Nuclear Estrogen Receptor ERα may not be Involved in Estradiol (E2) Induced Heart Valve Malformations

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Abstract

Congenital heart defects are relatively common and can prove fatal if left untreated. In utero exposure to estrogens or estrogen-mimicking compounds has been linked to heart valve malformations. One such estrogen-mimicking compound is bisphenol A (BPA), a very common component of plastic. This experiment investigated whether estrogen induced heart valve abnormalities were mediated through the ERα nuclear estrogen receptor. This was achieved by analyzing the health of heart valves of zebrafish embryos with or without the gene encoding the ERα nuclear estrogen receptor after exposure to a pathogenic concentration of estrogen. The results showed that fish with the ERα nuclear estrogen receptor were equally as likely to display heart valve malformations as those without the receptor. This work demonstrates that it is likely that estrogen causes heart valve abnormalities through a different pathway than the ERα receptor. In the future, it would be prudent to examine the role of elevated heart rate in causing heart valve malformations, as estrogen has also been shown to increase heart rate.

Introduction

In utero exposure to compounds that mimic estrogens is associated with an increased risk of heart valve defects (Soto and Sonnenschein, 2010). Approximately 0.8% of infants born in the United States have congenital heart defects. These defects accounted for 3.7% of all pediatric hospitalizations in 2009 (Mozaffarian et al., 2016). Calcific aortic valve disease is one of the most common forms of heart disease and requires over 100,000 heart valve replacements each year in the USA (Theodoris et al., 2021). Many of these cases are in patients with congenital heart defects which cause the aortic valve to form two leaflets instead of three, possibly because the abnormal mechanical stress and flow patients observed in these patients predisposes them to calcific aortic valve disease (Yutzey et al., 2014).

Estrogen is an essential hormone with many roles including regulating the development of female secondary sex characteristics, reproductive cycles, and sexual and maternal activity (Bondesson et al., 2014). Estrogen has also been found to regulate the dorsal-ventral limit of the hemogenic endothelial niche in zebrafish embryos by antagonizing VEGF signaling. Excess estrogen inhibits VEGF signaling leading to artery, hemogenic endothelium, and HSPCs to fail to form properly. When there is an shortage of estrogen, VEGF expression expands, causing hematopoietic stem and progenitor cell production in the vein. (Carroll et al., 2014). There are several different forms of estrogen, Estrone (E1), 17β-estradiol (E2), and Estriol (E3). E2 is the most powerful and common estrogen in reproductive age female vertebrates. Estrogens bind to two different receptors in zebrafish, nuclear estrogen receptors, and the G protein-coupled estrogen receptor (GPER). There are a few different nuclear estrogen receptors in zebrafish, ERα, ERβ1, ERβ2 (Cui et al. 2013). Nuclear Estrogen receptor ERα is encoded by the gene *esr1*. Esr1 has been found to have strong expression in the heart, especially in the valves during embryonic development (Gorelick et al., 2014). Estrogen receptors have been detected in the heart valves using the transgenic zebrafish line Tg(5xERE:GFP), which uses five estrogen receptor elements to drive GFP expression, (Gorelick and Halpern 2011). Receptors ERβ1 and ERβ2 both show the strongest expression in the embryonic liver (Gorelick et al., 2014).

Zebrafish treated with estradiol exhibited a 20% increase in heart rate. This effect was shown to be mediated by GPER as fish with loss of function mutations in their nuclear estrogen receptors still exhibited the effect. GPER transcripts were not found within the heart of zebrafish embryos, but was detected within the pituitary, implying that GPER acts centrally to regulate heart rate in the brain. Nuclear estrogen receptors were not found to colocalize with GPER in the brain,

suggesting that GPER works autonomously (Romano et al., 2017).

Miriam Semmar ’18 found that treatment of zebrafish with an inhibitor of Notch, a downstream product of the VEGF pathway, and estradiol produced pericardial effusions and death (2018). Sonya Jampel demonstrated high doses of E2 administered to Zebrafish embryos during the critical period for heart development resulted in pericardial effusions and the absence of heart valves in the atrioventricular canal. She also noted weak expression of GPER within the atrium and ventricle of the heart of zebrafish embryos at 62 hours post fertilization in contrast to the findings of Romano et al. (2019).

The mechanism by which estrogen and estrogenic compounds produce these effects is not yet understood. There are a few possibilities. It could be either a GPER or nuclear estrogen receptor mediated heart rate effect that causes flow rate to be suboptimal for valve formation. It could be a GPER direct growth effect, mediated by receptors in the heart. Finally, the effect of estrogen exposure could be mediated by ERα receptors expressed in the heart valves. Previous research in this lab has shown that direct GPER signaling is likely not responsible for the heart valve malformations found in response to E2 exposure, as a GPER agonist was not reliably found to produce heart valve defects and did not produce any effusions (Jampel, 2021). Surprisingly, control GPER knockouts showed an increased proportion of abnormal valves, suggesting that GPER may still have a role in the development of the atrioventricular valve even at normal levels of estradiol (Lopez, 2021).

Xenoestrogens, or estrogen-mimicking compounds, may also affect heart valve development. Notch is a gene that is highly expressed in the developing heart valves and has been identified as essential for their correct formation. An inverse agonist of ERα, XCT790, was identified by a machine learning program designed to identify small molecules that correct gene networks dysregulated in a model of heart disease. XCT790 was predicted to prevent osteogenesis and was found to be very promising as a treatment for heart defects, arresting and even reversing many disease markers in adult mice including reducing the thickness of aortic valves and pulmonary valves. XCT790 works at least partially through the inhibition of ERα, demonstrating that heart defects may be formed through the ERα signaling pathway (Theodoris et al., 2020).

BPA, a synthetic estrogen, is a common component of plastics and resins. BPA can leach from these materials and be absorbed by the human body, causing a range of adverse effects including cancer, diabetes, obesity, and fertility problems (Romano et al., 2017). Treatment of the heart with BPA, a synthetic estrogen, can produce an increase in heart rate, an arrythmatic effect, and pericardial effusions in zebrafish (Moreman et al., 2018). Treatment of zebrafish embryos with BPA has produced estrogen receptor activation in the heart and produced a collagen deficiency in the extra-cellular matrix of the heart. This alteration could have a role in the formation of calcific aortic valve disease as the extracellular matrix is a primary molecular pathway associated with calcific aortic valve disease, alteration of which constituting a biomarker for the disease. Structural defects of the atrioventricular valves and reduced ventricular beat rate and blood flow were also observed (Brown et al., 2019).

Esr1$^{+/-}$; Tg(fli1a:GFP) zebrafish obtained from the Gorelick lab were bred to produce the test subjects for this study. Zebrafish embryos are transparent and this strain has fluorescent heart endothelium, which allows for clear imaging of the heart valves. These fish will produce embryos homozygous and heterozygous for the esr1 knockout, as well as wildtype embryos as a control.

Zebrafish heart development begins around 12 hours post fertilization and by 48 hours the heart valves begin to form. Zebrafish hearts are simple, only containing two chambers in comparison to the 4 chambers of human hearts, but they resemble human hearts at 22 days of gestation, making them useful models for human heart development. Additionally, zebrafish receive oxygen by diffusion for the first week post-fertilization, allowing them to survive without a functional heart (Brown et al., 2016). However, changes in blood flow do alter the shape of the heart valve. Blood flow between the atrium and ventricle is generally bidirectional until the atrioventricular valve develops, preventing backflow. Notch and klf2a, both genes essential for the formation of heart valves activate in response to high reversing flow and restrict expression to regions experiencing high reverse flow. Decreasing blood viscosity and changing heart rate both reduced backflow and resulted in deformed heart valves (Vermot et al., 2009). Thus, changes in heart rate or viscosity of blood, possibly mediated by estrogen, may be sufficient to cause abnormal valve growth.

This study investigated the mechanism by which

heart valve defects are produced in Zebrafish. As these defects have been associated with E2 treatment, this project will be investigating whether the effect is produced by signaling from the ERα receptor.

Methods

Esr1$^{+/-}$; Tg(fli1a:GFP) zebrafish obtained from the Gorelick lab were bred to produce the test subjects for this study (Romano, 2017). This strain has fluorescent heart endothelium and all blood vessels, which allows for clear imaging of the heart valves. These fish produced embryos homozygous and heterozygous for the *esr1* knockout, as well as wildtype embryos as a control. Fish pairs were set up in tanks with dividers the day before breeding. Dividers were pulled the next morning around 11 AM. Eggs were collected in E3 embryo water between 1 and 4 hours post fertilization and unfertilized and moldy eggs were removed. Clutches were mixed to produce diverse experimental groups with equally proportional representation. At 22 hours post fertilization, the estrogen was administered and embryos were dechorionated in order to ensure full effect of the dose. E2 dosage varied from 8 to 12µM, diluted from a stock of 10 mg/mL. At 4 days post fertilization, non-fluorescent embryos were removed. Embryos without gross defects such as tail curl or pericardial effusion were anesthetized with 0.08% tricaine and set in 60mm dishes in which the bottom was replaced with a no. 1 coverslip. Embryos were transferred to the slide and embedded in 1.2% low melting agarose to immobilize fish for imaging. The embryos' atrioventricular valves were visualized and videoed using a Nikon A1 confocal microscope for two seconds using a 20x long working distance lens. Videos were denoised, randomized, and converted to AVI. The videos were scored independently by three students on a scale of 1-4, where 1 indicated normal leaflets, 2 indicated stubs, 3 indicated no leaflets, and 4 indicated that the student was unable to score the video. A majority of students had to agree on the score for it to be counted. The fish imaged were removed from their agar setting, dissolved using 25 mM NaOH heated at 95°C for 10 minutes, mixed, and then heated at 95°C for another 10 minutes. The solution was then neutralized using 40 mM Tris-HCl, and stored in a refrigerator. PCR was then run on the DNA using primers specific to the *esr1* knockout region. The DNA product was finally run in an 8% acrylamide gel in order to ascertain the genotype of each fish. This information was paired to the heart phenotype. Fischer's exact analysis was run on the data to ascertain significance. For more detailed methods, see appendix.

Results

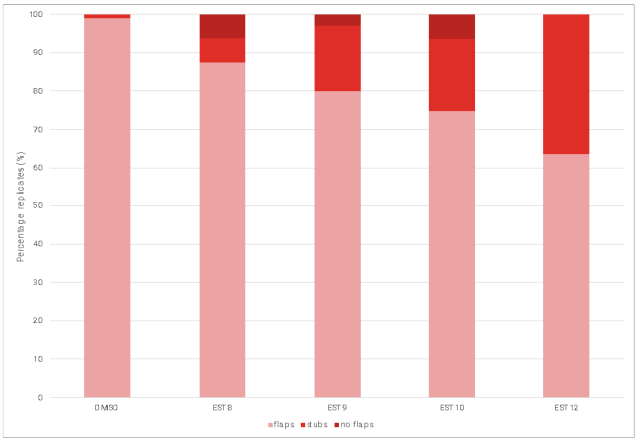

Figure 1: Dose curve phenotype data for DMSO (n=98), 8µM E2 (n=16), 9µM E2 (n=35), 10µM E2 (n=79), and 12µM E2 (n=22). Zebrafish used were mixed genotype. Fischer's exact analysis showed $p<0.01$

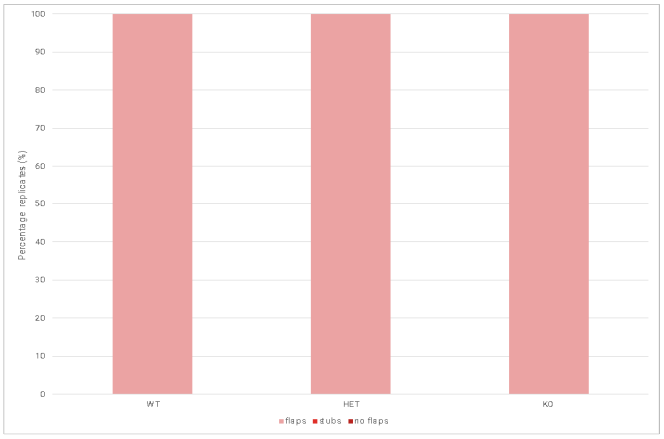

Figure 2: Composite data from DMSO treated fish from all genotypes, wildtype (n=9), heterozygous esr1 knockout (n=35), and homozygous *esr1* knockout (n=22)

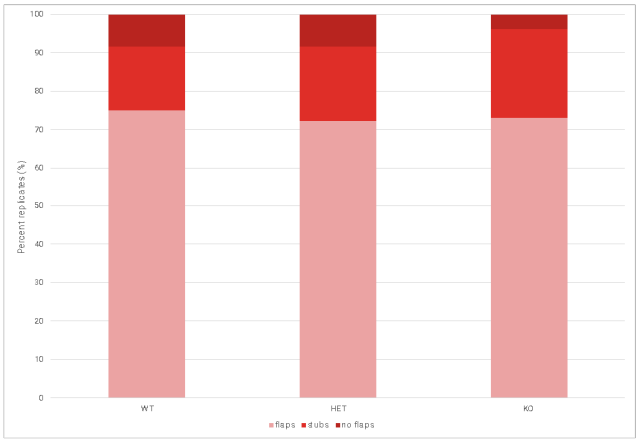

Figure 3: Composite data of valve phenotype dependent on esr1 genotype from 10μM E2 treated, wildtype (n=24), heterozygous esr1 knockout (n=36), and homozygous *esr1* knockout (n=26). Fischer's exact test showed p=0.944

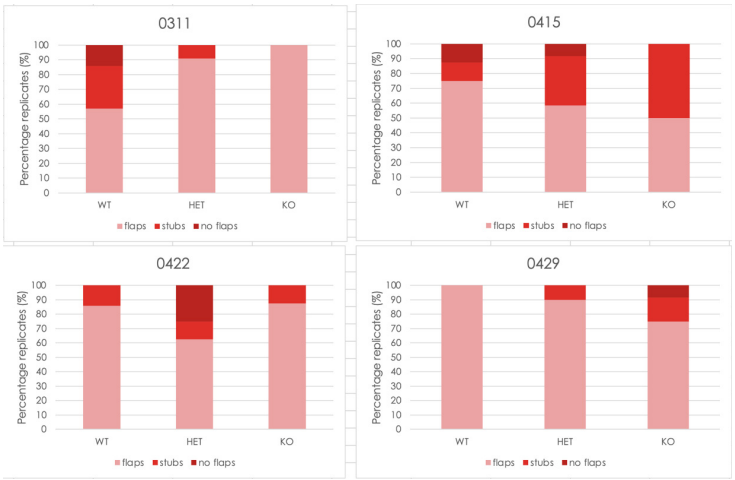

Figure 4: weekly breakdown of valve phenotype as dependent on *esr1* genotype

When all genotypes were combined, E2 treatment at all doses tested increased the proportion of abnormal valves. When treated with DMSO, only 1.0% of embryos showed flap malformation. 8μM E2 increased this proportion to 12.5%, 9μM to 20.0%, 10μM to 25.3%, and 12μM to 36.4% (figure 1). 12μM treated fish showed a high proportion of pericardial effusions and tail curl, so 10μM E2 was chosen for experimental treatment as it showed the highest proportion of malformed valves while maintaining a low proportion of gross defects.

Across all genotypes, embryos treated with DMSO showed no heart malformations, showing that the *esr1* knockout doesn't produce any heart valve or gross defects alone (figure 2). Embryos treated with 10μM Estradiol did not show genotype specific effects. 25% of wild type fish showed valve defects, 27.8% of *esr1* knockout heterozygotes, and 26.9% of *esr1* homozygous knockouts (figure 3). The difference between groups was not shown to be significant, showing that nuclear receptor ERα expression did not mediate E2 triggered heart valve malformation. Weekly results varied widely with no consistent trend (figure 4).

Discussion

This work confirmed previous work in the lab by Miriam Semmar '18 and Sonya Jampel '19 that excess E2 leads to malformation of the atrioventricular valves. It was found that a dose of 10μM of E2 was ideal to cause heart valve abnormalities without a high proportion of pericardial effusions or tail curl. It was clear this phenotype was due to the estradiol treatment as embryos treated with DMSO didn't exhibit any malformations.

This ideal dose of 10μM E2 was used to study whether genotype had any effect on valve phenotype. Statistically, there was no difference in the proportion of fish with abnormal valves between wildtype, $esr1^{+/-}$, and $esr1^{-/-}$ embryos. In weekly groups, there were differences observed, but these trends were not consistent and proved insignificant when data was compiled. Across all genotypes and weeks, DMSO treated embryos always had healthy leaflets, showing there was no difference in vehicle or treatment technique.

Some weeks did show abnormalities. Data could not be collected one week as most embryos showed gross malformations despite receiving the same 10μM dose of E2 as in other weeks. This raises the possibility that there may have been weekly unintended variation in estradiol dose or that some fish may have been more susceptible to the E2 treatment for reasons other than their *esr1* genotype. Additionally, due to the difficulty of embedding the embryos at the correct angle for imaging, there were many embryos that couldn't have their heart valves clearly imaged, adding uncertainty to the scoring process. To mitigate this uncertainty, three people scored each video and a majority had to agree for the score to be counted.

The results of this experiment show that heart malformations caused by E2 exposure are likely not mediated by the ERα nuclear estrogen receptor. As previous results from this lab have shown the effect is also likely not mediated by the GPER receptor, future

work should focus on isolating new targets that might mediate this response. One particularly promising target is heart rate. Past work by Vermot et al. showed that blood viscosity and heart rate affect backflow in the developing heart, activating expression of genes vital for heart valve formation. Estrogen has been shown to mediate increases in heart rate. It is possible these heart rate changes could impede valve development. While the results of this experiment seem to indicate that valve development is not mediated by the ERα nuclear estrogen receptor, more work is needed to confirm this result and investigate further the biological mechanism of malformation.

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Appendix

Fish Breeding and Doing Protocols

Setup

- Set up tanks
- Put in dividers
- Fill tanks with water up to 1 cm below the top of the divider to mimic shallow water
- Make sure to run reservoir water at the same time as not to drain water supply
- Put one male one female on either side of the divider
- Label tanks with breed content and pigment level
- We bred fish heterozygous for esr1 knockout
- Cover tanks
- MAKE SURE TO TURN RESERVOIR WATER OFF
- Push cart to the side and leave overnight

Egg Collection

- Pull dividers at 11 in morning to allow fish to mate
- After eggs are laid, remove fish from tanks
- 4 hours after eggs are laid, remove them from their tanks
- Take out inner filter
- Pour water over handheld filter
- Add DI water and swirl then pour over filter again
- Flip filter upside down over a petri dish then use the handheld egg water bottle to pour water over eggs to remove them from filter
- Fill petri dish only halfway
- Then remove unfertilized and moldy eggs from water

- Refill with egg water up to halfway then place in incubator at 28°C

Dosing
- Prepare estradiol and DMSO treatment using 4mL of E3 embryo water, 4 mL of 1x E3 + 2x PTU, and 14µL of DMSO and Estradiol, to the proper estrogen concentration
- Remove embryos from 28°C
- Get out net and wet
- Pour embryos into net, making sure to use the side without the embroidery
- Flip net over and dislodge embryos using treatment solution into smaller petri dish
- Label petri dishes (pink=EST10, green=DMSO)
- Dechorionate fish carefully and return to incubator

Confocal Protocol

Setup
- Prepare 0.08% tricane from 0.4% stock and put in petri dish
- Take out dosed fish—DMSO, 8, 9 µM estradiol
- Prepare agar (1.2%?) and put in heat block to melt
- Take confocal dish and form seal at bottom using agar

Immobilization of Fish
- Pipette 6-7 fish into 60 mm dish with 0.8% tricaine
- Check fluorescence and remove any non-fluorescent fish
- Pipette up (headfirst) and put into confocal dish
- Pipette up any excess liquid
- Pipette a blob of agarose in (bigger is better to increase setting time), sink any floaters, and arrange in line leaning slightly left
- Wait for Agar to set then add a couple drops of tricaine on top
- Make label
  - Treatment (ex. EST10 or DMSO) and color code (ex. Pink or green)
  - Diagram of fish location for reference
  - Fish and date (fli1a_220124)
  - File names (plate number and fish number, ex 1001)

Imaging
- Sign in to the sheet
- Turn on everything in the order on the sheet (turn off every laser but #3 and #6)
- Lens LWD 20x WI λs NA=0.95
- Add a drop of DI on top of lens
- Use lens paper to clean bottom of plate. Check if fish are to the left and put in confocal
- Turn on white light under DIA
- Raise lens to ~1500, focus/center heart ~1800 hit PFS
- Click (red) +green fluorescent to see through camera (turn off camera lock on microscope)
- turn down o488 (laser) to ~0.7 to get good brightness, can also turn down Hv for similar effect
- Check time tab and uncheck everything else to left, click “run now”
- Image>advanced denoising>apply to all frames
- Save again (tag _dn)
- To switch plates, lower by hand a bit then hit “quick escape”, switch plates

Clean Up
- After done, lower to ~200, remove plate, wipe DI water off lens, switch lens
- Turn off everything in the reverse order on the sheet

Scoring
- Transfer files to computer
- Make separate denoised folder
- Randomize files (see code below)

Code

```
##location of main folder
setwd(“folder Pathname”)
## randomize and re-name
filenames <- list.files(getwd())
num.videos <- length(filenames)
new.names <- sample(paste(“220204-”, 1:num.videos, “.nd2”, sep = “”)) ##can change so date name of dir.create(“Randomized220204”)
dir.create(“Randomized220204”)
file.copy(from = paste(filenames, sep = “”), to = paste(“Randomized220204/”, new.names, sep = “”))
## Key to match randomized to original
video.key <- data.frame(Original = filenames, Filename = new.names)
write.csv(video.key, file = “Video_Key220204.csv”, row.names = FALSE)
```

- Convert files to AVI using fiji
- Upload to google drive and make scoring sheet

- Score into flaps, stubbs, no flaps, and un-scorable
- Analyze composite data

Genotyping Protocol

DNA Extraction--hotshot

- Using a foreceps, fish were cut from their agar setting, being careful to keep the order and number intact
- 0.08% tricaine was added to fish and they were extracted from the dish one at a time using a micropipette with the tip cut off and put into a labeled pcr tube
- Excess liquid was suctioned off and 25 µL 25 mM NaOH was added to each tube.
- The tubes were heated at 95°C for 10 minutes in a PCR machine. They were then mixed until fish were dissolved and heated at 95°C for another 10 minutes. The reaction was then neutralized using 25 µL 40 mM Tris-HCl. Tubes were stored in the refrigerator
- PCR was run using primers specific to the esr1 knockout region
  - Esr1-HRM-F ACAACTCCTGCCTGTCAGT
  - Esr1-HRM-R TTTGTGCAGGTCCAGTGTGG

Gel

- An 8% acrylamide gel was prepared using the Mighty Small vertical electrophoresis mold
- 2.5 µL of 6x purple loading dye was added to each DNA sample and mixed.
- 7µL of each sample was loaded into each well, making sure to leave a unique number of wells before the ladder on each gel in order to ensure gels aren't confused in analysis.
- 2 µL of quick load 1 kb DNA ladder was added to each gel
- The gel was run at 50-60 V for a few hours or until bands migrated at least halfway down the gel
- Gels were removed from molds and developed for 15 minutes in dilute Ethidium Bromide
- Gels were then visualized and genotype was interpreted from sample length

Analysis

- Genotype information was paired to scoring data and Fischer's exact test was run in Stata to ascertain significance