Cell culture seeding density increases impact of Foxl2 overexpression on three key genes in ovarian development.

Autumn R. Joy[1], Tatyana Bazile[2], Melanie Borysewicz[3], Aerica Nagornyuk[1], and Turk Rhen[1], Ph.D.
Biology Department, University of North Dakota[1], Rensselaer Polytechnic Institute[2], Concordia College[3]

Abstract

RNA sequencing data has uncovered dimorphic patterns of gene expression relating to sex determination. Molecular interactions among these genes are still being described and uncovered. Here we use the common snapping turtle, a species with temperature-dependent sex determination, to explore the role of Foxl2 in regulating Amhr2, Cdkn1c, and Osr1, known cell-cycle inhibitors. In order to reduce the number of embryos used, this study also explored differences between one and two gonad cell cultures. Gene expression was higher within two-gonad versus one-gonad cultures. Foxl2 did not have any effect on expression within one-gonad cultures. However, in two gonad cultures Foxl2 increased expression of Osr1, and temperature-dependently increased expression of Amhr2 and Cdkn1c. This suggests that cell proliferation and communication interacts with environmental conditions during temperature-dependent sex determination.

Introduction

- The fate of the bipotential gonad, whether it forms an ovary or testis, is a critical decision in early vertebrate development. Sex determination influences individual fitness, susceptibility to certain pathologies, and population demographics of a species. In humans, this process is controlled broadly by sex chromosomes during fertilization, XX (female) or XY (male). However, most reptiles have not evolved sex chromosomes. Instead, they use temperature-dependent sex determination (TSD), where ambient temperature during a thermosensitive period (TSP) of embryonic development determines gonadal sex. In common snapping turtles, *Chelydra serpentina*, from Minnesota, exposure of embryos to a female-producing temperature (31°C) for only 6 days out of a 65-day incubation period is sufficient to produce 100% female offspring. This short TSP makes *Chelydra serpentina* an excellent animal model to study changes in gene expression during sex determination.
- The use of animal models to study sex determination includes the humane dissecting of turtle embryos and hatchlings to remove adrenal-kidney-gonad complexes. One aspect of this study was to assess if the number of gonads necessary for primary cell culture could be reduced from 8 gonads per well in 24-well cell culture plates to just 1 or 2 gonads per well in 96-well plates. .
- This study also relies upon previous data about sex determining genes. Dimorphic expression of several genes was detected using next generation sequencing by Rhen et. al. (2007, 2021) and Schroeder et. al. (2016). Forkhead Box L2 (Foxl2) is a transcription factor necessary for granulosa cell function and its expression was found to increase over the TSP during incubation at 31°C, a female producing temperature (Rhen et. al, 2007). Overexpression of Foxl2 would allow us to study its effect on expression of other genes that may play a role in ovarian development. We selected candidate target genes that show a positive correlation with Foxl2 expression in embryonic turtle gonads. The genes discussed here are involved in ovarian development and are known cell cycle inhibitors.
- Amhr2 is an important regulator of cell growth and is involved in oocyte maturation. It is known to have roles in granulosa cell development, and polymorphisms in in Amhr2 have been shown to influence ovarian stimulation outcomes (Chen et al, 2020).
- Cdkn1c is a known tumor suppressor and cell growth regulator. Mutations of Cdkn1c have been detected in sporadic cancers. In 2005, Nef et. al. found that Cdkn1c was overexpressed in XX mice gonads during development and suggest that expression of cell-cycle inhibitors is an important part of ovarian development.
- Odd-skipped related transcription factor 1, Osr1, is another cell cycle inhibitor related to tumor suppression and cell growth. Knockout of Osr1 in mice prevents kidney cell differentiation and the formation of the urogenital ridge from the intermediate mesdoderm (Wang et. al, 2005). Osr1 has also been identified as a marker for pre-granulosa cells in chickens and is expressed in the same cells as Foxl2 (Estermann et. al, 2020).

School of Medicine & Health Sciences
University of North Dakota

Methods

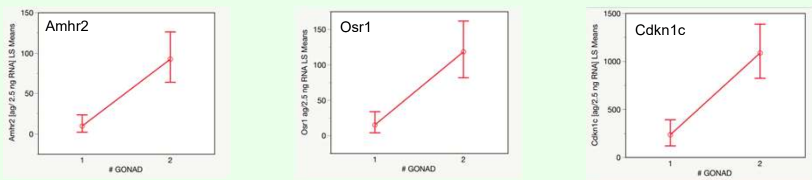
Collection and Cell Culture- 2020

Eggs were collected in the summer of 2020 within 24 hours of oviposition from a captive population of snapping turtles at the University of North Dakota. Eggs were washed with tepid water. Infertile eggs were identified and removed. Fertile eggs were then incubated at 25°C until embryos reached developmental stage 16.5 (Yntema, 1968). Embryos were euthanized by rapid decapitation and gonads were microdissected from the surface of the kidney-adrenal complex. One or two gonads were dissociated, and the single cell suspension was seeded in wells of a 96-well plate. Plates incubated for 48 hours to allow adhesion and proliferation (details of the dissociation and seeding procedure can be found in Bierstedt, 2021). After 2 days, embryos would have reached developmental stage 17 in ovo. Dissociated primary gonad cells were either transfected with a Foxl2-mCherry expression vector using Lipofectamine 3000 Transfection Reagent or exposed to a control Lipofectamine treatment. Cells were then incubated at 25°C or 31°C for two or three days. After incubation, media was removed and cells lysed in PicoPure extraction buffer. Cell lysate was removed from wells, transferred to cryotubes, incubated at 42°C for 30 minutes, and then samples were stored at -80°C.

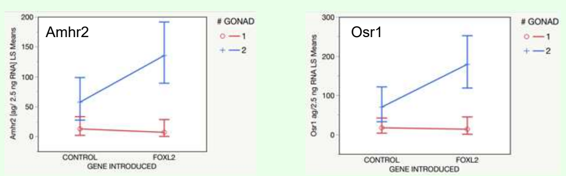
RNA Extraction, Reverse Transcription, and qPCR- 2021

Summer 2021, cell lysates were thawed and RNA was extracted with PicoPure RNA Isolation Kits, including on-column DNase treatment. RNA concentration was measured with a ThermoFischer NanoDrop One Spectrophotometer. Quantitative PCR with SsoAdvanced SYBRGreen PCR Reagent and beta-actin primers was performed on RNA solutions to test for genomic DNA. Then, 50 ng of total RNA per sample was used in 20μL reverse transcription reactions using random primer mixes and the Applied Biosystems High Capacity cDNA Reverse Transcription Kit. Primers for quantitative PCR were designed using Applied Biosystems Primer Express software with specifications of 70-100 bp amplicons. 10μL qPCR reactions were performed with SsoAdvanced SYBRGreen PCR Reagent and the forward and reverse primer for each gene. Concentrations of Amhr2, Cdkn1c, and Osr1 were measured in attograms of cDNA per 2.5 ng of total RNA input A standard curve with 10-fold serial dilutions of beta-actin PCR product ranging in $2.0*10^6$ to 0.2 ag per standard were used to quantify results. Melt curve analyses were added to the end of the qPCR protocol to ensure single product amplification. Gene expression data was analyzed using JMP 16. Square-root transformations of expression data were used to satisfy the homoskedasticity requirements of ANOVA.

Results



Figures 1,2,3: Concentration of Amhr2, Osr1, and Cdkn1c in one gonad versus two gonad cell cultures.



Figures 4,5: Concentration of Amhr2 and Osr1 by Foxl2 treatment group in one gonad versus two gonad cultures.

Gene	Amhr2	Cdkn1c	Osr1
ANOVA Factors	**Prob > F**	**Prob > F**	**Prob > F**
Number of Gonads in Cell Culture	**<.0001***	**<.0001***	**<.0001***
Temperature	**0.0404***	0.6387	0.3946
Length of Incubation at 31C	0.9797	0.4529	0.817
Introduction Foxl2 or control vector	0.1841	0.1656	0.0909
# of Gonads * Temperature	0.0693	0.6061	0.8
# of Gonads *Incubation Length	0.8045	0.9049	0.7391
Temperature * Incubation Length	0.4788	0.549	0.7014
# of Gonads * Temperature * Incubation Length	0.2294	**0.0451***	0.1193
# Gonads * Foxl2 or Control	**0.0384***	0.1168	**0.0404***
Temperature * Gene Introduction	0.2894	0.7332	0.9451
# of Gonads * Temperature * Gene Introduction	**0.0058***	**0.0447***	0.1737
Day of Incubation * Gene Introduction	0.9705	0.4307	0.4501
# of Gonads*Incubation Length*Gene Introduction	0.4208	0.9057	0.8576
Temperature*Incubation Length*Gene Introduction	0.5411	0.3255	0.677
# of Gonads*Temperature*Incubation Length*Gene Introduction	0.9456	0.718	0.9013
Who collected, pooled, and lysed cells	0.9742	0.0665	0.4437

Table 1: ANOVA results by gene.

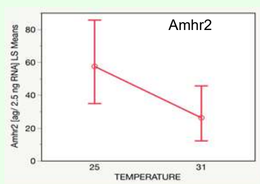
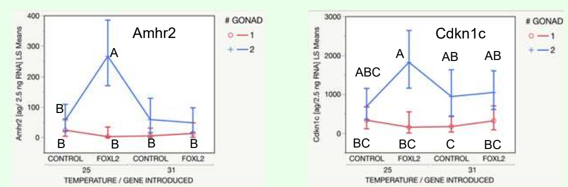


Figure 6: Concentration of Amhr2 by temperature of incubation.



Figures 6,7: Concentration of Amhr2 and Cdkn1c at 25ºC and 31ºC by Foxl2 treatment group, in one gonad versus two gonad cultures. Letters represent results of Tukey's HSD analysis.

Discussion & Conclusions

CONCLUSIONS AND DISCUSSION

- Overexpression of Foxl2 alone did not significantly alter expression of Amhr2, Cdkn1c, or Osr1, but Foxl2 interacted with the number of gonads and/or temperature to affect these genes.
- Expression of Amhr2, Cdkn1c, and Osr1 all increased with the number of gonads used in cell culture.
- Foxl2 transfection of two-gonad cultures increased the expression of Osr1 with no significant temperature interactions.
- Foxl2 transfection of two-gonad cultures induced higher expression of Amhr2 and Cdkn1c at the male-producing temperature, 25°C. Incubation at 31°C showed no significant difference from one-gonad cultures.
- Temperature had a significant effect on Amhr2 due to high expression in Foxl2 transfected two-gonad samples.

This study demonstrates intricate interactions between thermal conditions, cell density, and sex determination.

Foxl2 is a potential regulator of Amhr2, Cdkn1c, and Osr1. This aligns with known principles that define cell proliferation as an important part of sex differentiation and gonadal organization.

Further investigations into gonadal cell communication, proliferation, and density could improve the in vitro representation of in vivo and in ovo molecular pathways of sex determination.

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