity of adams9 for the proper development and upkeep of the zebrafish ovary and possible role in establishing/maintaining an adult stem cell niche.

To discern whether the intersex phenotypes are a result of transdifferentiation or cystic disease, future work should focus on the migration of PGCs in embryos as well as the proliferation in juveniles and sexual fate decisions of young adults. Identifying colocalizing expression of growth
factors, hormonal pathways, and transcription factors specific to gonad in males vs. females
adamts9 mutants, should give further insight into what molecular pathways adamts9 utilizes.
Conditional knockouts in zebrafish and mice within cells specific to the gonadal ridge would
provide insights into the sex specific function of adamts9 in the gonad, without complication
underlying morphogenic abnormalities.

Early death of adamts9−/− may be due to irregular insulin signaling and/or development of organs
that express adamts9 at levels like the ovary, including the kidney and heart (Brunet, et al.,
2015). Adamts9(−/−) mutant zebrafish have more visible lipid accumulation than wildtype fish
(Fig 6X). The expression of members of the IGF family and their receptors should be quantified
in within organs of the adamts9−− knockout to guide further inquiry. In the murine adamts9(+/−)
heart, accumulations of versican/aggrecan were observed in the cardiac chamber (Kern et al.
2010), and adamts9 methylation is linked to cystic renal disease in humans (Brunet et al. 2015).
Investigation into Adamts9 phenotypes in these organs should be analyzed through dissection
and H&E staining.

Finding factors that increase pluripotency or maintain differentiation in stem cells are important
to improve regenerative therapies (Roushandeh et al., 2017). The IGF system is known to be
important for adult mesenchymal stem cells proliferation, self-promotion, self-renewal that
enhances the onset of differentiation (Youssef, et al 2017). Investigation to whether adamts9 is
expressed by WT mesenchymal stem cells and investigating the relationship between Adamts9,
IGF and receptors in the zebrafish gonad would begin to uncover adamts9s role in regeneration
in relation to stem cells of ovarian stroma, kidney, and heart.
Table 1. Guide RNA (gRNA) and primer sequences with the paired restriction enzyme. Bold and underlined are the restriction enzyme recognition motifs. Highlighted is gRNA4, the only gRNA to successfully generate mutations. PCR conditions for fw4/rv4: Annealing temp 57 C, Extension time 45 seconds. Initial denaturation was for 2:00 minutes, bacterial clones need 10:00 minutes initial denaturation. Since only one gRNA worked, only AseI was used for digestion of genomic DNA as well as amplified cDNA.

<table>
<thead>
<tr>
<th>Guide RNA</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Restriction Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>gRNA1</td>
<td>GGTACCTTACTCTGAGTC</td>
<td>TTCGTCACCCGACTCCGTT</td>
<td>CCCCGATTAACTTGACCCAC</td>
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<tr>
<td>gRNA2</td>
<td>GGTTCCTGACGACGTC</td>
<td>GCACTGCTCGTTACACCC</td>
<td>CAGCACAACCCACTACAGG</td>
</tr>
<tr>
<td>gRNA3</td>
<td>GGTGAAAAACTCTGCATTG</td>
<td>TAAGGACCCCAGCATTGGA</td>
<td>CGTTCGACGGGACAAAT</td>
</tr>
<tr>
<td>gRNA4</td>
<td>GGGAGGTGGCGATATATG</td>
<td>AACACAAAGTGTAAGCCCT</td>
<td>ATGGGGCTGAGTGTCCAAA</td>
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</tbody>
</table>
Table 2. cDNA primer sequences and PCR conditions. Primers were designed so PCR product would span multiple exons to reduce the chance of amplifying genomic DNA.

<table>
<thead>
<tr>
<th>cDNA Primer</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>PCR product Length</th>
<th>Annealing Temp (°C)</th>
<th>Extension Time (seconds)</th>
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</thead>
<tbody>
<tr>
<td>ADAMTS9-7</td>
<td>AATGAGCTGGATGGACCCAC</td>
<td>CACCATAACCTTGTCGAGG</td>
<td>442 bp</td>
<td>61.4</td>
<td>45</td>
</tr>
<tr>
<td>ADAMTS9-8</td>
<td>AGGTCTAGCTGAGCTGGTA</td>
<td>ACCGGCTCATCAAGCAAACA</td>
<td>291 bp</td>
<td>57</td>
<td>30</td>
</tr>
</tbody>
</table>
Table 3: Genotype ratios from heterozygous in crossings. Expected a Mendelian ratio of 25% WT, 50% (+/-) and 25% (-/-). At 1 mpf (month post fertilization) only 11% of 90 fish screened were homozygous mutants, which was reduced to only 4% when older than 3 mpf.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Expected Value</th>
<th>1 mpf</th>
<th>&gt;3 mpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+/+ )</td>
<td>25%</td>
<td>38.90%</td>
<td>40.20%</td>
</tr>
<tr>
<td>(+/- )</td>
<td>50%</td>
<td>50.90%</td>
<td>55.70%</td>
</tr>
<tr>
<td>(-/- )</td>
<td>25%</td>
<td>11.1%*</td>
<td>4.1%*</td>
</tr>
</tbody>
</table>

N= 90 1162
**Table 4.** Survival study of fish after fertilization (24 hrs, 7 days, and 14 days). At 14 days WT fish also had reduced survival due to rearing conditions, but (+/-) in cross had significantly more death at this time. 24 hpf: WT, 31 pairs, 81 clutches; (+/-), 18 pairs, 25 clutches. 7dpf: WT, 15 pairs, 37 clutches; (+/-), 10 pairs; 16 clutches. 14dpf: WT, 5 pairs, 4 clutches; (+/-) 5 pairs, 6 clutches.

<table>
<thead>
<tr>
<th>Age</th>
<th>WT in cross</th>
<th>(+/-) in cross</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hpf</td>
<td>94.2%</td>
<td>89.9%</td>
</tr>
<tr>
<td>7 dpf</td>
<td>93.4%</td>
<td>94.26%</td>
</tr>
<tr>
<td>14 dpf</td>
<td>77.9%</td>
<td><strong>51.33%</strong></td>
</tr>
</tbody>
</table>
Figure 1. Targeted and heritable genetic modification of a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 9 (adamts9) gene in zebrafish.

(A) Schematic drawing shows organization of introns and exons of adams9 and location of CRISPR/Cas9 targets indicated by arrows. The gRNA4 targeting exon 7 was successful in inducing mutations.


(B) Restriction enzyme analysis of PCR products amplified from genomic DNA of tail fin-of wildtype or adams9 mutants. PCR products were subjected to AseI restriction enzyme digestion. Homozygous (-/-) samples lost the recognition site and are not digested. Heterozygous (+/-) samples have both a mutated and WT allele, resulting in 3 distinct bands when ran on an agarose gel.

(C) Sequence of adams9 genomic DNA identified two mutant lines. One mutant line had 10 nucleotide deletions (∆10; TATTAATGAG), while another mutant line had eleven (∆11; TTGCAGTATTA). Underlined is deleted nucleotide sequence compared to WT sequence.
Figure 2. Generation of homozygous mutant zebrafish.

Microinjection of gRNA and Cas9 mRNA into 400 wildtype (WT) embryos within 30 after fertilization. Embryos were grown into adulthood (F0) where they were crossed with wildtype (WT) fish. Their embryos were grown into adulthood (F1) and screened the same way. Mutations were isolated through bacterial cloning and sequence. Individuals heterozygous with the same mutation were crossed to generate homozygous offspring (F2) and subsequent generation, which were used in all following experiments. Two separate mutant lines were generated, each with a unique frameshift mutation.
Figure 3. Mutations in *adams9* cDNA.

(A) Representative PCR products amplified from cDNAs of wildtype (WT) or *adams9*(−/−). Samples were collected from brain tissue.

(B) Comparison of DNA sequence of *adams9* mutant to *wt*. Underlined is deleted nucleotide sequence compared to the WT sequence.
Figure 4. Confirming *adams9* Knockout

(A) Schematic drawings of various functional domains of zebrafish Adamts9 protein and predicted truncated proteins of each mutant line. The Δ10 mutation starts nonsense sequence at aa 557 and stopped at 596aa, whereas Δ11 mutation had a premature stop at aa 555. In both cases the mutation lost the active site of Adamts9 (aa 573). Corresponding positions of two commercial antibodies against the prodomain (RP1) and metalloprotease (RP5) domains of human ADAMTS9 were shown.

(B-D) Hematoxylin and Eosin (H&E) (Row B) and immunohistochemistry staining (Rows C-D) of stage IV fully-grown immature follicles from WT or *adams9* mutant zebrafish using antibodies against the prodomain of human ADAMTS RP1 (C) (Triple Point Biologics, catalog# RP1-ADAMTS9, has 41% sequence identity to zebrafish Adamts9) or RP5 (D) (Triple Point Biologics, catalog# RP5-ADAMTS-9, has 81% sequence identity to zebrafish Adamts9). H&E Sections were adjacent to those used for immunohistochemistry to show the structure of the oocyte (B-b’). Low (uppercase letters) magnification images of a representative stage IV follicle. Boxed areas are 40x enlarged (lowercase letters). High magnification (40x) representative immunostaining of stage IV follicles from *adams9*+/− with the antibody against human ADAMTS9 RP1 (c’) and RP5 (d’). Zona pellucida is indicated by *, Arrow points to follicular cells.
Figure 5. Characterizing Fertility and Sex Ratios

(A) Average number of embryos collected over 2 weeks. Adamts9(+/-) fish were paired together to generate homozygous fish. After spawning regularly for a week, embryos were collected and counted for two weeks during the daily water change before adults were fed. Number of pairs are represented by n, an average of 4 samples were collected from each pair. Wildtype (WT) fish were subjected to the same treatment as controls.

(B) WT and (+/-) fish have a 2:1 ratio of male to females in fish older than 3 mpf. Most homozygous fish were male and fertile, only 3 females were found but they were not able to spawn. There was also a group of fish found that did not have normal gonad morphology and instead had empty membrane bound compartment (Intersex). WT total n= 118, male= 79, female= 39; (+/-) total n= 192, male=128, female= 64; (-/-) total n= 45, male= 37, female= 3, Intersex= 5.
Figure 6. Comparison of external appearance and gonadal morphology of WT and adamts9−/− fish.

(A-E) Columns are representative of male adamts9+/+ (A- A. IV) and adamts9−/− (B- B. IV); female adamts9+/+ (C- C. IV) and adamts9−/− (D- D. IV); and intersex fish (E- E. IV).

(A. I- E. I) Top view of male adamts9+/+ (A. I) and adamts9−/− (B. I); female adamts9+/+ (C. I) and adamts9−/− (D. I); and intersex fish (E. I). Arrow Heads: spinal malformity in mid-body and caudal peduncle.

(A. II- E. II) Comparison of open abdominal cavity. Testis are difficult to see between swim bladder and intestine in male adamts9+/+ (A. II) and adamts9−/− (B. II) fish. Ovaries take up most of the space in female adamts9+/+ (C. II), but adamts9−/− females have longer, thinner ovaries (D. II). Strikingly, adamts9−/− intersex fish appear empty (E. II).

(A. III- E. III) Comparison of dissected gonad. Testis of adamts9+/+ (A. III) and adamts9−/− (B. III) fish are similar. Ovaries of adamts9+/+ female (C. III) are highly vascularized and opaque with thousands of eggs within the organ, adamts9−/− females have smaller ovaries with less visible eggs, lots of undifferentiated tissue (D. III). Intersex fish have membranous sac lacking all internal structure (E. III). Large eggs= (White)* and Blood vessel= (white) arrow.

(A. IV- E. IV) Paraffin histology of gonad with H&E. WT (A. IV) and adamts9−/− (B. IV) testis, spermatozoa are mature (dark staining) and have tails (not shown). Histological comparison of WT (C. IV) and adamts9−/− ovary (D. IV). Large eggs (*) are visible in both samples but homozygous females have less large eggs and more interstitial space. Histology of the intersex gonad reveals there is no internal cellular structure and is at a smaller magnification (1x) than other histology panels (4x) (E. IV).
Figure 7. Paraffin sections stained with H&E of intersex fish

(A) Dissected intersex gonad has is thin and transparent with no visible internal structures.

(B) Shows the organ is devoid of internal structure, except along periphery are cysts.

(C) A closer look at peripheral spermatogenic cysts. Spermatogonia and spermatozoa are present.

(D) 40x of spermatozoa within intersex spermatogenic cysts. The spermatozoa are mature with tails
Figure 8. JB4 sections stained with H&E of intersex fish.

(A) Shows that the majority of the organ is devoid of structure but has areas that contain eggs starting to collect vitellogenin.

(B) A closer look at the cells show eggs are surrounded by tissue that does not resemble that of WT ovarian nuclear staining.

(C) 40x of stage I oocyte.

(D) 40x of surrounding tissue that resembles developing spermatogenic cysts.
References


November 7, 2016

National Institutes of Health  
Center for Scientific Review  
6701 Rockledge Drive  
Room 1040, MSC 7710  
Bethesda, MD  20892

Dear Sir or Madam:

The vertebrate animal use described in the following application submitted to the NIH was reviewed and is congruent with an IACUC-approved animal use protocol:

Title of Application: "Regulation and Functions of Adamts9 During Ovulation"

Name of Principal Investigator: Yong Zhu, Ph.D.

Name of Institution: East Carolina University

Congruency Approval Date: November 7, 2016

Animal Use Protocol Expiration Date: (D185d) February 6, 2017

This institution is fully accredited by AAALAC and has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare. The Assurance Number is A3469-01.

Sincerely yours,

Susan McRae, Ph.D.  
Chair, Animal Care and Use Committee

SM/jd  
cc: ECU Office of Sponsored Program
Appendix B: IACUC Approval of Research

February 3, 2017

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

Dear Dr. Zhu:

Your Animal Use Protocol entitled, "Studies of Hormones and Receptors in Zebrafish" (AUP #D185e) was reviewed by this institution's Animal Care and Use Committee on February 3, 2017. The following action was taken by the Committee:

"Approved as submitted"

*Please contact Aaron Hinkle at 744-2997 prior to hazard use*

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

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure
EAST CAROLINA UNIVERSITY
ANIMAL USE PROTOCOL (AUP) FORM
LATEST REVISION NOVEMBER, 2013

Project Title:
Studies of Hormones and Receptors in Zebrafish

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<tr>
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<th>Principal Investigator</th>
<th>Secondary Contact</th>
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<tbody>
<tr>
<td>Name</td>
<td>Yong Zhu</td>
<td>Fadi Issa</td>
</tr>
<tr>
<td>Dept.</td>
<td>Biology</td>
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</tr>
<tr>
<td>Office Ph #</td>
<td>252-328-6504</td>
<td>252-328-5446</td>
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<tr>
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<tr>
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<td><a href="mailto:ISSAF14@ECU.EDU">ISSAF14@ECU.EDU</a></td>
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For IACUC Use Only

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Zebrafish SOP attached
Exception to Guide: 10 adult fish/liter instead of 5
I. **Personnel**

A. **Principal Investigator(s):**
   Yong Zhu

B. **Department(s):**
   Biology

C. **List all personnel (PI’s, co-investigators, technicians, students) that will be working with live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:**

<table>
<thead>
<tr>
<th>Name/Degree/Certification</th>
<th>Position/Role(s)/Responsibilities in this Project</th>
<th>Required Online IACUC Training (Yes/No)</th>
<th>Relevant Animal Experience/Training (include species, procedures, number of years, etc.)</th>
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<tbody>
<tr>
<td>Yong Zhu/PhD</td>
<td>Professor/PI/Supervisor</td>
<td>Yes</td>
<td>Have relevant animal experience and training with fish since 1986, know well zebrafish husbandry requirements, spawning protocol, and microinjection procedure etc., responsible for training all collaborators and associates working in the lab</td>
</tr>
<tr>
<td>Edmund J Stellwag/PhD</td>
<td>Associate Professor/Co-investigator/co-supervisor</td>
<td>Yes</td>
<td>Over 20 yrs with fish, 10 yrs with zebrafish, helping training associates</td>
</tr>
<tr>
<td>Fadi Issa/PhD</td>
<td>Assistant Professor/Co-investigator/co-supervisor</td>
<td>Yes</td>
<td>Over 10 yrs with fish, helping training associates</td>
</tr>
<tr>
<td>Myon Lee/PhD</td>
<td>Associate Professor/Co-investigator/co-supervisor</td>
<td>Yes</td>
<td>Three year with zebrafish, train directly by PI (Zhu), helping training associates</td>
</tr>
<tr>
<td>Dongteng Liu</td>
<td>Visiting PhD student/Student/Research Assistant</td>
<td>Yes</td>
<td>6 years with fish, one year with zebrafish, train by PIs (Zhu)</td>
</tr>
<tr>
<td>Xinjun Wu</td>
<td>PhD student/Student/Research Assistant</td>
<td>Yes</td>
<td>2 years with zebrafish, train by PIs (Zhu)</td>
</tr>
<tr>
<td>Dong Suk Yoon</td>
<td>Postdoctoral fellow/co-investigator/Research Associate</td>
<td>Yes</td>
<td>One year with zebrafish, train by PI and co-PI (Zhu &amp; Lee)</td>
</tr>
<tr>
<td>Zayer Thet</td>
<td>MS student/Student/Research Assistant</td>
<td>Yes</td>
<td>3 years with zebrafish, train by PI and a graduate student (Zhu &amp; Shaner)</td>
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<tr>
<td>Name</td>
<td>Role</td>
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<tr>
<td>Nichole Carter</td>
<td>MS student/Student/Research Assistant</td>
<td>Yes</td>
<td>2 years with zebrafish, train by PI (Zhu)</td>
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<tr>
<td>Paul Delmus Bridgers</td>
<td>MS student/Student/Research Assistant</td>
<td>Yes</td>
<td>2 years with zebrafish, train by PI</td>
</tr>
<tr>
<td>Thomas Miller</td>
<td>MS student/Student/Research Assistant</td>
<td>Yes</td>
<td>2 years with zebrafish, train by PI &amp; Co-PI</td>
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<tr>
<td>Emma R Daughtrey</td>
<td>MS student/Student/Research Assistant</td>
<td>Yes</td>
<td>2 years with zebrafish, train by PI &amp; Co-PI</td>
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<tr>
<td>Katie Clements</td>
<td>MS student/Student/Research Assistant</td>
<td>Yes</td>
<td>2 years with zebrafish, train by PI &amp; Co-PI</td>
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<tr>
<td>Matthew Wayne Jr</td>
<td>UG student/Student/Research Assistant</td>
<td>Yes</td>
<td>0 year with zebrafish, train by PI</td>
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<td>Chilton</td>
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<tr>
<td>Marcus Jermaul</td>
<td>UG student/Student/Research Assistant</td>
<td>Yes</td>
<td>0 year with zebrafish, train by PI</td>
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<td>Jawanza Williams</td>
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<tr>
<td>Jennifer Lesniak</td>
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<td>Yes</td>
<td>0 year with zebrafish, train by PI</td>
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<td>Amanda G Rogers</td>
<td>UG student/Student/Research Assistant</td>
<td>Yes</td>
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<td>Elizabeth L Ryan</td>
<td>UG student/Student/Research Assistant</td>
<td>Yes</td>
<td>0 year with zebrafish, train by PI</td>
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<tr>
<td>Alexandria Ivana</td>
<td>UG student/Student/Research Assistant</td>
<td>Yes</td>
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<td>Warren</td>
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<td>Jalen Malik Barnes</td>
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<td>Yes</td>
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<td>Christopher Shaquell</td>
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<td>Yes</td>
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II. Regulatory Compliance

A. Non-Technical Summary

Using language a non-scientist would understand, please provide a clear, concise, and sequential description of animal use. Additionally, explain the overall study objectives and benefits of proposed research or teaching activity.