# Organotypic Modeling Platform for Adverse Outcome Pathways of Male Reproductive and Developmental Processes

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## Abstract

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In recent decades male reproductive health and fertility problems have increased. Male reproductive dysfunction can be a result of exposure to chemicals during critical periods of testicular development. Studies have shown that testis development is one of the most sensitive developmental processes and often drives chemical risk assessment. The testis, however, is difficult to study and methods for assessing toxicity remain limited. This study addressed gaps in toxicity testing for this organ by building upon a co-culture method and framework for assessing toxicity outcomes in the developing testis. The aims of the study were to 1. develop a systems biology platform for integrating normal and adverse responses across testis development in rodents *in vivo* and *in vitro*; 2. leverage a previously developed co-culture method to quantify baseline characteristics of the mouse culture; and 3. use the mouse co-culture to evaluate effects of cadmium treatment during a critical window of susceptibility.

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## Background

### *Trends in Male Fertility*

In recent decades the number of men facing reproductive health and fertility problems has increased. Total fertility rate has fallen significantly in a number of locations around the world including Europe, Japan and the United States while there has been a steady rise in testicular germ cell cancer worldwide (Skakkebaek et al., 2016). Additionally, a comprehensive literature review of sperm concentration and male fertility trends for the past 33 years identified a significant decline in North America, Europe, Asia and Africa (Sengupta et al., 2016). Male reproductive dysfunction can be a result of exposure to chemicals during critical periods of testicular development. Multiple analyses of reproductive toxicity databases have reported that the testis is among the most sensitive organs to exogenous exposure and often drives the lowest observable adverse effect level (LOAEL) for risk assessment (Parks Saldutti et al., 2013). Despite the need for methods of toxicity testing for this sensitive organ, few exist due to the great complexity and timing of developmental processes.

### *Windows of Susceptibility and Adverse Outcome Pathways*

Early life is a sensitive window during which exposure to chemicals can disrupt normal development. Mounting evidence supports the hypothesis that there are critical periods of growth and development spanning the *in utero* and adolescence life stages during which susceptibility to environmental chemical exposures is heightened. In addition to increased sensitivity, early life exposures can also damage long term health, compromising wellbeing and functional endpoints across the life course. (World Health

Organization, 2000). Decreased range of function is also known as an adverse outcome and can be assessed in a framework outlining an exposure pathway leading to a disease over the course of a lifetime. An adverse outcome pathway (AOP) framework can be used to interpret and translate exposure in early life to diseases in adulthood to improve the safety assessment of potential environmental toxicants (Ankley et al., 2010; Burden et al., 2015). The AOP wiki site currently includes an example of an AOP network based on five reproductive and developmental toxicity AOPs. This approach demonstrates how an AOP framework can be used for model system development and refinement (Knapen et al., 2015). Figure 1 outlines an AOP framework for a toxicant exposure leading to a whole organism response with a specific example for testis development. The AOP example for testis development shows how cadmium exposure has the potential to disrupt endocrine function, increase reactive oxygen species and cause inflammation leading to a cellular response culminating in decreased organ function.

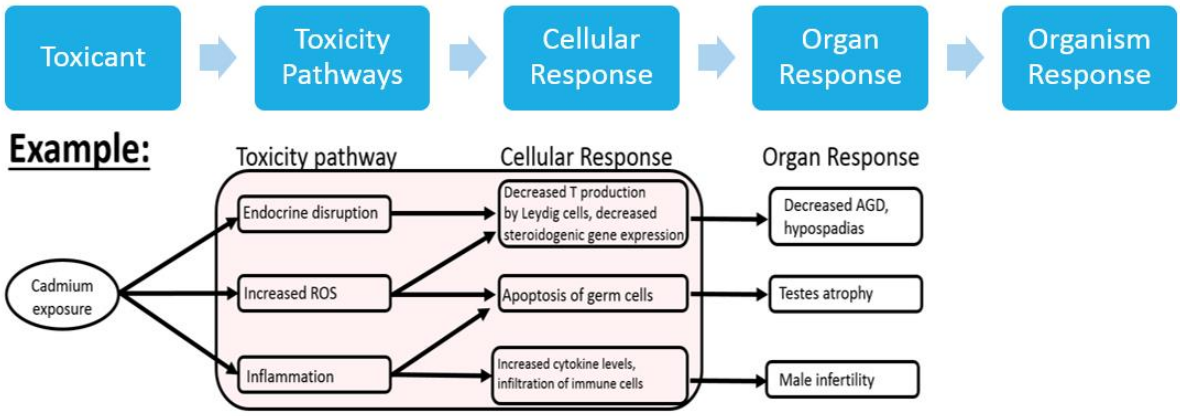


**Figure 1:** The top figure was adapted from Burden, et al. 2015 and the example figure was adapted from Harris Dissertation Defense, 2015. An adverse outcome pathway (AOP) framework can be used to interpret and translate exposures to diseases later in life. This AOP example for testis development shows how cadmium exposure has the potential to disrupt endocrine function, increase reactive oxygen species and cause inflammation leading to a cellular response culminating in decreased organ function.

### *In Vitro Models for Developmental Toxicity*

Identifying reproductive toxicants is an important step in controlling environmental risk factors that have the potential to impact reproductive health and development. There is a need for high throughput and high content methods of screening the large number of chemicals that have not yet been assessed for reproductive toxicity (Judson et al., 2009). Testing every chemical in an animal model would be too time intensive and is not economically feasible (Parks Saldutti et al., 2013). Second to chronic bioassays, the *in vivo* toxicity tests for reproductive and developmental endpoints use the largest number of animals for assessment (e.g. prenatal developmental toxicity studies examines ~250 fetuses per test group) and yet these study designs are often criticized for their low power to detect reproductive toxicity endpoints such as infertility, testicular dysgenesis syndrome, or transgenerational effects (Blystone et al.; Hotchkiss et al., 2008).

In 2007, the National Research Council recognized this need by proposing a vision using *in vitro* methods to evaluate adverse effects on developmental pathways (The National Academies Press, 2007). Methods and approaches for *in vitro* tests have continued to evolve and have been refined since this report came out (Judson et al., 2010; Judson et al., 2011; Rusyn & Daston, 2010). Recently, ToxRef DB was utilized to identify 774 chemicals that were evaluated for male reproductive toxicity. Identified compounds were examined using the U.S. EPA's *in vitro* high-throughput screening database (ToxCast DB) to investigate bioactivity across molecular and cellular endpoints (Leung et al., 2015). While this is a great advance in the development of high-throughput

models for the assessment of testicular toxicants, high-content systems for assessing perturbations help to gain insight regarding the mechanisms of toxicity.

**Thesis Goals**

**Specific Aim 1:** Develop a systems biology platform for interpreting normal and adverse responses for testicular developmental changes across the life stage in rodents *in vivo* and *in vitro*.

- Hypothesis: Biological changes observed *in vitro* will correspond to key developmental events observed *in vivo*. These correlations will be dependent on developmental timing.

**Specific Aim 2:** Quantify baseline characteristics of a high-content *in vitro* testicular mouse co-culture to evaluate the extent to which the model system captures key developmental processes of *in vivo* testicular development.

**Specific Aim 3:** Quantify adverse effects of cadmium in the testicular mouse co-culture to evaluate impacts during a critical window of susceptibility.

- Hypothesis 1: The dose response to cadmium will be life stage specific.
- Hypothesis 2: Cadmium will disrupt differentiation and proliferation of germ and Leydig cells.
- Hypothesis 3: Cadmium will decrease testosterone production from Leydig cells.

**Thesis Introduction**

Chapter 1 outlines how the systems biology platform was created and how it was utilized to anchor endpoints characterized in the *in vitro* models. Specifically, a literature search was carried out to build a framework for investigating temporal and species

comparisons of proliferation, steroid regulation and spermatogenesis processes throughout rodent testis development. Previously, a three-dimensional testicular co-culture system was created and optimized to include immature rat testes (Wegner et al., 2013; Yu et al., 2009; Yu et al., 2005) and the baseline characteristics were characterized throughout time (Wegner et al., In Progress). The current project focused on modifying the previously created co-culture system to include immature mouse testes tissue and the baseline characteristics were quantified over time for 16 days. Both the rat and mouse baseline characteristics were anchored and mapped next to the *in vivo* timeline allowing for a temporal, species and *in vivo/in vitro* comparisons.

Chapter 2 describes a preliminary study in which the mouse *in vitro* testis cell co-culture was treated with cadmium and the dose response was characterized. Cadmium is a pollutant that is ubiquitously distributed in the environment. Occupational exposure to cadmium is the most common source of exposure, but exposure for the general public also occurs through cigarette smoke, food and water (Fowler, 2009; Yu et al., 2008). This is a pertinent public health issue because cadmium exposure has been associated with many negative health outcomes including impacts on male reproductive function (Yu et al., 2008). Cadmium exposure and testicular toxicity has been studied by assessing functional endpoints. However, specific mechanisms of toxicity, especially during male reproductive development, have yet to be characterized. This preliminary study modified a previously developed three-dimensional testicular co-culture system (Wegner et al., 2013; Yu et al., 2009; Yu et al., 2005) to investigate the mechanisms of toxicity associated with cadmium exposure in a model of *in vitro* testicular development.

## Chapter One: Male Reproductive and Developmental Framework for the Assessment of Adverse Outcome Pathways


### Abstract

Background: In recent decades the number of men facing reproductive health and fertility problems has increased. Male reproductive dysfunction can be a result of exposure to chemicals during critical periods of testicular development. Multiple studies have shown that testis development is one of the most sensitive developmental processes and often drives chemical risk assessment. The testis, however, is difficult to study and methods for assessing toxicity remain limited.

Objectives: In this work, we addressed gaps in toxicity testing for this sensitive organ by building upon a three-dimensional testicular co-culture system and framework for assessing toxicity outcomes in the developing testis.

Methods: The present study developed a systems biology platform for integrating normal and adverse responses across testis development in rodents *in vivo* and *in vitro* and leveraged a previously developed primary co-culture method to quantify baseline characteristics of the mouse culture *in vitro*.

Results: Our framework allowed for the comparison of testicular developmental processes across time and rodent species *in vivo*. We were able to anchor *in vitro* results in the *in vivo* timeline allowing for temporal, species and *in vivo/ in vitro* comparison.

Conclusions: Based on our temporal *in vivo* timeline, we are confident we are capturing similar windows of development in the rat and mouse co-culture systems. The developmental processes captured in the co-culture systems are similar to processes in human testicular development. Our high-content co-cultures for toxicity testing are capturing sensitive developmental pathways and have broad impacts for human risk assessment.

## Introduction:

### *Trends in Male Fertility*

In recent decades the number of men facing reproductive health and fertility problems has increased. Total fertility rate has fallen significantly in a number of locations around the world including Europe, Japan and the United States while there has been a steady rise in testicular germ cell cancer worldwide (Skakkebaek et al., 2016). Additionally, a comprehensive literature review of sperm concentration and male fertility trends for the past 33 years identified a significant decline in North America, Europe, Asia and Africa (Sengupta et al., 2016). Male reproductive dysfunction can be a result of exposure to chemicals during critical periods of testicular development. Multiple analyses of reproductive toxicity databases have reported that the testis is among the most sensitive organs to exogenous exposure and often drives the lowest observable adverse effect level for risk assessment (LOAEL) (Parks Saldutti et al., 2013). Despite the need for methods of toxicity testing for this sensitive organ, few exist due to the complexity and the timing of developmental processes that occur during its differentiation and maturation.

### *Windows of Susceptibility and Adverse Outcome Pathways*

Early life is a sensitive window during which exposure to chemicals can disrupt normal development. Mounting evidence supports the hypothesis that there are critical periods of growth and development spanning the *in utero* and adolescence life stages during which susceptibility to environmental chemical exposures is heightened (Faustman et al., 2000). In addition to increased sensitivity, early life exposures can also damage long term health, compromising wellbeing and functional endpoints across the life course. (World Health Organization, 2000). Decreased range of function is also known as an adverse outcome and can be assessed in a framework outlining an exposure pathway leading to a disease over the course of a lifetime. An adverse outcome pathway (AOP) framework can be used to interpret and translate exposure in early life to diseases in adulthood to improve the safety assessment of potential environmental toxicants (Ankley et al., 2010; Burden et al., 2015).

Recent human studies point towards early dysgenesis of the fetal testis as a critical biological link between male reproductive disorders, including changes in sperm differentiation, hypospadias, infertility, and testicular cancer. This constellation of male reproductive disorders is termed testicular dysgenesis syndrome (TDS) (Wohlfahrt-Veje et al., 2009). The etiology of these disorders is unknown, however genetic factors can be identified in ~15% of infertility cases (Krausz, 2011). Human susceptibility and variability heavily influence how environmental exposures affect health and well-being. As such, there is a great need for increased research efforts to "define the range and causes of susceptibility to adverse effects of chemicals in the population" (Rusyn, Gatti, et al., 2010).

***Reproductive Toxicity Testing***

Identifying reproductive toxicants is an important step in controlling environmental risk factors that have the potential to influence reproductive development. There is a need for high-throughput and high-content methods of screening the large number of chemicals that have not yet been assessed for reproductive toxicity (Judson et al., 2009). High content screening is needed to investigate multiple time points and allows for the analysis of different parameters at the single cell level (Moutsatsos et al., 2016). Testing every chemical in an animal model would be too time intensive and is not economically feasible (Parks Saldutti et al., 2013). Second to chronic bioassays, the *in vivo* toxicity tests for reproductive and developmental endpoints use the largest number of animals for assessment (e.g. prenatal developmental toxicity studies examines ~250 fetuses per test group) and yet these study designs are often criticized for their low power to detect reproductive toxicity endpoints such as infertility, testicular dysgenesis syndrome, or transgenerational effects (Blystone et al.; Hotchkiss et al., 2008).

In 2007, the National Research Council recognized the need for improved toxicity testing by proposing a vision using *in vitro* methods to evaluate adverse effects on developmental pathways (The National Academies Press, 2007). Methods and approaches for *in vitro* tests have continued to evolve and have been refined since this report came out (Judson et al., 2010; Judson et al., 2011; Rusyn & Daston, 2010). Recently, the ToxRefDB was utilized to identify 774 chemicals that were evaluated for male reproductive toxicity. Identified compounds were examined using the U.S. EPA's *in vitro* high-throughput screening database (ToxCast DB) to investigate bioactivity across molecular and cellular endpoints (Leung et al., 2015). While this is a great advance in the

development of high-throughput models for the assessment of testicular toxicants, high-content systems for assessing perturbations help to gain insight regarding the of the mechanisms of toxicity.

Creating an *in vitro* culture system capable of capturing complete spermiogenesis reliably is challenging for many reasons including isolating, purifying and creating an optimal biochemical and biophysical niche (Parks Saldutti et al., 2013). Current *in vitro* methods used to investigate testis development include the pop-off assay, the 2-chamber culture system, and three-dimensional (3-D) models (Parks Saldutti et al., 2013). Our lab has created a novel 3-D system which provides a significant improvement over existing 2-D substratum approaches (Yu et al., 2003; Yu et al., 2005). Our 3-D testicular co-culture system includes Sertoli, Leydig, germ cells and macrophages suspended in a matrigel extracellular matrix. The 3-dimensionality of this culture system improves consistency of system responses, Sertoli cell attachment and increases cell-cell communication (Yu et al., 2005).

## Current Project

### *Systems Biology Approach to Toxicity Testing*

Utilizing the systems biology approach is an important building block in understanding and parsing out concepts from varying levels of biological analysis (Styczynski et al., 2016). Three-dimensional cell cultures capable of high-content analyses are an important component in the systems biology framework of understanding developmental processes sensitive to perturbation during very specific time points. We have developed a systems biology platform for interpreting normal and adverse responses

across testicular development in rodents *in vivo* and *in vitro.* The platform allowed us to look at our results across species at different levels of detail culminating in a bigger picture for translation of potential human adverse outcomes.

Specifically, our systems biology approach includes mouse *in vivo* transcriptomic global gene expression pathway dynamics throughout testicular development (S. H. Wegner et al., 2015), a rat *in vitro* co-culture created and optimized in 2005 (Wegner et al., In Progress; Yu et al., 2009; Yu et al., 2005), rat *in vivo* transcriptomic global gene expression pathway dynamics (Wegner et al., In Progress), and a mouse *in vitro* co-culture which is described in the current study.

Publicly available data were used to quantify mouse *in vivo* transcriptomic gene expression pathway dynamics throughout testicular development (S. H. Wegner et al., 2015). This was done in order to provide a quantitative description of normal pathway dynamics in mouse testicular development. The goal was to provide a context for interpreting perturbed pathways with exposure to exogenous compounds. The method consisted of identifying genes significantly changed throughout testis development in mice. These genes were clustered by their direction of change and assigned a gene ontology (GO) term. Temporal pathway dynamics of enriched terms were quantified based on average expression of intensity for all genes associated with a given term (S. H. Wegner et al., 2015). Included as examples from the published paper are the dynamics characterizing temporal changes in steroid regulation, spermatogenesis, meiosis and mitosis pathways shown in Figure 2. Pathways associated with spermatogenesis and meiosis were shown to increase during development and remain in adulthood allowing consistent regeneration of mature sperm. Conversely, processes specific to developmental

pathways were shown to be highest during gestation and early post-natal time points. These processes related to steroid regulation and mitosis are unique to development and decline once mature testicular physiology has been achieved.

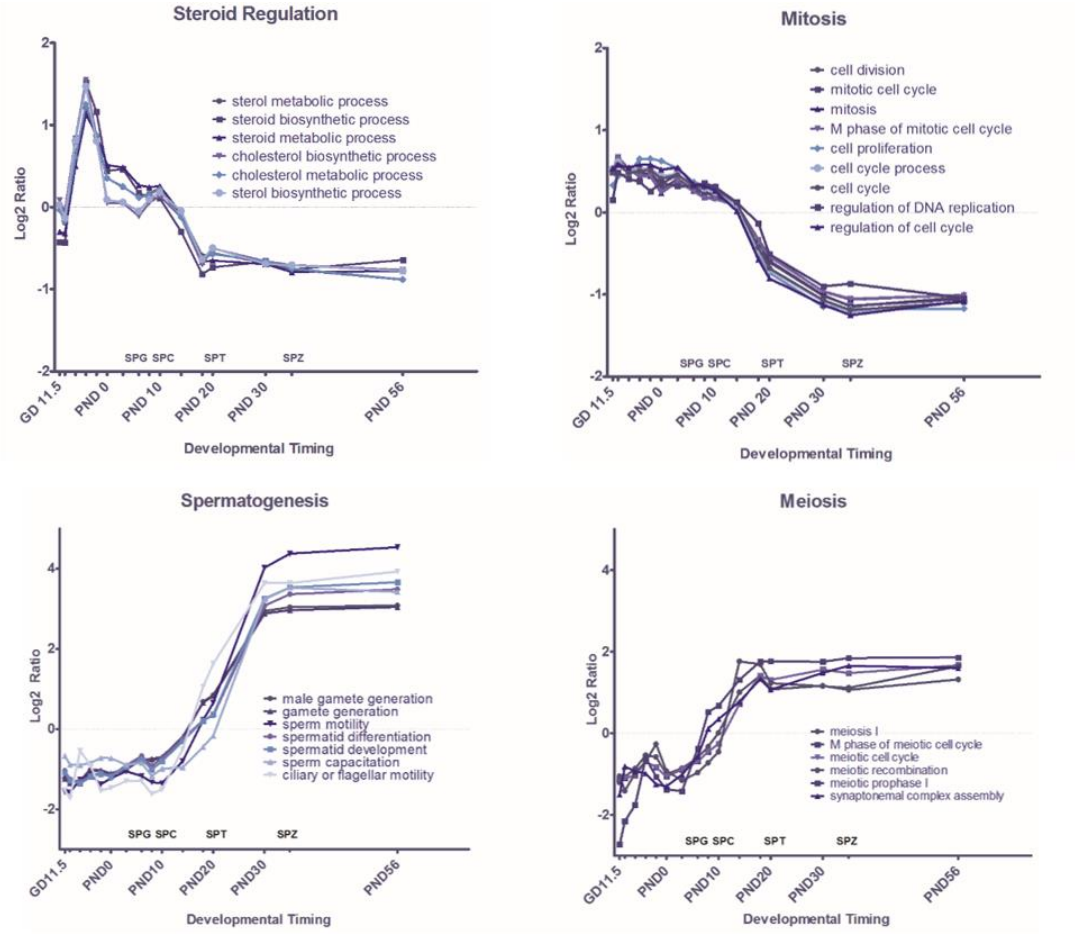


**Figure 2:** Genes were clustered by their direction of change and assigned a gene ontology (GO) term. Temporal pathway dynamics of enriched terms were quantified based on average expression of intensity for all genes associated with a given term (S. H. Wegner et al., 2015). Highlighted from this paper are the developmental dynamics associated with pathways characterizing temporal changes in steroid regulation, spermatogenesis, meiosis and mitosis.

The rat *in vitro* co-culture was optimized in 2005 (Yu et al., 2005) and since then baseline dynamics have been characterized (Wegner et al., 2013; Yu et al., 2009). The novel 3-dimensional system provides a significant improvement over existing 2 dimensional substratum approaches (Yu et al., 2003; Yu et al., 2005). The co-culture system includes Sertoli, Leydig, germ cells and macrophages suspended in a 3-dimensional niche provided by the matrigel extracellular matrix. The 3-dimensionality of this co-culture system was shown to improve germ cell proliferation and survival, enhance cell survival pathways and reduce cell stress and apoptosis pathways (Yu et al., 2005).

In order to evaluate the ability of this model to accurately predict male reproductive toxicants, several medium throughput cytotoxicity and cell viability assays have been optimized for use in this co-culture. The assays were applied to screen a diverse panel of over 80 drugs and environmental chemicals of interest, including cadmium and mercury (Yu et al., 2003; Yu et al., 2008), androgen like compounds (Amory et al., 2014) and several phthalate esters (Harris et al., 2015; Harris et al., 2016).

To compare rat *in vivo* and *in vitro* gene expression dynamics involved in key developmental processes a transcriptomic analysis was carried out to identify important similarities and differences (Wegner et al., In Progress). Figure 3 shows heat maps of relative gene expression intensity *in vivo* and *in vitro* for a set of genes associated with a specific GO term. Shown below are heat maps for Leydig cell differentiation, male germ cell proliferation and steroid metabolism. Genes were clustered based on the expression *in vivo* on post-natal days 6,8 and 10 and *in vitro* on days 0,1,3,5 and 7.

In many of the pathways observed, genes associated with the developmental processes that were most highly expressed *in vivo* were also among the most highly expressed in the GO term *in vitro*. Also observed were gene expression trends that were less consistent *in vivo* and *in vitro*. For example, the most highly expressed genes associated with spermatogenesis were more variable between the *in vivo* and *in vitro* models. Additionally, *in vitro* gene expression trends indicated an increase in stress response genes, with more greater expression among GO terms for oxidative stress and apoptosis (Wegner et al., In Progress).

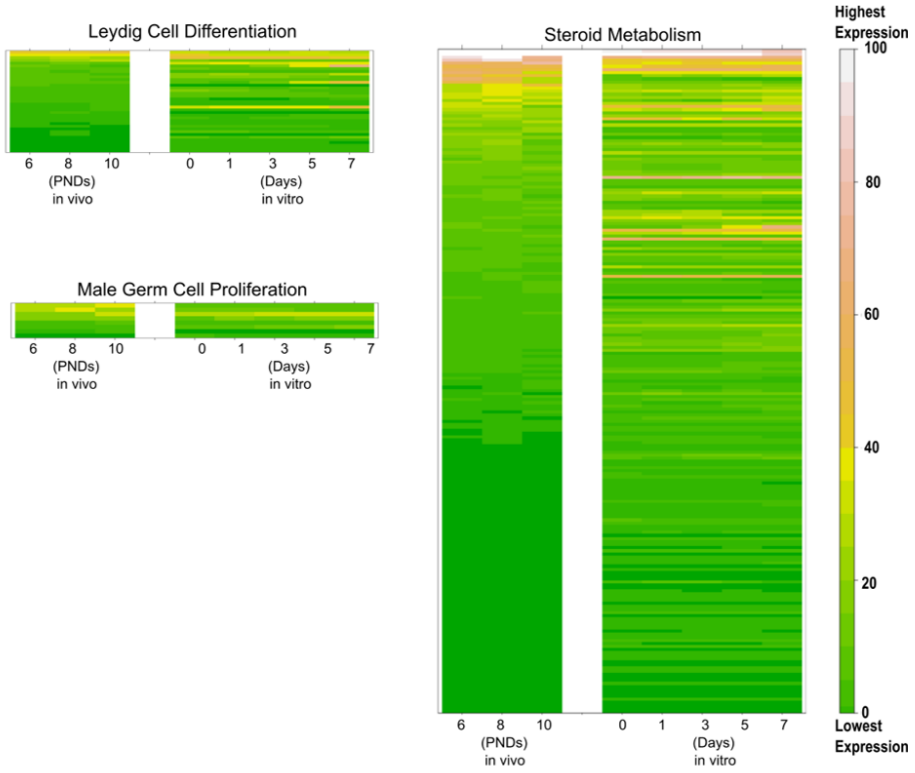


**Figure 3:** Heat maps of relative gene expression intensity for a set of genes associated with a specific GO term for Leydig cell differentiation, male germ cell proliferation and steroid metabolism. Genes were clustered based on the expression *in vivo* on post-natal days 6,8 and 10 and *in vitro* on days 0,1,3,5 and 7 (Wegner et al., In Progress).

### *Mouse In Vitro Testicular Co-Culture*

As previously addressed, our lab established an *in vitro* three-dimensional testicular co-culture system by isolating testis tissue from immature rats (Wegner et al., 2013; Wegner et al., In Progress; S. Wegner et al., 2015; S. H. Wegner et al., 2015; Yu et al., 2009; Yu et al., 2005). In the present study, the culture system was modified to include mouse tissue because both rats and mice are commonly used models for assessing toxicity. Assessing endpoints in each co-culture system allows for the opportunity to compare these two commonly used rodent models.

Additionally, mouse models allow for the introduction of genetic diversity into the culture system. One example of how genetic diversity can be incorporated into toxicity studies is through the use of the Collaborative Cross (CC). The eight CC founder strains were selected from a list of over 100 potential mouse strains because they maximize genetic diversity and utility for investigating traits of widespread interest (Rusyn, Gatti, et al., 2010). The generation of the CC established a "translational platform for systems genetics" that will reduce the limitations of research done in inbred strains by increasing the genetic diversity in these animals (Rusyn, Gatti, et al., 2010). In fact, these eight strains encompass approximately 90% of the known allelic diversity across all 1-Mb intervals spanning the entire mouse genome (Rusyn, Gatti, et al., 2010). There has already been some characterization of collaborative cross strains that are sensitive or resistant to testicular toxicity (Habeebu et al., 2001) and those that have deleterious features related to male reproduction (Odet et al., 2015). Such information can then be used in mechanistic research to define critical elements of adverse response and in risk assessments to modify factors used for kinetic and dynamic uncertainty.

The present study utilized one of the CC founder strains, C57BL6/J to begin to investigate the *in vitro* dynamics of testicular development using mice. Quantifying the baseline characteristics of the mouse *in vitro* culture will allow for temporal, species and *in vivo/in vitro* comparisons of developmental timing in the co-culture model.

## Methods

### *Development of framework*

A literature search was carried out in order to build a detailed timeline of testicular development in rats and mice. The timeline, shown in Figure 4, was built to include proliferation and maturation, spermatogenesis and steroid regulation pathways mapped temporally from post-natal day 0-37 in both species. For each pathway, colors in the timeline correspond to different developmental processes, which is described in the key. The timeline was used to anchor baseline characteristics observed in our rat and mouse co-cultures and to interpret our results within the context of normal testes development in rodents.

### *Testicular Co-Culture Preparation and Maintenance*

Male C57BL6/J mice purchased from Jackson Labs were time mated in our facility under a protocol approved by the UW Institutional Animal Care and Use Committee. Testicular tissue was isolated on postnatal day 9 and digested into a single cell suspension. Detailed methods of the plating process were previously described in Wegner et al. 2013 and Yu, et al 2005. Cells were plated in 24 well plates (Corning, Primeria multiwell 24 well) with a density of 0.8 $x10^6$ cells/mL. A total of 0.5 mL cell

suspension/well was overlaid with 10 μL of chilled Matrigel (BD Biosciences) (for a final concentration of 200 μg/mL) extracellular matrix which was added immediately to the center of each well. After the addition of matrigel, each plate was gently rocked back and forth to provide a 3-D scaffold. Co-cultures were incubated at 37˚C with 5% CO2 and maintained for up to 16 days in culture replacing the entire medium 48 hours post plating and half of the medium every two days.

***Live/Dead/Nuclei Imaging***

We utilized an iCys Research Imaging Cytometer with an Olympus 1X71 microscope and iCys 3.4 software to image live cells, dead cells and nuclei in our co-cultures on days 3, 7 and 16 *in vitro*. Living co-cultures were incubated for 15 minutes at 37˚C with fluorescence dyes that labeled live cells with Calcien AM (Life Technologies) at a concentration of 1 μM in PBS. Dead cells were labeled with 2.5 μg/mL propidium iodide (Life Technologies), and nuclei were labeled with 10 μg/mL Hoechst 33342 (Sigma). Cell staining and morphology were visualized with the iCys Research Imaging Cytometer at 20x magnification. Images presented are representative of 3 biological replicates.

***Western Blotting***

On days *in vitro* 2, 3, 6, 7, 15 and 16 cells were harvested in 1X cell lysis buffer (Cell Signaling Technology) with Protease Inhibitor I (Calbiochem) to reduce protein degradation and Phosphatase Inhibitor Cocktail Sets I and II (Calbiochem) to preserve post translational protein modifications. Protein was isolated by 3 freeze/thaw cycles

followed by centrifugation to pellet membranes and cell debris. Protein concentration of the supernatant was determined using a protein assay kit (Bio-Rad Laboratories) and all samples were brought to equal concentrations with 1X cell lysis buffer. Samples were prepared for Western blotting with 4X sample buffer and reducing agent (Life Technologies). Samples were loaded in 10% Bis-Tris NuPage precast minigels (Life Technologies) and proteins separated by size by running gels at 200V for approximately 45 minutes in running buffer containing 500 μL Antioxidant solution (Novex, Life Technologies). Protein was transferred to polyvinylidene difluoride nylon membranes (Bio-Rad Laboratories) for immunoblotting at $4^{o}$ C for 100 minutes at 100V. Membranes were rinsed in tris-buffered saline (TBS) pH 7.4-7.6, then blocked for 1 hour with 5% nonfat dry milk with 0.1% TTBS. Membranes were then rinsed 3 times with TTBS and incubated overnight with primary antibody with 5% milk or 5% BSA with TTBS. Primary antibodies used include SCP3 (ab97672, mouse monoclonal) for early meiosis, 3betaHSD (ab75710, mouse monoclonal) for Leydig cells, c-kit (sc-168, rabbit polyclonal) for germ cells, vimentin (sc-6260, mouse monoclonal) for Sertoli cells, PCNA (MAb424, mouse monoclonal) for proliferation with a 1:1000 dilution factor and Actin (Sigma, mouse monoclonal) with a 1:5000 dilution factor. The following day, membranes were washed 3 times in TTBS and incubated for 2 hours with secondary antibody conjugated to horseradish peroxidase diluted in 5% milk in TTBS. Secondary antibodies included anti-rabbit secondary antibody (Cell Signaling Technology Inc.) or anti-mouse secondary antibody (BD Pharminogen) with a dilution factor of 1:5000. After secondary antibody incubations, membranes were washed 5 times for 5 minutes with TTBS and incubated with enhanced chemiluminescence detection reagent (GE

Lifescience) for 2 min, then exposed to X-ray films (Bioexpress). Films were developed and digitally scanned. Band intensity was quantified by densitometry using ImageJ software. Expression intensity of each probe was normalized to intensity of corresponding actin loading controls. Data presented here reflects at least 1 biological replicate.

***Testosterone Assay***

To measure testosterone concentrations through time in the co-culture, medium was collected on days *in vitro* 2,3,6,7,15 and 16. Testosterone concentrations in each media sample determined with a dilution factor of 1:1 by an ELISA assay for total testosterone according to the kit protocol (Testosterone ELISA Kit, Neogen Corporation #402510). Samples outside the range of detection were diluted 1:10 and re-run in the assay. Testosterone concentrations measured in the media were normalized to the protein content of corresponding cell lysate (determined by BioRad Protein Assay as described above). Data are presented in terms of fold change in total ng testosterone / mg protein relative to control. Data presented here reflect at least 3 biological replicates.

***Immunofluorescence***

Medium was removed and the co-culture was washed once with PBS then fixed with 4% paraformaldehyde (Sigma) for 30 minutes. The 4% paraformaldehyde was removed then washed in PBS and stored at 4˚C until ready for immunofluorescence imaging. Fixed cells were washed 3 times with PBS, then blocked for 1 hour with PBS blocking buffer containing 5% goat serum (Gibco) and Triton-X 100 to permeablize cell

membranes. Cells were then incubated at 4˚C overnight with the primary antibody SCP3 (ab97672, mouse monoclonal) and PBS antibody dilution buffer containing 5% BSA and triton X 100. Samples were washed 3 times in PBC and incubated in Alexa flour tagged goat anti-rabbit and goat anti-mouse secondary antibodies (Life Technologies) diluted 1:750 in antibody dilution buffer for 2 hours. Samples were then washed three times, with Hoechst dye 33342 in the final wash to stain nuceli. Labeled cells were visualized at 20x magnification and color channels were processed and combined using Metamorph software to create an overlay of the 3-color and immunofluorescence images.

***Morphology***

The co-culture was monitored throughout time by capturing images on an Olympus inverted microscope operated in phase contrast mode with a Photometrics Cool Snap Cf camera system using Roper Scientific imaging software. Images were captured for all 16 days in culture with a sample set shown here. Images shown here reflect at least 3 independent biological replicates.

**Results**

There is a large amount of data in the literature characterizing developmental dynamics of testicular development in mice and rats (Davidoff et al., 2004; Haschek et al., 2013; Kerr et al., 1988; Lee et al., 1975; Malkov et al., 1998; Mendis-Handagama et al., 2001; O'Shaughnessy et al., 2002; Orth, 1982; Picut et al., 2015; Vergouwen et al., 1991). Part A. in Figure 4 consolidates evidence from the broader literature to generate a timeline of testicular development that can help anchor the *in vitro* results to *in vivo*

developmental processes that are sensitive to toxicant perturbation. The literature search timeline focuses on three main biological processes; proliferation and maturation, spermatogenesis and steroid regulation. *In vivo* biological processes are compared in a temporal fashion for both rats and mice for all three processes. As shown in Part B of Figure 4, we can use the timeline to anchor baseline characteristics we observed in the rat (Wegner et al., In Progress) and mouse co-culture from the current study to interpret our results within the context of normal testes development in rodents.

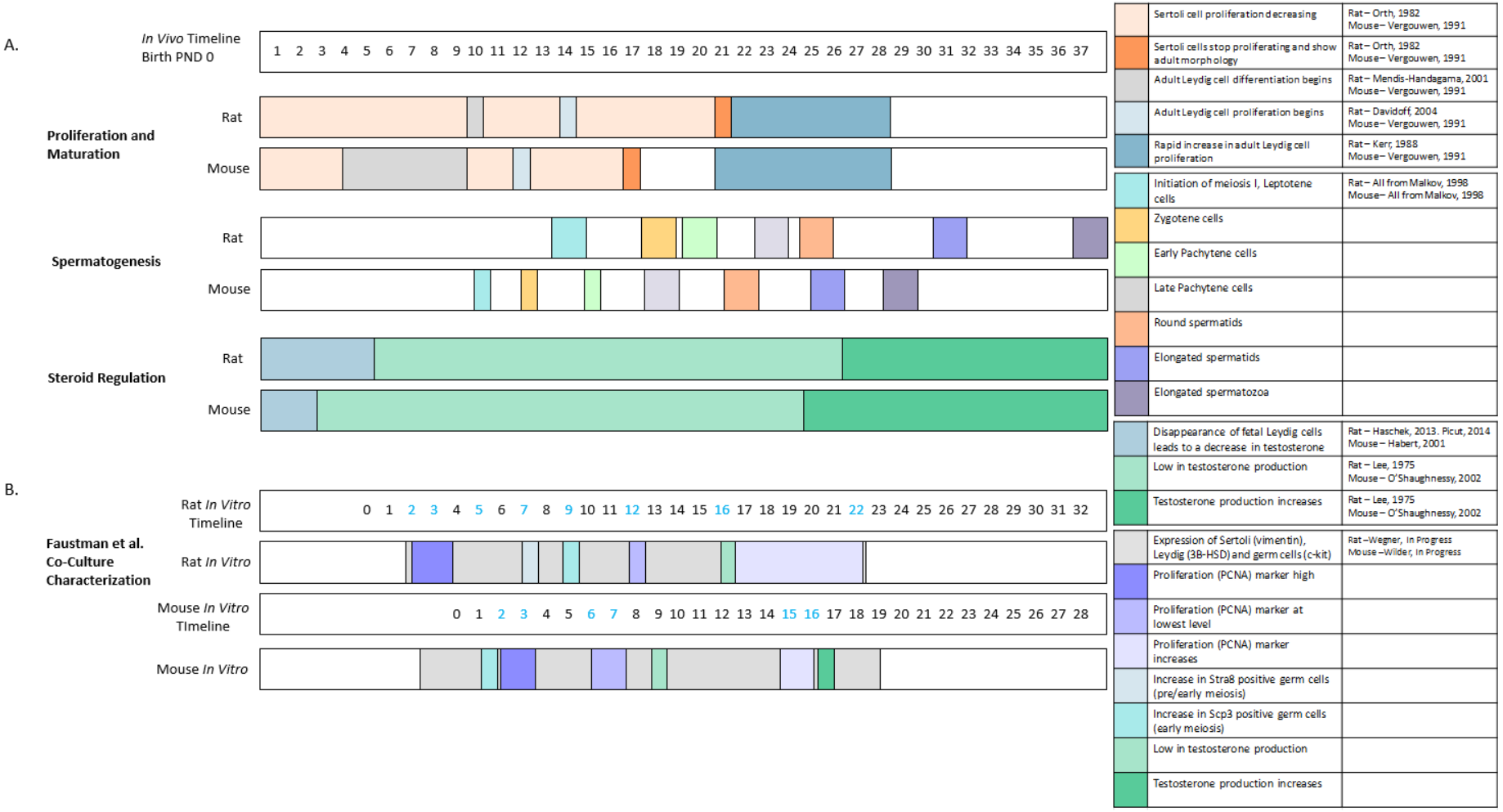


Figure 4: *In vivo* and *in vitro* timeline comparison. Part A. in the figure corresponds to *in vivo* timing. Each color corresponds to a developmental process which is described in the key along with a reference. Rat and mouse *in vitro* timing is indicated in Part B with harvest days identified by blue numbers in the timeline. *In vitro* data for the rat comes from Wegner et al. In Progress while the mouse *in vitro* data was analyzed in the current study.

***Temporality***

A temporal framework allows for the interpretation of the *in vitro* co-culture models throughout time. Additionally, a temporal context has direct implications for the assessment of toxicant impacts during critical windows of susceptibility. Specific examples of how dynamic processes play an important role in the development of the testes *in vivo* are shown in Part A of Figure 4.

Proliferation and maturation processes happening *in vivo* during this window include temporal changes in the number and type of cell types found in the developing testes. Sertoli cells are the most abundant cell type in the testis when pups are born and continue proliferating until they show adult morphology on post-natal day (PND) 21 in rats (Orth, 1982) and PND 17 in mice (Vergouwen et al., 1991). Within a few days of birth, fetal Leydig cells disappear in rats (Haschek et al., 2013; Picut et al., 2015) and mice (Habert et al., 2001). Adult Leydig cells begin to differentiate around PND 10 (Mendis-Handagama & Ariyaratne, 2001) with rapid proliferation around PND 21 in rats (Kerr & Knell, 1988). In mice, adult Leydig cells begin to differentiation on PND 4 with rapid proliferation also occurring around PND 21 (Vergouwen et al., 1991).

Spermatogenesis is initiated with meiosis I in rats on PND 14-15 and in mice on PND 10 with elongated spermatozoa appearing on PND 37 in rat and PND 28 in mice (Malkov et al., 1998).

The disappearance of fetal Leydig cells leads to a subsequent decrease in testosterone production from about PND 6 – 27 in rats with the lowest concentration of 0.37 +/- 0.07 ng/ml serum (Lee et al., 1975) and PND 4 – 25 in mice with the lowest concentrations of 0.5 pmol/ testis (O'Shaughnessy et al., 2002). Shortly after adult Leydig

cells begin to rapidly proliferate, testosterone production increases on PND 27 in rats with concentrations increasing to around 1 ng/ml serum (Lee et al., 1975) and PND 25 in mice with concentrations increase to around 100 pmol/testis (O'Shaughnessy et al., 2002).

### ***In vivo* vs. *in vitro* comparison**

*In vitro* endpoints are anchored in the *in vivo* framework for interpretation of developmental processes throughout testis development. By using the *in vivo* framework, we can assess 'normal' vs. 'toxicant' response during this developmental life stage in the *in vitro* model.

*Proliferation and Maturation*

Proliferating cellular nuclear antigen (PCNA), a marker of cells in the DNA synthesis (S) phase of the cell cycle, was measured using a Western blot analysis. The levels of PCNA were normalized to the levels of beta actin measured for each sample. PCNA was shown experimentally to be high *in vitro* during the first few days in culture indicating Sertoli cells were still proliferating. PCNA then decreased as Sertoli cells reached maturity then increased again as Leydig cells began to proliferate. This trend is seen both in the rat (Wegner et al., In Progress) and mouse *in vitro* co-cultures with Figure 5 indicating this trend in the mouse model.

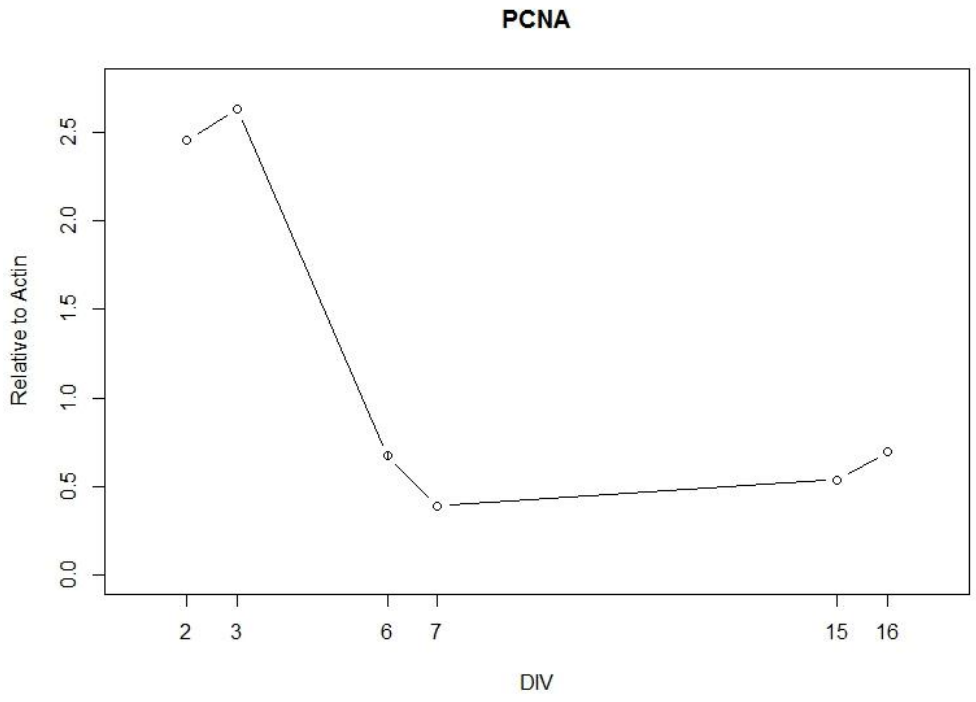


Figure 5: Proliferating nuclear antigen (PCNA) was assessed a Western blot for the mouse *in vitro* co-culture. PCNA was normalized to beta actin. The trend indicates a drop off in PCNA abundance when Sertoli cells may be reaching maturity on days *in vitro* 6/7 and a slight increase when adult Leydig cells may begin to proliferate in our culture on days *in vitro* 15/16. While, this data is from two biological replicates, each time point has one data point.

Live, dead and total nuclei were quantified in the culture on days *in vitro* 2, 3, 6, 7, 15 and 16 using an iCys Research Imaging Cytometer. As shown in Figure 6, the viable cell population appears to have different morphological features. On day *in vitro* 3, the cell population is larger and the nuclei are less condensed. On day *in vitro* 16, however, the viable cells are much smaller with more compact nuclei indicated by brighter cells. We hypothesize that the new cell population on day *in vitro* 16 are the adult Leydig cells. The next step is to investigate this hypothesis with a Leydig cell type specific immunofluorescence marker.

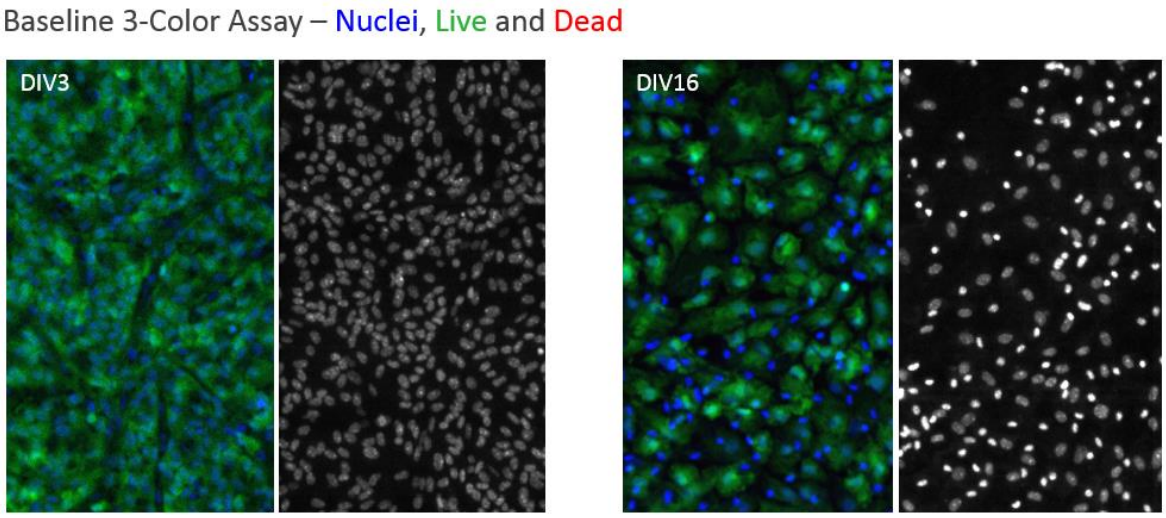


Figure 6: The mouse co-cultures were imaged on days *in vitro* 3, 7 and 16 with days 3 and 16 shown above. In the color images on the left, live cells were stained with calcein AM and appear as green. Dead cells were stained with propidium idodide (PI) and would appear as red though, no dead cells appear in these images. Nuclei were stained with Hoe 33342 and appear as blue. The black and white images show nuclei with no color added. Images were taken with 20X magnification.

*Spermatogenesis*

Spermatogenesis was measured in our culture by a Western blot to quantify both synaptonemal complex protein 3 (Scp3), which is a marker specific for early meiotic processes and a receptor tyrosine kinase (C-kit), a marker for germ cells. As shown in Figure 7, Scp3 was measured throughout time in the culture and normalized to beta actin. On day *in vitro* 2, a slight peak in Scp3 was detected. This is around the same time as the first wave of spermatogenesis is initiated with meiosis I *in vivo* which may indicate we are capturing this process in the *in vitro* culture. Evidence of germ cells throughout time in our culture was measured using the marker C-kit. Levels of C-kit normalized to beta actin remained relatively stable throughout time in the *in vitro* culture as shown in Figure 8.

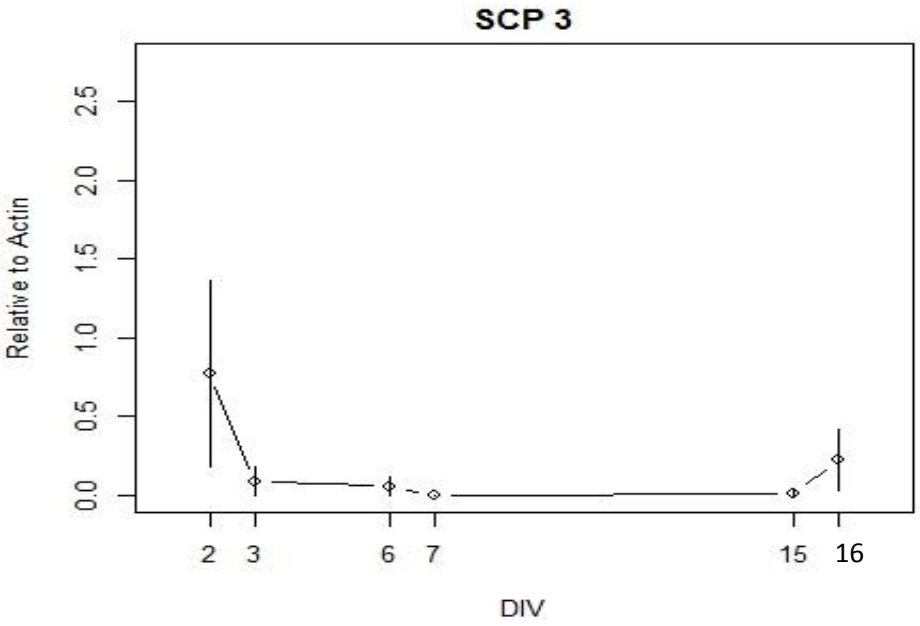


Figure 7: Synaptonemal complex protein 3 (Scp3) is a marker specific for early meiotic processes. On day *in vitro* 2, a slight peak in Scp3 was detected. This is around the same time as the first wave of spermatogenesis is initiated with meiosis I *in vivo* which may indicate we are capturing this process in the *in vitro* culture. This data is from three biological replicates with two data points per time point.

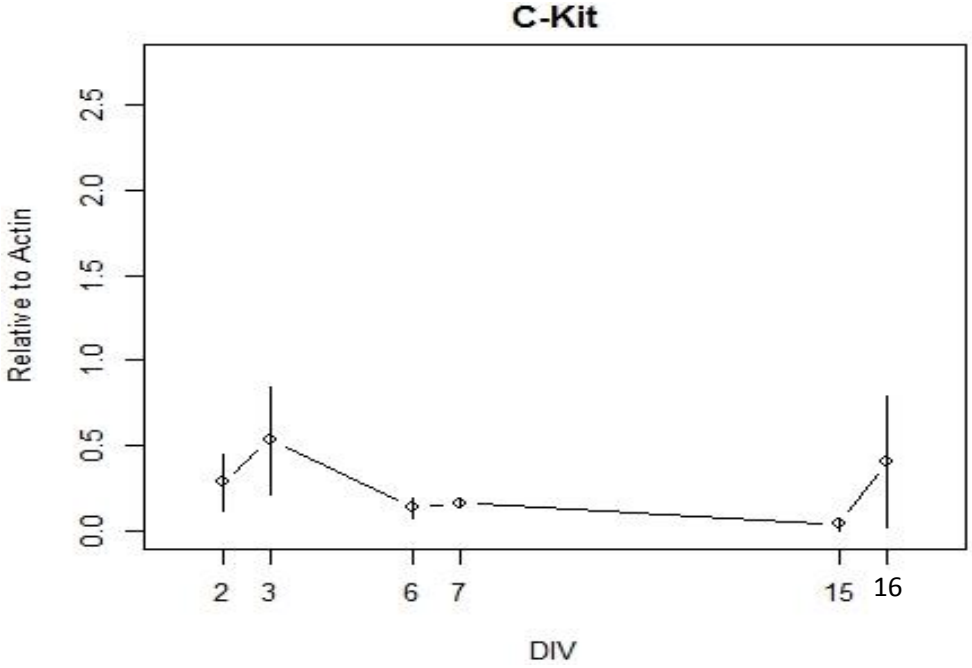


Figure 8: Receptor typrosine kinase C-kit is a marker specific for germ cells. Levels of C-kit normalized to beta actin remained relatively stable throughout time in the *in vitro* culture. These data are from three biological replicates with two data points per time point.

In addition to the Western blots, and to semi-quantitatively investigate the presence of spermatogenesis related proteins, we stained the culture with an immunofluorescence marker for Scp3 as shown in Figure 9. This image is an overlay of a 3-color assay with live cells shown in green, nuclei shown in blue and an immunofluorescence image taken to visualize Scp3 shown in red. The images taken on the laser scanning cytometer were overlaid to create this picture to get a sense of the morphology and spatial location of cells undergoing early meiotic processes in our culture on day *in vitro* 16.

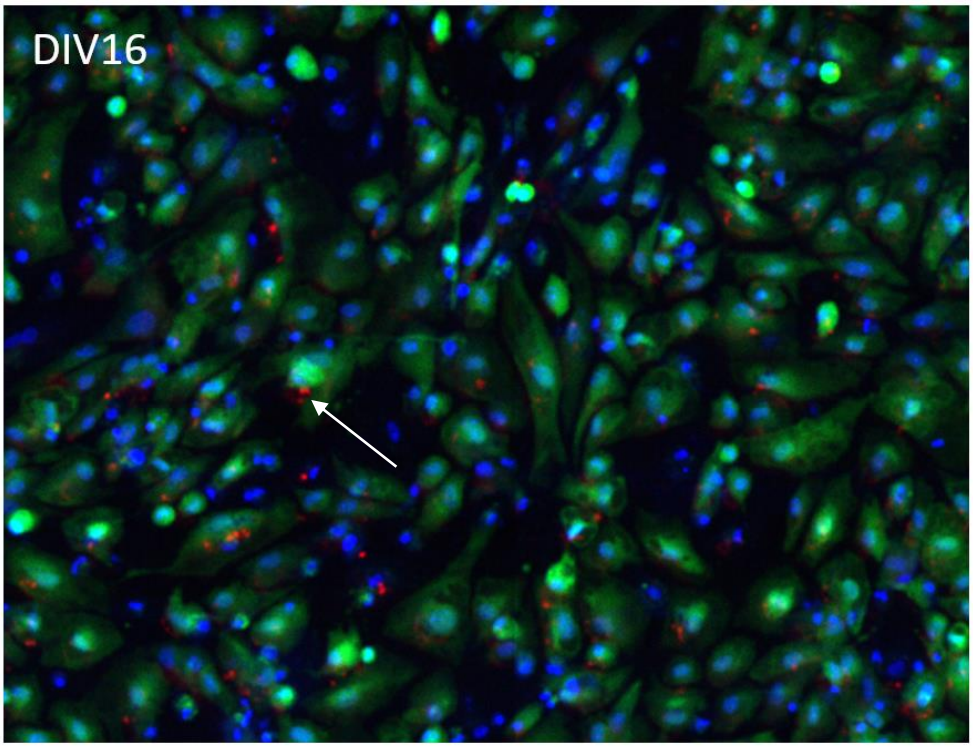


Figure 9: This is an overlay of images taken during a 3 color assay, the blue stains nuclei and the green stains live cells. The cells were fixed and stained with the immunofluorescence marker SCP3, shown in red, for early meiosis. The white arrow indicates an example of a SCP3 in the co-culture. This shows some evidence of spermatogenesis processes in our co-culture model on day *in vitro* 16. Images were taken with 20X magnification.

Another method of imaging was employed to visualize changes to morphology throughout time in the culture using a basic microscope. Shown in Figure 10 are microscopic images of the culture taken throughout time with 20X magnification. On days *in vitro* 15 and 16 the cells are shown migrating towards each other to form multiple clusters in the 3-D niche.

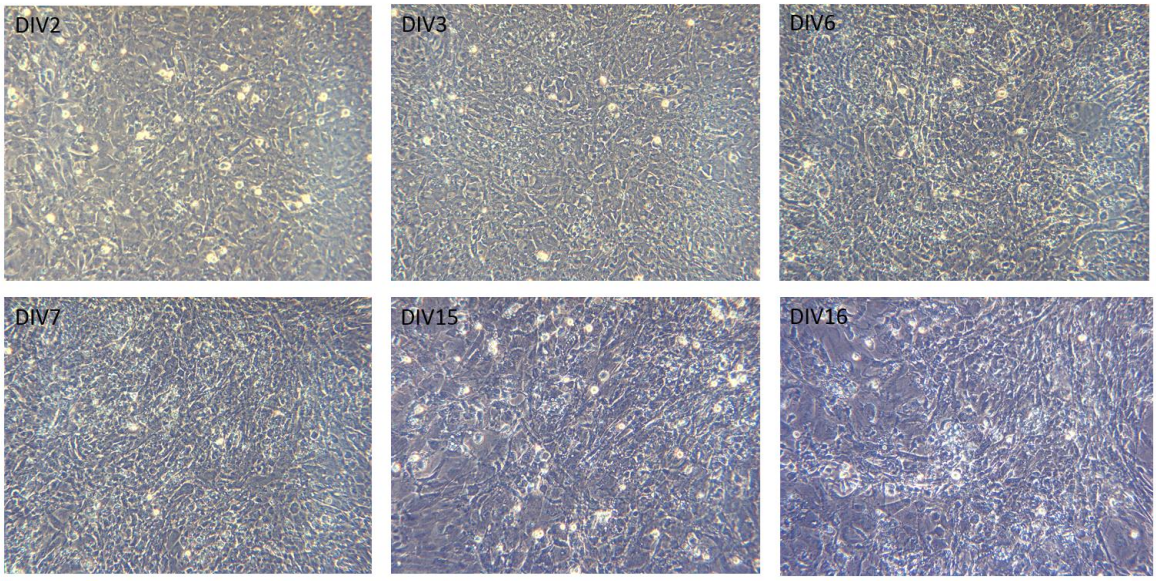


Figure 10: Images captured on a basic microscope show how the morphology and the location of the cells move throughout time in the culture at 20X magnification. On days 15 and 16 the cells are shown migrating towards each other to form multiple clusters in the 3-D niche.

*Steroid Regulation*

Testosterone production was measured throughout time in the culture by harvesting media and normalizing to the total amount of protein in each well to give a final concentration in ng of testosterone per mg of cellular protein. As shown in Figure 11, testosterone production in the culture is found to be higher on days *in vitro* 2 and 3. During this time we hypothesize that fetal Leydig cells are regressing but still producing some testosterone in our culture. The levels continue to decrease as fetal Leydig cells

become more scarce in the culture. This nadir in testosterone is followed by a rapid proliferation of adult Leydig cells which begin to start producing testosterone. This developmental process is potentially being captured by our culture system indicated in the trend we see in testosterone production throughout time.

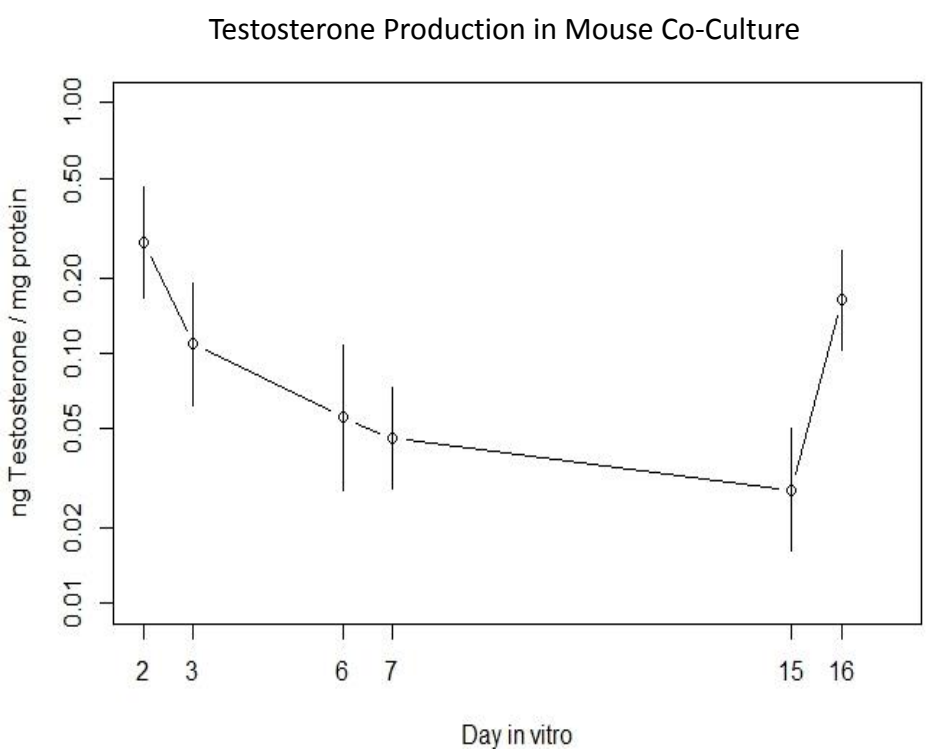


Figure 11: Testosterone production throughout time. Testosterone harvested from each well was normalized to total protein from that well. Testosterone production was highest on days *in vitro* 2 and 3 when we hypothesize there are fetal Leydig cells producing testosterone in the culture. The level of testosterone production decreased with the decrease in the number of fetal Leydig cells on days *in vitro* 6 and 7. Then we observed an increase in testosterone production as there is a potential increase in adult Leydig cell proliferation in the co-culture on days *in vitro* 15 and 16. This data is based on a 3 biological replicates.

To semi-quantitatively show the presence of Leydig cells throughout time in the culture we used a Western blot to measure the presence of 3-beta hydroxysteroid

dehydrogenase (HSD), a key enzyme involved in steroidogenesis. This protein was found to be expressed in culture throughout time as shown in Figure 12.

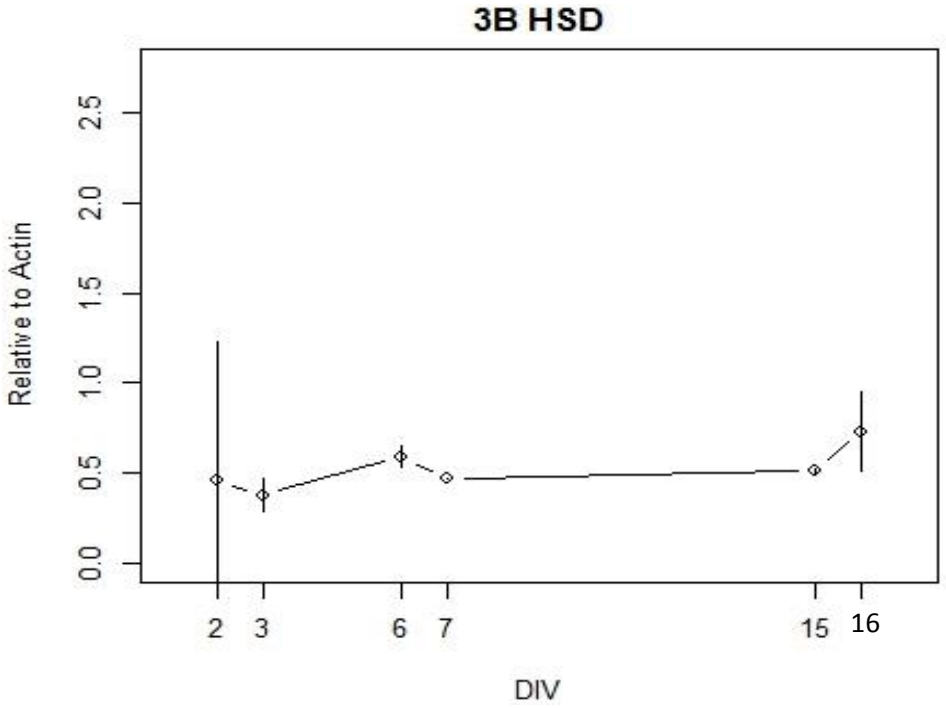


Figure 12: Presence of Leydig cells was semi-quantitatively measured throughout time in the culture by a Western blot for the protein 3-beta HSD which is a key enzyme involved in steroidogenesis. 3-beta HSD was normalized to beta-actin. While, these data are from two biological replicates, each time point has one data point.

***Interspecies Comparison***

Mapping interspecies developmental timing is important for life stage comparisons across commonly used model systems. Interspecies comparisons are important for the interpretation of results across different toxicity testing model systems and for extrapolation of potential adverse outcomes in humans. For the three developmental pathways mapped in the systems biology timeline, we are capturing a very similar developmental life stage in both mice and rats. For all three processes the mouse

timing is slightly shifted with processes consistently happening earlier but overall the timelines show very similar developmental timing.

## Discussion

### *Co-Culture*

Captured in our co-culture models are processes relevant for assessing male reproductive and developmental toxicity. The co-culture was characterized at specific time points based on the hypothesis that sensitive developmental processes would be happening in the culture during those days *in vitro*. In this initial assessment of these time points we do believe we are capturing processes that may be sensitive to toxicant perturbations. These conclusions are drawn from the perturbations we saw in morphological and testosterone production changes.

While we are encouraged by these initial studies it is important to recognize there are reproductive and developmental processes we cannot recreate or capture in our co-culture models. Processes we cannot capture in our developmental system include spermatid formation. We have some evidence of spermatogenesis processes but we are investigating how far along the spermatogenesis process these cells progress in our culture. Thus far only one group has been able to replicate human spermatid production (Tesarik et al., 2000).

### *Timeline*

The developmental *in vivo* timeline was constructed from many different publications including different strains and endpoint measurements. This timeline is meant to be a relative map of processes happening in rat and mouse testicular

development over time. Currently, there are few resources to visualize the many processes occurring during testicular development. This is why we see utility in developing a systems based temporal and species comparison to visualize this sensitive process.

### *Similarities to Mouse In Vivo Transcriptomics*

There are specific examples from our mouse *in vitro* culture which align with mouse *in vivo* transcriptomic pathway analysis previously done in our lab (S. H. Wegner et al., 2015). The mouse developmental pathway analysis *in vivo* supports the timeline by depicting increased pathway expression of mitosis related genes through the time period when we would expect the somatic Sertoli and Leydig cell populations to be proliferating. The *in vivo* transcriptomic analysis also supports the *in vitro* observations that the spermatogenesis pathway is underway, beginning with an increase in the early meiosis marker Scp3 day *in vitro* 2. Gene expression for the steroid regulation developmental pathways are expressed more abundantly while fetal Leydig cells remain in the testis. Steroid regulation decreases with the disappearance of fetal Leydig cells and further decreased by PND 30 as the testis reaches maturity.

### *Broader Impacts for Human Risk Assessment*

Since our co-culture models are able to capture key developmental pathways and responses to toxicant perturbation, we can use this model as a high content method of assessing toxicants with the potential to perturb testicular development in humans. There is evidence supporting the hypothesis that testicular developmental pathways are well conserved in mammals. Specifically, transcriptomic developmental pathway analysis

among mice, rats and humans have shown that a key group of gene associated with meiotic processes are highly conserved across these species (Chalmel et al., 2007). Additionally, a study comparing cellular events in human and mouse testicular development reports that the temporal sequence is similar but of course longer in humans (Ostrer et al., 2007).

The results of this project are particularly relevant for shedding light on the uncertainty factors used in risk assessment to translate results from *in vitro* cell cultures and rodent models to human populations. Risk assessment uncertainty factors include a multiplication factor of ten for evaluating no observable adverse effect levels (NOAELs) from studies *in vitro* to those performed *in vivo*, from rodents to humans, and from adults to children. The systems biology platform and *in vitro* results across two species begin to address these uncertainty factors by mapping mechanistic information regarding specific biological processes that define life stage susceptibility and comparisons of biological responses *in vitro* and *in vivo* and between two species. Using this platform, we have anchored the *in vitro* results to *in vivo* processes to better understand the implications of changes in our cell culture across life stages at the organism level. Ultimately, more platforms like this need to be developed to integrate *in vitro* results into the risk assessment framework. The comparisons made between *in vivo* and *in vitro* development are important for extrapolation across commonly used models of toxicity testing and for the facilitation of AOP evaluations.

## Future directions

In order to make gene expression pathway dynamic comparisons to the mouse *in vivo* analysis, we plan to analyze *in vitro* transcriptomics during time points of interest. Additionally, we will incorporate collaborative cross mouse strains in order to increase genetic variability in the culture system. In order to visualize and quantify proteins and specific cell types in our co-culture quantitatively, we will optimize the imaging of immunofluorescence markers using the iCys Research Imaging Cytometer. Finally, the ultimate goal of the culture system is to use it as a high content method for screening compounds having the potential to cause reproductive and developmental harm. When we assess the toxicity of compounds of interest in our test system we will analyze the production of cytokines with a customized cytokine panel and introduce a cytosolic re-dox reporter which will indicate reactive oxygen species present in our culture.

## Conclusions

Based on our temporal *in vivo* timeline, we now have a frame that will allow us to evaluate similar windows of development in the rat and mouse co-culture systems. Additionally, our high-content co-cultures for toxicity testing are capturing sensitive developmental pathways relevant for human risk assessment.

## Chapter 2: Evaluation of Cadmium Treatment on an *In Vitro* Model of Testis Development

**Abstract:**

Background: Cadmium is a pollutant that is ubiquitously distributed throughout the environment. Exposure to cadmium is most common in occupational settings but exposures can also occur through cigarette smoke, food and water. (Yu et al., 2008). This is a pertinent public health issue because cadmium exposure has been associated with many negative health outcomes including impacts on male reproductive function (Yu et al., 2008). Cadmium exposure and testicular toxicity has been studied by assessing functional endpoints. However, specific mechanisms of toxicity, especially during male reproductive development, have yet to be characterized.

Objectives: This preliminary study modified a previously developed three-dimensional testicular co-culture system with tissue isolated from immature mice to investigate the mechanisms of toxicity associated with cadmium exposure during testicular development.

Methods: To evaluate the three dimensional testicular co-culture response to cadmium, the co-culture was treated on days *in vitro* 2, 6 and 15, and effects were measured 24 hours later. These time points were chosen based on sensitive developmental processes shown to be happening *in vivo* in the systems biology framework. Cadmium was introduced to the culture in 2.5, 5 and 10 µM concentrations.

Results: In our preliminary study we observed a dose and life stage dependent disruption in testosterone production, cytotoxicity, cell viability and morphology.

Conclusions: The quantitative results have been interpreted within our systems biology platform and demonstrate the potential of our model to capture adverse outcomes in proliferation and testosterone production pathways in this *in vitro* model of male reproductive development.

## Introduction

### *Cadmium and Testicular Toxicity*

Cadmium is a pollutant that is ubiquitously distributed throughout the environment. Exposure to cadmium is most common in occupational settings but exposures can also occur through cigarette smoke, food and water (Yu et al., 2008). This is a pertinent public health issue because cadmium exposure has been associated with many negative health outcomes including impacts on male reproductive function (Yu et al., 2008). Cadmium exposure and testicular toxicity has been studied by assessing functional endpoints. However, specific mechanisms of toxicity, especially during male reproductive development, have yet to be characterized. This preliminary study modified a previously developed three-dimensional testicular co-culture system with tissue isolated from immature mice to investigate the mechanisms of toxicity associated with cadmium exposure during testicular development.

**Current Study**

Previously, our lab established an *in vitro* three-dimensional testicular co-culture system by isolating testis tissue from immature rats (Wegner et al., 2013; Wegner et al., In Progress; S. Wegner et al., 2015; S. H. Wegner et al., 2015; Yu et al., 2009; Yu et al., 2005). In the present study, the culture system was modified to include mouse tissue because both rats and mice are commonly used models for assessing toxicity. Additionally, cadmium dose response was evaluated in the rat co-culture allowing for the opportunity to compare these two commonly used rodent models.

The treatment time points were based on a detailed framework characterizing mouse testis development in a temporal fashion, shown in Figure 4. Based on preliminary studies, we believe we are capturing adult Leydig cell differentiation and proliferation as well as the beginning of the spermatogenesis process in our co-culture model. Cadmium treatment on day 2 was chosen for the potential to disrupt the differentiation of adult Leydig cells and meiosis I, the first step in the spermatogenesis process. Treatment on days 6 and 15 were chosen for the correspondence to adult Leydig cell proliferation and subsequently, impacts to testosterone production. We hypothesized that the dose response to cadmium would be life stage specific, there would be impacts on; early spermatogenesis processes, adult Leydig cell differentiation and proliferation, and testosterone production.

Cadmium chloride ($CdCl_2$) treatment doses were chosen based on the previously characterized cadmium dose response in the rat testes co-culture system (Yu et al., 2008). In that study, Yu, et al. tested concentrations 0, 5, 10, 20 and 40 µM $CdCl_2$ and samples were harvested for endpoint analysis 24 hours later. Cadmium treatment lead to a dose-

dependent decrease in cell viability with an $LC_{50}$ of 10 µM. This assay served as a basis for our dose rationale where we chose to treat with 2.5, 5 and 10 µM $CdCl_2$.

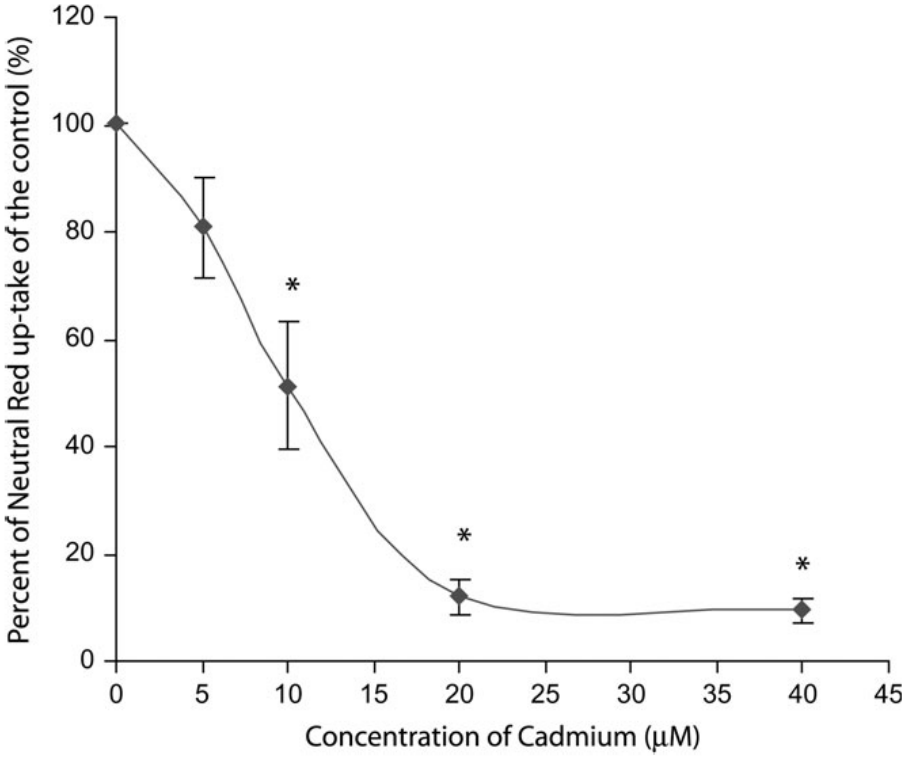


Figure 13: In the Yu, et al. publication it was reported that there was a dose dependent decrease in cell viability in the rat co-culture. The co-cultures were treated with 0,5,10,20 and 40 µM $CdCl_2$ and assessed 24 hours later for neutral red uptake, a measure of cell viability. This study served as our basis for the treatment doses we chose in the current study.

**Methods**

Detailed methods of the mouse *in vitro* co-culture are discussed above. Treatment time points were chosen based on a temporal assessment of key pathways of testis development comparing proliferation and maturation, spermatogenesis and steroid regulation processes, shown in Figure 4. Treatment doses were picked based on a previously characterized dose response in the rat co-culture model. The same endpoints

as discussed above were characterized 24 hours after $CdCl_2$ treatment; 3-color assay, testosterone production, Western blots and morphology.

### *LDH Assay*

In addition to the endpoints discussed above, cytotoxicity was measured with an LDH assay. Cytotoxicity was assessed using CytoTox 96® non-radioactive cytotoxicity assay kits (Promega Corporation). Triplicates per treatment were performed for each assay plate. Samples were incubated with 50 µL LDH substrate mix under dark conditions. After 30 minutes, reactions were terminated with stop solution and absorbance at 490nm was read giving an indication of the amount of LDH for each sample. Plates were read on a spectrophotometric plate reader (SpectraMAX190; Molecular Devices). Media blank corrected absorbance values were normalized to percentage of an LDH max (untreated control wells in which all cells were lysed, giving the maximum reading of total LDH at a particular time point).

## Results

### *Testosterone Production*

Impacts on testosterone production in the mouse co-culture were dose and time dependent (Figure 14). There was a significant decrease in testosterone production on day *in vitro* 3 with the introduction of 10 µM $CdCl_2$. We hypothesize that at this time in the co-culture testosterone is being produced by the fetal Leydig cells. Almost no change was seen in the co-culture on day *in vitro* 7. This may be because during this time there are the lowest numbers of testosterone producing Leydig cells in the culture. While not

significant, there was a slight decrease in testosterone production on day *in vitro* 16 with increasing $CdCl_2$ dose. At this time point, we hypothesized adult Leydig cells would be rapidly proliferating and hence testosterone production in the culture would increase.

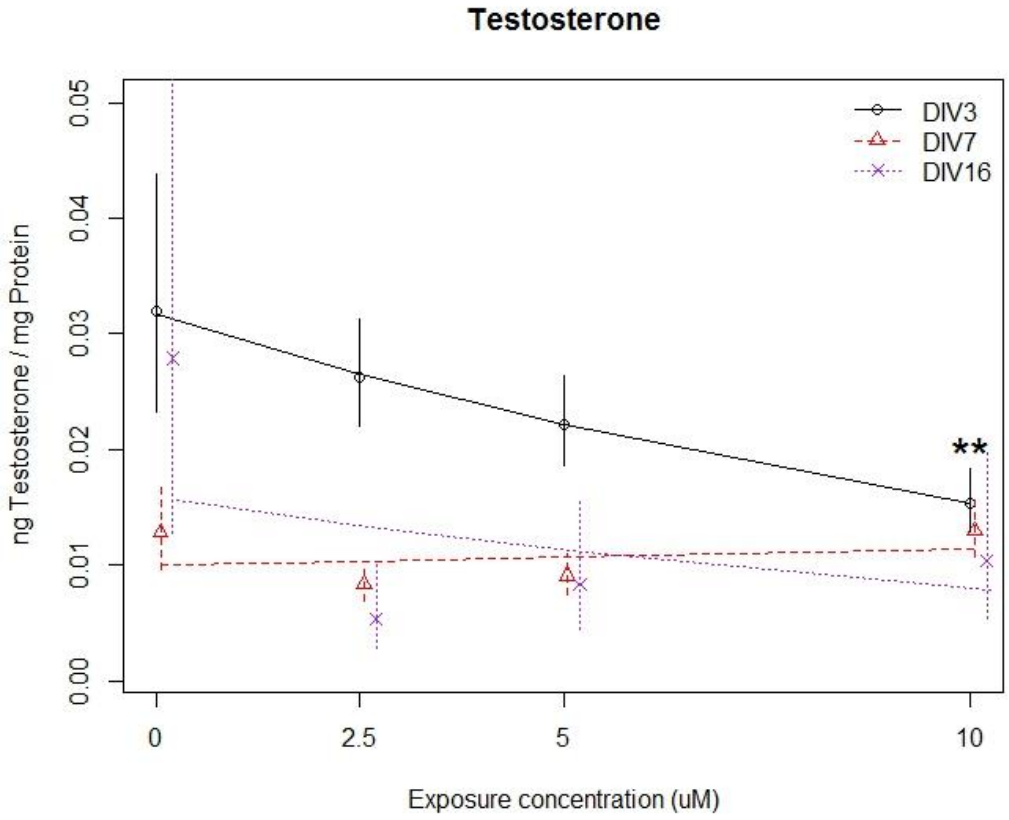


Figure 14: The impact on testosterone production was life stage and dose dependent. There was a significant decrease in testosterone production on day *in vitro* 3 with the introduction of 10 µM $CdCl_2$. Almost no change was seen in the co-culture on day *in vitro* 7. While not significant, there was a slight decrease in testosterone production on day *in vitro* 16 with increasing $CdCl_2$ dose. At this time, we hypothesized adult Leydig cells would be rapidly proliferating and hence testosterone production in the culture would increase. Cadmium has been known to decrease testosterone production. Data are representative of 3 biological replicates. Significance indicators were based on a post hoc analysis compared to the control. Significance level $p<0.001$ indicated by ** and $p<0.05$ indicated by *.

***Cytotoxicity***

Cytotoxicity was measured with an LDH assay, shown in Figure 15. Cytotoxicity was also life stage and dose dependent. We observed a significant dose response for all three time points. The most dramatic response was seen day *in vitro* 16 with all three doses, 2.5, 5 and 10 µM $CdCl_2$ showing a significant response. The two highest doses, 5 and 10 µM $CdCl_2$ were significant on days *in vitro* 7 and 3.

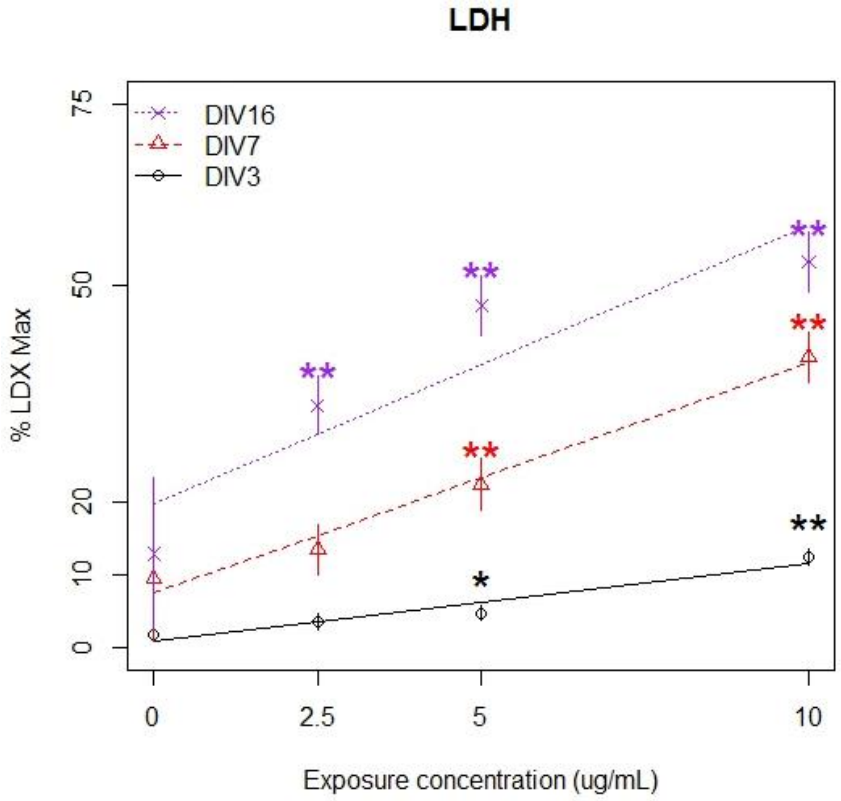


Figure 15: Cytotoxicity measured by LDH release was assessed on days *in vitro* 3, 7 and 16, 24 hours post treatment with 0, 2.5, 5 and 10 µM $CdCl_2$. Cytotoxicity was also life stage and dose dependent. The most dramatic response was seen day *in vitro* 16 for all three doses, 2.5, 5 and 10 µM $CdCl_2$ with a significant response. The two highest doses, 5 and 10 µM $CdCl_2$ were significant on day *in vitro* 7 and 3. These data are based on 3 biological replicates. Significance indicators were based on a post hoc analysis compared to the control. Significance level $p<0.001$ indicated by ** and $p<0.05$ indicated by *.

### *Statistical Analysis*

A mixed effect model was used to determine the dose response for the LDH and testosterone production plots. Samples were analyzed in a biological triplicate for all three time points. In the mixed effect model, dose was treated as a continuous variable and the biological replicates were assigned as random effect variables. For the post-hoc analysis, dose was used as a categorical variable in order to compare to the control sample.

### *Morphology*

Morphology was assessed 24 hours post $CdCl_2$ treatment. Visual observations suggest that the number of dead cells and germ cells detached from Sertoli cells increases in a time- and dose- dependent fashion. Germ cells detached from Sertoli cells are indicated with white arrows in Figure 16. It appears that the greatest number of detached cells are seen on day *in vitro* 16 with the introduction of 10 µM $CdCl_2$.

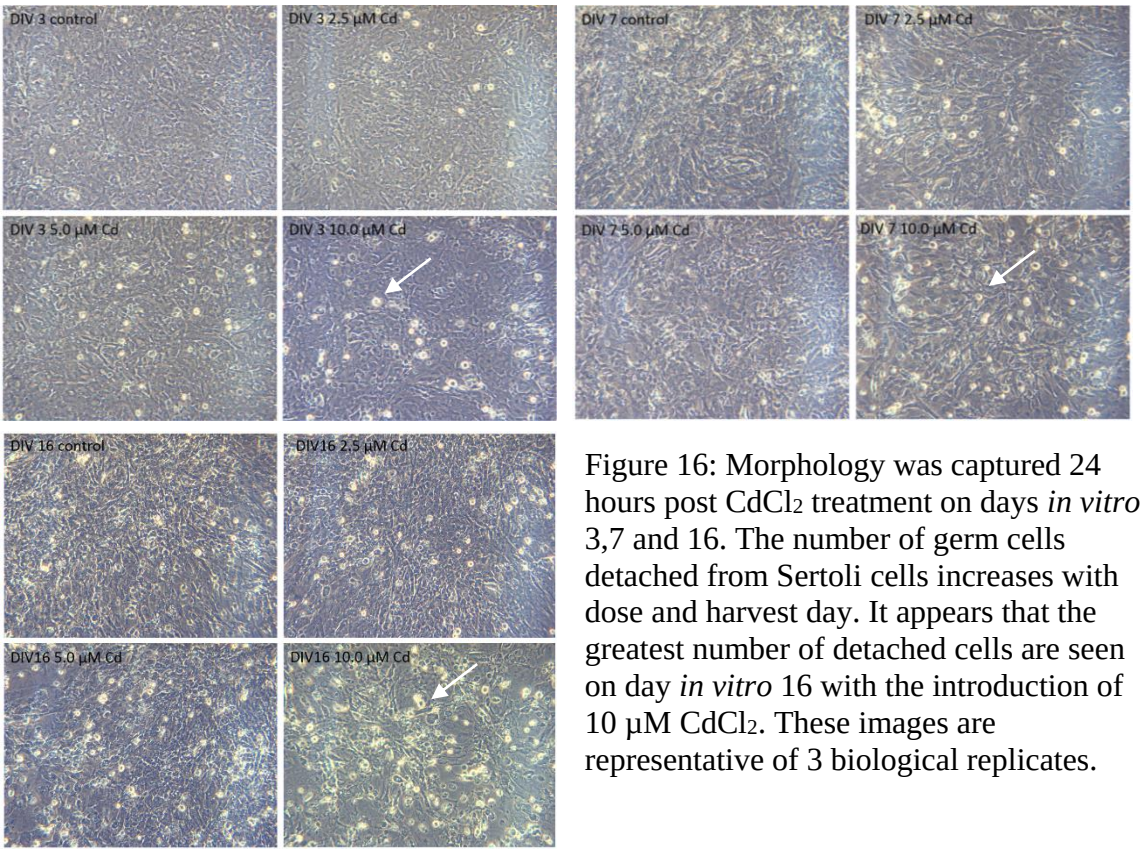


Figure 16: Morphology was captured 24 hours post $CdCl_2$ treatment on days *in vitro* 3,7 and 16. The number of germ cells detached from Sertoli cells increases with dose and harvest day. It appears that the greatest number of detached cells are seen on day *in vitro* 16 with the introduction of 10 µM $CdCl_2$. These images are representative of 3 biological replicates.

***Live/Dead/Nuclei Imaging***

To further investigate the number of dead cells in the co-culture, a 3-color assay was used. In Figure 17, live cells appear green, dead cells appear red and nuclei are shown as white on a black background and white. The number of dead cells appears to increase over time with the greatest number dead cells seen on day *in vitro* 16.

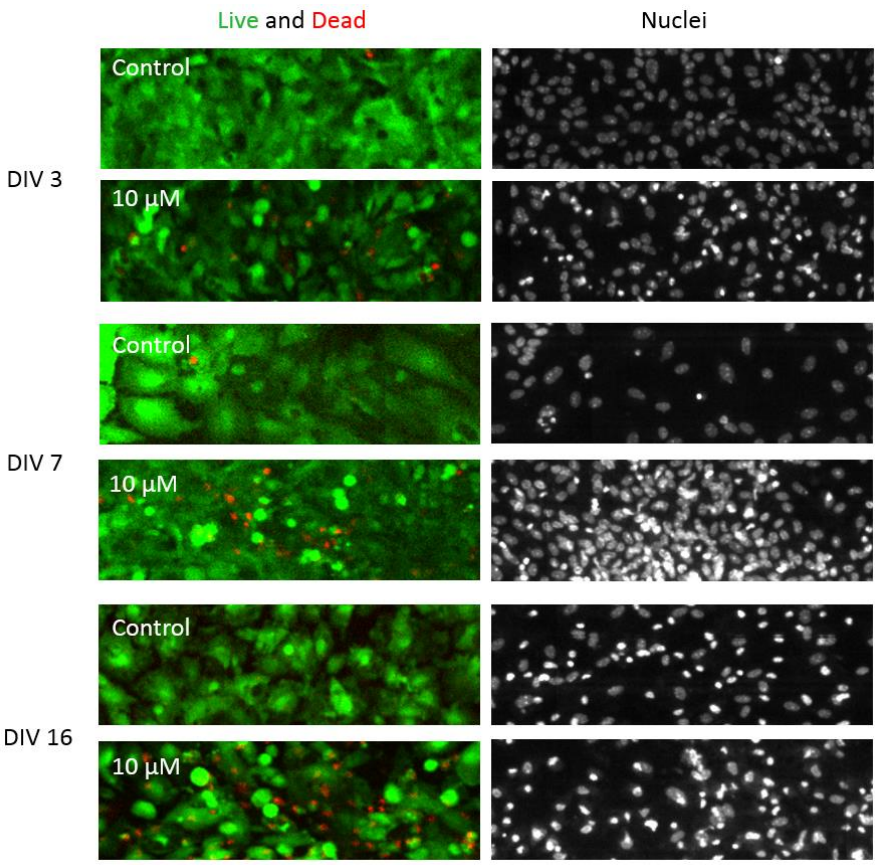


Figure 17: A 3-color assay was utilized to investigate the number of live, dead and total nuclei in response to $CdCl_2$ treatment on days *in vitro* 3,7 and 16. The number of dead cells increases with dose and harvest day. It appears that the greatest number of dead cells are seen on day *in vitro* 16 with the introduction of 10 µM $CdCl_2$. These images are representative of 3 biological replicates.

**Discussion**

In this preliminary study utilizing the mouse testicular co-culture system we report a life stage and dose dependent response to cadmium. This observation is indicated by increasing cytotoxicity as shown in the LDH assay and an increasing number of dead cells shown in the 3-color assay images and an increasing number of germ cell detached from Sertoli cells shown in the morphology images with increasing dose of $CdCl_2$. Our findings are consistent with what is reported in the literature, for example Wong et al.

reported functional endpoints such as testicular weight and fertility rate were adversely effected as the exposure occurred at later life stages in immature rat studies (Wong et al., 1980).

Impacts to testosterone production were observed in a time sensitive manner. The most dramatic impact was seen on day *in vitro* 3 when we believe fetal Leydig cells are still present in the co-culture. As they decrease in number to their lowest concentration on days *in vitro* 6 and 7, we see almost no change in testosterone production in the co-culture. We also hypothesize we are capturing adult Leydig cell differentiation and proliferation processes in our co-culture system. As the adult Leydig cells begin to rapidly increase on day *in vitro* 16, there is an increase in testosterone production in the co-culture. While not significant, there is some evidence of impacts of $CdCl_2$ on testosterone production on day *in vitro* 16. The dose dependent decrease may be related to cadmium effects known to inhibit testosterone production in *in vivo* studies using mice (Monsefi et al., 2010).

We also observed similar morphological perturbations in the mouse testes co-culture to when compared to the rat testes co-culture after treatment with $CdCl_2$ (Yu et al., 2008). As the dose increased, the number of gonocytes disconnected from Sertoli cells increased in both the rat and mouse testes co-culture. Cell viability in the mouse testes co-culture will be compared to the rat testes co-culture after a 24 hour exposure in further comparative analysis.

**Future Directions**

Future directions for this study include optimizing immunofluorescence markers indicative of specific cell types within the culture. The ability to image and quantify the

abundance of the different cell types found within the culture will greatly improve our ability to inform specific mechanisms of action of toxicants introduced to the culture system.

We also anticipate characterizing the 10% benchmark dose concentrations ($BMC_{10}$) for the cytotoxicity response. In order to do this the EPA's Benchmark Dose Modeling approach will be used.

**Conclusion**

The cell viability, testosterone and LDH results have been interpreted within our systems biology platform and demonstrate the potential of our model to capture adverse outcomes in proliferation and testosterone production pathways in this *in vitro* model of male reproductive development. In conclusion, we are encouraged that the three-dimensional mouse testicular co-culture system is capable of capturing sensitive developmental processes susceptible to cadmium perturbation. We will continue to optimize the co-culture for future use as a high content system for toxicity testing for male reproductive and developmental perturbation.

**Thesis Conclusion**

Our high-content testes co-culture system and systems biology framework capture sensitive developmental pathways that are relevant to human development and allow the results to be characterized over developmental time and across species. Based on our temporal *in vivo* timeline, we have shown multiple lines of evidence that we are capturing similar windows of development in the rat and mouse testes co-culture systems. The

characterization of similarities and differences in the critical windows of development between species is important for translating results from animal studies to human health risk assessment. This approach also allows for the comparison of cellular and transcriptional changes across development and species which can be used to inform adverse outcome pathway development and translation to risk assessment. Additionally, this system captured the perturbation of sensitive developmental processes following exposure to cadmium, demonstrating the applicability of this system and framework for the detection of reproductive and developmental toxicity and interpretation of the biological changes in the context of normal development. The expansion of this system from rat to mouse cultures allows for the incorporation of genetic variability and susceptibility factors by leveraging the collaborative cross mouse strains. As we continue to optimize our culture to explore genetic variability factors, this culture system will become an even more high-content *in vitro* system. The culture system, in tandem with our systems biology framework, will be capable of characterizing not only the adverse outcomes to toxicant exposures but also identifying critical windows of susceptibility and genetic factors that modify the dose response relationship which drives risk assessment.

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