The Sex of the Cell Dictates its Response

Carlos Ganesh Penaloza

Graduate Center, City University of New York

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The Sex of the Cell Dictates its Response

By

Carlos G. Peñaloza

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Date
Executive Officer
Dr. Laurel Ann Eckhardt, Biology Doctoral Program

Date
Chair of Examining Committee
Dr. Zahra Zakeri, CUNY at Queens College

Dr. Richard Lockshin, St. John’s University College

Dr. Cathy Savage-Dunn, CUNY at Queens College

Dr. Wilma Saffran, CUNY at Queens College

Dr. Betty Diamond, North Shore LIJ Feinstein Institute Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK
ABSTRACT

THE SEX OF THE CELL DICTATES ITS RESPONSE

By

Carlos G. Peñaloza

Adviser: Professor Zahra Zakeri

Male and female differences in frequency of occurrence in disease have perplexed scientists for some time. This in part derives from limitations in the systems in which one can evaluate sex differences. At the organismal level, differences can be hidden by a myriad of extensive and complex factors. Additional limitations exist since most biomedical studies are performed almost exclusively on male subjects, as the female hormonal milieu is intrinsically more variable and too troublesome for routine inclusion in research protocols. Research documenting sex differences continues to grow, and while most researchers suggest that sex hormones are at the core of these differences, more evidence is suggesting other innate biological factors are at play.

To address these limitations, we asked whether differences could be induced and assessed in a controlled cellular system. We then asked whether any aspect of sex dimorphism could be attributed to chromosomal rather than hormonal differences; and finally established a role for differential DNA methylation in sex differences.

We found that male and female cells responded differently to cell death inducers as measured by cell viability. We then evaluated gene expression between the sexes, and found that many genes were differentially expressed \textit{in vivo}. These differences persisted in our cultures, affording us the ability to further characterize these sex differences.
Furthermore, we found that cell death induction led to dimorphic gene expression; at instances having opposite effects on cells, where one sex is repressed, and the other is induced.

We then evaluated DNA methylation to characterize the differential gene regulation. Blocking DNA methylation globally, using 5-Aza-2-deoxycytidine, led to a loss of gene expression differences between the sexes and found that differences in methylation patterns correlated directly with differences in gene expression.

Here we provided a model system, whereby we can test for individual differences resulting in sex dimorphisms at the cellular level. It is critical to continue to expand our knowledge in this area, as this work can be extrapolated and applied to other instances where differences are being measured between the sexes; providing more tools to better characterize those conditions.
OVERVIEW

Male and female differences in frequency of occurrence in disease have perplexed scientists for some time. This in part derives from limitations in the systems in which one can evaluate sex differences. At the organismal level, differences can be hidden by a myriad of extensive and complex factors. Additional limitations exist since most biomedical studies are performed almost exclusively on male subjects, as the female hormonal milieu is intrinsically more variable and too troublesome for routine inclusion in research protocols. Research documenting sex differences continues to grow, and while most researchers suggest that sex hormones are at the core of these differences, more evidence is suggesting other innate biological factors are at play.

To address these limitations, we began by asking whether differences could be induced and assessed in a controlled cellular system, and whether sex hormones dictated these differences. We then asked whether any aspect of sex dimorphism could be attributed to chromosomal rather than hormonal differences; and finally established a role for differential DNA methylation in sex differences.

To this end, we used a cell culture model system; induced and evaluated sex differences while controlling for hormones, using the mouse development, to control for endogenous hormonal influences. We found that male and female cells responded differently to cell death inducers ethanol and camptothecin, as measured by cell viability. Importantly cells devoid of endogenous sex hormones (ED10.5) responded in a sex specific manner. This represented the first report, where sex differences are observed at the cellular level, devoid of sex hormones. We evaluated the effect of endogenous sex hormones in our system using cells from later developmental stages, as these cells have been exposed to endogenous sex hormones, and presumptively
differential imprinting between the sexes. We found in the absence and presence of endogenous sex hormones, these differences persist.

We then evaluated gene expression between the sexes, and found that in a gene array containing over 22,000 genes, 51 were found to be differentially expressed. Included were genes belonging to metabolic and transcriptional processes, as well as other less documented genes. Importantly, we found that members of the cytochrome p450 family of metabolic enzymes were dimorphically expressed, as well as a DNA methyltransferase subunit, namely Dnmt3l. Differences in gene expression persisted in our cell cultures, thereby affording us the ability to further manipulate our model to better characterize sex differences.

Furthermore, we found that cell death induction, by ethanol and camptothecin led to dimorphic gene expression. Of importance, the direction of gene regulation was sex dimorphic, at instances having opposite effects on cells, where one sex is repressed, and the other is induced. To characterize further these differences, we looked at response elements, possibly controlling the expression of these genes. We found that individually the genes contained response elements, but only CpG islands were shared by most differentially expressed genes. This, together with initially finding Dnmt3l dimorphically expressed, suggested that these genes could be under the regulation of DNA methylation.

We evaluated DNA methylation in two ways; blocking de novo DNA methylation globally and profiling DNA methylation status of the promoters in dimorphically expressed genes. Blocking DNA methylation globally, using 5-Aza-2-deoxycytidine, led to a loss of gene expression differences between the sexes. Next we looked at methylation patterns within our genes of interest, and found differences in methylation patterns and that these correlated directly with
differences in gene expression; specifically greater methylation pattern differences resulted in greater gene expression differences.

We now asked whether DNA methylation played a role in ethanol induced death. DNA methylation blockage eliminated cell viability differences in response to ethanol. To address the loss of differences, we looked at the expression profiles of alcohol dehydrogenases, aldehyde dehydrogenases and Cyp2e1 for possible modulators of sex differences as these are key regulators of alcohol metabolism. We found that the Cyp2e1 levels were the only one modulated, and that differences in cell viability mirrored differences in Cyp2e1 levels. We blocked various alcohol metabolic processes and looked at cell viability, gene expression and correlated that: alcohol induced death was as a result of reactive oxygen species (ROS) generation, potentially from Cyp2e1 activity, and that differences in Cyp2e1 resulted in the sex differences observed in cell viability. Here for the first time, a sex specific cellular response has been described as derived from differential DNA methylation. Identifying upstream effectors leading to differences in DNA methylation differences that can alter cellular behavior, can further clarify the differences in disease manifestation, and potentially shed light for diseases whose response is dimorphic.

Here we provided a model system, whereby we can test for individual differences resulting in sex dimorphisms at the cellular level. It is critical to continue to expand our knowledge in this area, as this work can be extrapolated and applied to other instances where differences are being measured between the sexes; providing more tools to better characterize those conditions.
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INTRODUCTION

Reports on sex differences in disease manifestation continually increase, yet the underlying causes are still not well documented. Many reports suggest that sex hormones are responsible for the dimorphisms, yet other findings indicate otherwise. Male and females are physiologically different and thus, at the cellular and molecular levels, differences can be seen. So far, few have studied diseases whose expression is dimorphic to the extent of understanding the underlying mechanisms contributing to the dimorphic response. As will become apparent within the various sections of this thesis, the differences observed result from many factors including, but not limited to sex hormones, genetics and/or a combination of factors. In this thesis, we will examine cellular differences between male and female cells in the absence or presence of sex hormones and identify potential underlying mechanisms affecting the differential response. Often, the dissection of genetic and hormonal factors is not possible and unconventional approaches had to be developed, as was the case with the experimental designs outlined in detail within the subsequent sections of this thesis. For this reason, we explored differences in susceptibilities of various cell types, emphasizing the potential impacts of cell type, development, hormonal influences, and genetic differences, to better understand what factors may be contributing to the observed differences.

In this introduction, we first explore the presence of sex differences in disease, then the biology of sex and finally, the factors that can influence the differences in disease manifestation between the sexes. We conclude the introduction by describing the model system created and used for our studies, describing some underlying reasons for the skewed sex differences.
Sex Differences in Disease Manifestation, Progression and Severity

Differences in disease manifestation, progression and severity have been described for many diseases, including cardiovascular diseases (Rossouw 2002, Pérez-López, Larrad-Mur et al. 2010, Maric-Bilkan and Manigrasso 2012, Regitz-Zagrosek, Mahmoodzadeh et al. 2012, Regitz-Zagrosek, Seeland et al. 2012, Townsend, Miller et al. 2012, Fairweather, Cooper Jr et al. 2013), cancers (McCann 2000, Townsend, Miller et al. 2012, Majek, Gondos et al. 2013), autoimmune (Voskuhl 2011, Amur, Parekh et al. 2012, Fairweather, Petri et al. 2012, Oertelt-Prigione 2012, Zandman-Goddard, Peeva et al. 2012, Yurkovetskiy, Burrows et al. 2013), and neurodegenerative diseases (Schultz, Braak et al. 1996, Marceglia and Priori 2007, Grimm, Lim et al. 2012, Spence, Wisdom et al. 2013). The causes of the sex differences are still misunderstood and poorly documented. Below is a short list of sex-related differences that have been reported in death caused by disease (Table I1), new instances of sex-skewed diseases (Table I1), and the female to male ratios reported for these diseases (Table I2).

**Death and Incidence for Dimorphic Diseases**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Death Female</th>
<th>Death Male</th>
<th>Incidence Female</th>
<th>Incidence Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Disease</td>
<td>40%</td>
<td>60%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td>41%</td>
<td>59%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic Respiratory Disease</td>
<td>43%</td>
<td>57%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alzheimer's Disease</td>
<td>74%</td>
<td>26%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung Cancer</td>
<td>40%</td>
<td>60%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorectal Cancer</td>
<td>42%</td>
<td>58%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table I1:** List of death and incidence for sex dimorphic diseases. Differences are measured by differences in death caused by the disease, or by incidence reported for the disease, between the sexes. (Harrison 2010, Dorak and Karpuzoglu 2012)
Sex Differentially Manifested Diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Ratio F:M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic pulmonary fibrosis</td>
<td>7:11</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>2:1</td>
</tr>
<tr>
<td>Dermatomyositis</td>
<td>2:1</td>
</tr>
<tr>
<td>Thrombocytopenia purpura</td>
<td>2:1</td>
</tr>
<tr>
<td>Myasthenia gravis</td>
<td>2:1</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>3:1</td>
</tr>
<tr>
<td>Systemic sclerosis</td>
<td>4:1</td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>4-6:1</td>
</tr>
<tr>
<td>Graves’ disease</td>
<td>7:1</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>9:1</td>
</tr>
<tr>
<td>Sjogren syndrome</td>
<td>9:1</td>
</tr>
<tr>
<td>Hashimoto's thyroiditis</td>
<td>3:5:1</td>
</tr>
</tbody>
</table>

**Table 12:** List of dimorphic disease manifestation between the sexes. The list keeps growing, and reported numbers are conservative. There is a higher predisposition of diseases to females when compared to males (Harrison 2010, Dorak and Karpuzoglu 2012).

In the next sections, we explore each category of reported differences in disease manifestation, and describe what factors can lead to the skewed differences.

- **Cardiovascular Disorders**

There is a strong male predisposition to frequency and clinical manifestation of atherosclerosis, particularly coronary heart disease (Pérez-López, Larrad-Mur et al. 2010, Fairweather, Cooper Jr et al. 2013) and other diseases. Coronary deaths in men consistently exceed those in age-matched women by 2.5-4.5 fold until the age of 75 (Kalin and Zumoff 1990, Wu and von Eckardstein 2003, Lawton 2011, Dunlay and Roger 2012). While the onset of coronary diseases is biased toward males, once the disease develops, females have 2-fold greater chance of mortality (Vaccarino, Parsons et al. 1999, Weidner 2000). These differences suggest the existence of intrinsic sex differences with regard to cardiovascular diseases, provoking considerable interest on the role of sex hormones in cardiovascular diseases. Although female
mice have been shown to have lower mortality and better preserved cardiac function after myocardial infarction and in simulated infarction in cardiomyocytes (which may be as a result of differences in estrogen receptor α (ERα) dominance in females (Hulley, Grady et al. 1998, Manson, Hsia et al. 2003)), failure to see cardio-protective effects of estrogen in clinical trials (Hulley, Grady et al. 1998, Manson, Hsia et al. 2003) led to the new focus on androgens and other sex-specific tissue and cellular characteristics that mediate response to various stimuli (Wu and von Eckardstein 2003, Pérez-López, Larrad-Mur et al. 2010, Spoletini, Caprio et al. 2013). Monocyte-derived macrophages, having important roles in the development of atherosclerosis, also have more androgen receptors in males than in females; exposure to androgens induces the up-regulation of 27 atherosclerosis-related genes, while androgens have no effect on the activity of genes directly associated with female sexual characteristics (Ng, Quinn et al. 2003). These findings indicate that the presence of androgen receptor may be a key mediator of sex differences in cardiovascular biology.

- **Cancers**

Similar findings have been reported in the area of lung cancer. Although lung cancer has been a leading cause of cancer mortality among men, progressively it has been affecting women at higher rates (McCann 2000, Wisnivesky and Halm 2007, Townsend, Miller et al. 2012, Majek, Gondos et al. 2013). One study done to evaluate the absolute risk of lung cancer in a high risk population with similar smoking habits has shown that females run a 2.7 fold higher risk of developing lung cancer compared to males (Bain, Feskanich et al. 2004, Henschke and Miettinen 2004), although other studies with more than 60,000 women and 20,000 men failed to show any sex biased predisposition to lung cancer (Bain, Feskanich et al. 2004). Similarly in 32 of 35
cancer sites, males had higher incidence rates with three exceptions, namely: thyroid, gallbladder, and anus cancer, which had higher incidence in females. The greatest differences occur in laryngeal and hypopharyngeal cancer, which are about 6 fold higher in males (Dorak and Karpuzoglu 2012).

Multiple studies indicate that females are more susceptible to the carcinogenic effects of cigarettes than men, as a result of genetic, metabolic and hormonal factors (Russo, Hasan Lareef et al. 2003, Krieger, Löwy et al. 2005).

Among the detoxifying enzymes, members of the cytochrome p450 family are regulated in a sexually dimorphic manner (Mollerup, Ryberg et al. 1999, Coon 2005). Cyp1A1 is up-regulated in females more than male smokers, inducing the partial metabolism of carcinogens from cigarettes resulting in DNA adducts in females preventing further breakdown of active carcinogens (McCanlies, O’Leary et al. 1998, Mollerup, Ryberg et al. 1999, Salnikova, Belopolskaya et al. 2013) that can result in further detrimental effects, as will be discussed later in this introduction.

- **Autoimmune Disorders**

Similarly, many autoimmune diseases affect women more than men, but severity of disease is not different. Female predominance occurs in thyroid diseases (i.e. Hashimoto’s and Graves’) (Lockshin 2006), and some rheumatic diseases (i.e. lupus and rheumatoid arthritis) (Giltay, van Schaardenburg et al. 1999, Yacoub Wasef 2004, Schwartzman-Morris and Putterman 2012) as well as hepatic diseases (i.e. autoimmune hepatitis and primary biliary cirrhosis) (North, Graff et al. 2012). Male predominance characterizes other rheumatic diseases, such as ankylosing spondylitis, Reiter syndrome, and vasculitis (Enlow, Bias et al. 1982, Giltay, van Schaardenburg...
et al. 1999, Lee, Reveille et al. 2007, Chen, Chen et al. 2011). Other autoimmune diseases (i.e., juvenile-onset diabetes, inflammatory bowel disease, and idiopathic thrombocytopenic purpura) are sex neutral (Lorenzi, Cagliero et al. 1985, Mylvaganam, Ahn et al. 1989, Bottini, Gloria-Bottini et al. 2000, Mackner, Vannatta et al. 2012). If immune response is inherently different between men and women, sex discrepant responses to vaccination, infection and immunomodulation should occur. A few studies on sex differences in vaccination show slightly higher antibody titers in women but no other sex difference in clinical protection by, or adverse reactions to vaccination (Aaby, Martins et al. 2010, Flanagan, Klein et al. 2011). No sex differences were seen in antibody response to measles vaccination (Mitchell 1999), or vaccination against mumps (Rebiere and Galy-eyraud 1995, Poethko-Müller and Mankertz 2012).

Sex hormones are, at best, weak explanations for high female to male ratios in sex dimorphic immune response. An animal model suggests that estrogen may permissively allow survival of forbidden autoimmune clones (immune cells that can react against self), which may provide a link between hormonal differences and sex-dimorphic autoimmune disease. In some mouse strains, healing is sexually dimorphic, the dimorphism being under control of estrogens (Heber-Katz, Chen et al. 2004, Gilliver, Ruckshanthi et al. 2008, Engeland, Marucha et al. 2013).

Other than hormonal influences, it has been proposed that genetic control of autoimmunity is another source of the dimorphism. Genes on X and Y chromosomes are possible causes of sex discrepancies. CD40 ligand, some interferon-related genes, and other immunologically relevant genes are on the X or Y chromosome (Schrott, Waters et al. 1993, Chitnis, Monteiro et al. 2000, Libert, Dejager et al. 2010, Abdulaziez, Abdulmaksud et al. 2012). Differences in the levels of
gene activity, presence/absence or having half as much or twice as much of certain genes can possibly explain the sex dimorphic disease expression. Genes in the Y chromosome are present only in males, while genes on the X-chromosome have the potential of being expressed more in females than in males. These genetic differences can lead to different ways of responding to stress, and for that matter, immune response. Susceptibility to lupus resides on the Y chromosome in the male-predominant BXSB mouse model (Schrott, Waters et al. 1993, Yacoub Wasef 2004, Abdulaziez, Abdulmaksud et al. 2012), supporting the notion that dimorphism can occur as a result of genetic control of autoimmunity.

- **Neurodegenerative Disorders**

Neurodegenerative diseases often affect the sexes differently. Schizophrenia is sexually dimorphic in expression, with male onset occurring 3-4 years earlier than females (Aleman, Kahn et al. 2003, Able, Drake et al. 2010, Falkenburg and Tracy 2012). There is an increase of incidence in women around the time of menopause (between ages 45 and 54) (Häfner, Riecher-Rössler et al. 1993), and some studies showed lower levels of 17β-estradiol in women showing symptoms of schizophrenia, compared to healthy women of the same age (Häfner, Riecher-Rössler et al. 1993, Begemann, Dekker et al. 2012). Furthermore, 17β-estradiol selectively protects females and not male neurons from glutamate induced stress, with ERα having a sexually dimorphic neuro-protective effect (Bryant and Dorsa 2010). Taken together, these studies suggest the possibility of sex hormones playing a role in the differences of disease onset between the sexes.

Similarly a female bias in Parkinson’s disease, as well as Alzheimer’s, has been discussed, but is not definitive (Shulman 2007, Miller and Cronin-Golomb 2010). Sex difference in glutathione
metabolism is a possible underlying mechanism leading to the dimorphism in Alzheimer’s Disease (Liu, Harrell et al. 2005). Amyotrophic Lateral Sclerosis (ALS), another neurodegenerative disease causing the progressive loss of brain and spinal motor neurons, has been described to be sexually dimorphic. In a rat model system using overexpression of SOD1, the onset of ALS was faster in male rats than in the female counterparts (Wang, Deng et al. 2009). Evidence for an effect of hormone replacement therapy is also controversial (Krieger, Löwy et al. 2005, Konstandi, Cheng et al. 2013, Majek, Gondos et al. 2013). A rodent model shows that sustained exposure to estrogen can modify brain response to estradiol, desensitizing neuroendocrine responses, suggesting an explanation for a possible sex bias in these neurodegenerative diseases (Arimoto, Wong et al. 2013). Multiple sclerosis also shows a sex bias in that twice as many females as males are afflicted with the disease (Crabtree-Hartman 2010). This difference may be caused by a protective effect of testosterone in young men, and a protective effect of estradiol in pregnant females (Swartz, Fee et al. 2007). Multiple sclerosis symptoms are reduced at high levels of estrogen and exacerbated at low levels of estrogen, as seen when comparing flares during different stages of menstrual cycle and pregnancy (Zorgdrager and De Keyser 1998, Gold and Voskuhl 2009, Holmqvist, Hammar et al. 2009).

From these reports, it is clear that sex differences in disease manifestation and progression exist, and that while the leading factors are not well documented, it is probable that these differences arise from differences in the biology of sex and/or in gene expression differences affecting biologic processes. In the next sections, we explore sex differences in biology, including chromosomal, developmental and genetic differences.
Sex vs. Gender

Gender and sex have been used synonymously, but they hold very different meanings. The distinction between sex and gender lies in the biology of the organisms. Biologically the presence of a Y-chromosome in mammals is indicative of a male organism, representing the sex of the organism. The absence of the Y-chromosome is indicative a biologic female organism. Gender on the other hand, while debatable in as to its biologic meaning (primarily referring to behavior or self-identity), is independent of biologic sex. When looking at disease manifestation and the biology of disease, it is important to consider the sex of the organism. Based on this differentiation, the remainder of this thesis will reflect the term sex and not gender. This section represents the determination of biologic sex, which we feel is the basis of the differences in diseases presented above.

The Biology of Sex – Sex determination

Male and female differences are initiated by chromosomal differences that result in dimorphic gene expression profiles and secondary messengers, ultimately resulting in both anatomical and physiological males and females. We know much about the molecular differences between the sexes that lead to these differences. One cannot exclude them from playing a role in sex differences in disease and stress response, since these are great differences. We will focus our attention on mammalian sex determination.
- **Primary Sex Determination**

Sex determination in mammals originates by chromosomal composition, which leads to the formation of sex organs (ovaries and testicles). The sex organs further affect the development of the organism, in the form of secondary sexual characteristics and secretion of sex hormones (Wilhelm, Palmer et al. 2007, Angelopoulou, Lavranos et al. 2012, Gilbert 2013, Johnen, González-Silva et al. 2013).

Primary sex determination, the determination of the gonads, is strictly chromosomal in mammals and not influenced by the environment. In mammals, males are generally XY in chromosomal composition, whereas females are XX. During gametogenesis, males can provide a single X chromosome or Y chromosome, while females provide only an X chromosome gamete (Gilbert 2013, Johnen, González-Silva et al. 2013). During fertilization, if the egg (X) is fertilized by an X chromosome-containing sperm, the individual is female, while if it is fertilized by a Y chromosome-containing sperm it becomes male. The Y chromosome carries a gene that encodes a testis-determining factor. This factor organizes the gonad into testis rather than an ovary; hence an individual with five X chromosomes carrying only one Y chromosome would still be male. An individual with just one X chromosome and no Y chromosome will develop into a female. Both male and female gonads develop from the bipotential gonad (Wilhelm, Palmer et al. 2007, Angelopoulou, Lavranos et al. 2012, Gilbert 2013, Johnen, González-Silva et al. 2013, Wainwright, Jorgensen et al. 2013). The aforementioned mechanism of sex determination differs in non-mammalian vertebrates. Varying levels of sex determining transcripts dictate the sex of the individual, as well as secondary characteristics (Wilhelm, Palmer et al. 2007). Below we discuss some of the prominent genes involved in sex determination.
- Sry: the Y-chromosome sex determinant

In initiating the sex determination process in mammals, the Sry gene, present on the Y chromosome, produces a message that is translated into the Testis Determining Factor (TDF) (Wallis, Waters et al. 2008, Gilbert 2013, Johnen, González-Silva et al. 2013), triggering subsequent male determining stages of development, discussed below. This is only speculation, as Sry has been only correlated with testis formation, due to its absence in females. In a mouse system where Sry has been deleted, many sex effects have been found, whereby XY mice have female phenotypes and XX + Sry are chromosomally female but with male phenotypes (Chen, Williams-Barris et al. 2013, Gilbert 2013, Johnen, González-Silva et al. 2013). In addition, there have been other studies where Sry has been linked to possible cell migratory signals, for cells to develop into the male gonads (Wallis, Waters et al. 2008, Kashimada and Koopman 2010, Gilbert 2013).

- Sox9: autosomal sex reversal

The Sox9 transcript precedes the expression of Sry and is also linked to testis development, as its absence leads to female phenotype. This transcript is also found in the gonadal ridge in the same area as Sry (Jakob and Lovell-Badge 2011, Gilbert 2013). Differences in the number of copies of this gene lead to XY individuals with female phenotypes, hermaphrodite phenotypes, or XX individuals with male phenotypes (Jakob and Lovell-Badge 2011, Gilbert 2013). Mutation of SOX9 and SF1 (Splicing Factor) binding sites reduce ex vivo transactivation of the pri-miR-202 promoter, demonstrating that pri-miR-202 may be a direct transcriptional target of SOX9/SF1 during testis differentiation. Expression of the primary transcript of miR-202-5p/3p is low in XY gonads in a conditional Sox9-null mouse model, suggesting that pri-miR-202 transcription is
downstream of SOX9, a transcription factor that is both necessary and sufficient for male sex determination (Wainwright, Jorgensen et al. 2013).

- **Dax1**: potential ovary determining gene on the X-chromosome

As could be deduced from this previous section, **Sry**, produced in males, stimulates testis formation. **Dax1** regulates ovary development. When co-expressed they need to be expressed differentially between the sexes to promote differentiation between the sexes. In the presence of **Sry**, the transcripts compete, and **Sry** wins, leading to testis development. In the presence of duplications of **Dax1**, **Dax1** wins over **Sry** leading to female phenotype and ovary development (Ludbrook and Harley 2004, Karkanaki, Praras et al. 2007, Bull 2008, Angelopoulou, Lavranos et al. 2012, Arnold 2012, Gilbert 2013).

Regulation of the genes mentioned above, as well as others, leads to the actual development of gonads in the organism; most of these genes appear to function only at this time. Sex hormones regulate the development of the gonad. However, they may set other patterns of gene activity, which could persist and render male and female cells different.

**- Gonad Development**

Once sex is determined, as described above, the gonadal rudiment is considered bipotential as it can differentiate into testis or ovaries determined by chromosomal composition. Prior to differentiation, mammalian gonads develop through a bipotential stage, at which time they have neither female nor male characteristics (Bernard and Harley 2007, Karkanaki, Praras et al. 2007, Bull 2008, Herpin and Schartl 2009, Jacob and Lovell-Badge 2011, Angelopoulou, Lavranos et al. 2012, Gilbert 2013). During this bipotential stage, gonad rudiments form adjacent to the developing kidneys. The ventral portions of gonadal rudiments are composed of the genital
ridge epithelium, which proliferates into loose connective mesenchymal tissue above it, forming the sex cords. Germ cells migrate into the gonad and are surrounded by the sex cords (Wilhelm, Palmer et al. 2007, Gilbert 2013). The decision to adopt the male fate is based not only on whether male genes, such as Sox9, are expressed, but also on the active repression of female genes, such as wingless-type MMTV integration site family, member 4 (Wnt4). Because loss of Wnt4 results in the up-regulation of Glial Activating Factor (Fgf9), the relationship between these two signaling factors is not symmetric: loss of Fgf9 in XX Wnt4(-/-) gonads does not rescue their partial female-to-male sex-reversal (Jameson, Lin et al. 2012).

In the absence of a Y chromosome, the gonadal primordia develop into ovaries; the ovaries then produce estradiol, which enables the development of the Müllerian duct into the uterus, oviducts, and upper end vagina (Karkanaki, Praras et al. 2007). The germ cells are toward the outside of the surface of the gonad. The initial sex cords of XX gonads degenerate, producing a new set of sex cords that stay near the outer surface of the organ and are now called cortical sex cords. The germ cells now become the ova, and the cells surrounding cortical sex cords differentiate into the granulosa cells (Bull 2008, Angelopoulou, Lavranos et al. 2012, Gilbert 2013). The mesenchyme cells of the ovary differentiate into the theca cells. Thecal and granulosa cells form the follicles that envelop the germ cells and secrete steroid hormones. In females, the Müllerian duct remains intact, and it differentiates into the oviducts, uterus, cervix, and upper vagina (Karkanaki, Praras et al. 2007, Arnold 2012, Gilbert 2013).

If the Y chromosome is present, testes form and secrete two major hormones; anti- Müllerian duct hormone (AMH), which destroys the Müllerian duct; and testosterone, which masculinizes the fetus, stimulating the formation of the penis, scrotum, and other portions of the male anatomy.
and inhibiting other female secondary characteristics (Kobayashi, Stewart et al. 2011, Gilbert 2013). In XY individuals, the sex cords continue to proliferate, fusing and forming the thinner rete testis (Kobayashi, Stewart et al. 2011, Gilbert 2013). The sex cords, now called testis cords, lose contact with the surface epithelium and become separated by a thick extracellular matrix called the tunica albuginea. Germ cells are found in the cords within the testes (Gilbert 2013). At puberty, the cords hollow out to form the seminiferous tubules, and the germ cells begin to differentiate into sperm. During fetal development, the interstitial mesenchyme cells of the testes differentiate into Leydig cells, which make testosterone (Wilhelm, Palmer et al. 2007).

Estrogen is needed for the complete development of both the Müllerian and Wolffian ducts. In females, estrogen secreted from the fetal ovaries appears sufficient to induce the differentiation of the Müllerian duct into its various components: the uterus, oviducts, and cervix (Kondo, Nanda et al. 2009, Barske and Capel 2010, Jacob and Lovell-Badge 2011, Angelopoulou, Lavranos et al. 2012, Arnold 2012, Gilbert 2013). In males, estrogen is necessary for fertility, as it is responsible for water absorption, concentrating sperm, inducing longer lifespan of sperm as well as more concentrated ejaculations. Blood levels of estrogen in females are much greater than in males, but in the rete testis in males, estrogen can reach higher levels, indicative of its functional localization (Karkanaki, Praras et al. 2007, Wilhelm, Palmer et al. 2007, Wallis, Waters et al. 2008, Arnold 2012, Gilbert 2013).

Gonad development triggers secondary sex determination, which ultimately results in secondary male and female characteristics, discussed below.
- Secondary Sex Determination

As previously indicated, gonadal development leads to secondary sex determination. The penis, seminal vesicles, and prostate glands are determined by hormones secreted by the testis (Bernard and Harley 2007, Karkanaki, Praras et al. 2007, Bull 2008, Angelopoulou, Lavranos et al. 2012, Gilbert 2013). In females, the vagina, cervix, uterus, oviducts, and mammary glands are determined by hormones produced by female hormones. In the absence of gonads, the resulting phenotype is that of a female, regardless of chromosomal composition, indicating that the hormones of the mother are sufficient for the initial differentiation, and or that the default sex is female (Quinn and Koopman 2012, Gilbert 2013).

The formation of the male secondary sex determination or male gonad phenotype involves the secretion of two testicular hormones, namely anti-Müllerian duct hormone by the Sertoli cells, which causes the degeneration of the Müllerian duct, and testosterone by the Leydig cells, which causes the Wolffian duct to differentiate into the epididymis, vas deferens and seminal vesicles. The existence of these independent systems of masculinization is demonstrated by genetic males with androgen insensitivity syndrome (Karkanaki, Praras et al. 2007, Bull 2008, Herpin and Schartl 2009, Jacob and Lovell-Badge 2011, Gilbert 2013). These mutated males lack the testosterone receptor protein, and cannot respond to the testosterone made by their testes, therefore develop the female phenotype, albeit without uterus and fallopian tubes (derivatives of the Müllerian tubes) despite their XY chromosomal composition (Viger, Silversides et al. 2005, Quinn and Koopman 2012, Gilbert 2013).

Testosterone may not be the masculinizing hormone in certain tissues, such as the male urethra, prostate, penis or scrotum. These are masculinized by 5α-dihydrotestosterone. The formation of
external genitalia is under the control of dihydrotestosterone, whereas Wolffian duct
differentiation is controlled by testosterone itself. Genetic males having deficiency in the
enzyme responsible for the conversion of testosterone to dihydrotestosterone, 5α-reductase, are
male internally, but develop female secondary characteristics (Johansen, Jordan et al. 2004,
Schulz, Richardson et al. 2004, Gilbert 2013).

Estrogen is needed for the complete development of both the Müllerian and the Wolffian ducts.
In females, estradiol secreted from the fetal ovaries appears sufficient to induce the
differentiation of the Müllerian duct into its various components: the uterus, oviducts, and cervix.
The extreme sensitivity of the Müllerian duct to estrogenic compounds is demonstrated by the
teratogenic effects of diethylstilbesterol (DES), a powerful synthetic estrogen that can cause
infertility by changing the patterning of the Müllerian duct (Bernard and Harley 2007, Mork and

Among the differences that arise as a result of sex differentiation, the one with largest impact is
the difference in hormonal levels, which dictates many differences between the sexes (Gilbert
2013). This endocrine difference has been considered by many to be the main cause of the sex
dimorphism observed in disease expression and stress response (Kalin and Zumoff 1990,
2012, Townsend, Miller et al. 2012). In the next section, we will discuss the endocrine system,
its components, and indicate the components of the endocrine system that differ between the
sexes.
Hormonal Action and Biologic Sex

There are two mechanisms of hormone action on all target cells. Non-steroid hormones (mostly water soluble) do not enter the cell but bind to plasma membrane receptors, generating a chemical signal (second messenger) inside the target cell (Tuohimaa, Bläuer et al. 1996, Yen, Ando et al. 2006). Several different second messenger chemicals, including cyclic AMP, have been identified (Rey 2005, Viger, Silversides et al. 2005, Wilhelm, Palmer et al. 2007, Gilbert 2013). Second messengers activate other intracellular chemicals to produce the target cell response. Steroid hormones, which pass through the plasma membrane act in a two-step process. Once inside the cell, steroid hormones bind to the nuclear membrane receptors, producing an activated hormone-receptor complex that enters the nucleus. The activated hormone-receptor complex binds to DNA and activates specific genes, increasing production of specific proteins (Mani, Portillo et al. 2009, Gilbert 2013). By and large, the roles of both steroid and non-steroid hormones revolve around gene regulation which, can result in many differences between the sexes, if dosage dependent. In subsequent sections, we discuss examples where hormone dosage differences result in different responses, giving credence to the role of differential hormone levels between the sexes, and disease manifestation.

Since hormones exert their roles when linked to their respective receptors, differences in receptors and levels of these hormones between the sexes can lead to the differences previously described in disease (Kalin and Zumoff 1990, Riecher-Rössler, Häfner et al. 1994, Rossouw 2002, Krieger, Löwy et al. 2005, Gilliver, Ruckshanthi et al. 2008, Crabtree-Hartman 2010, Pérez-López, Larrad-Mur et al. 2010, Dorak and Karpuzoglu 2012, Fairweather, Petri et al. 2012, Maric-Bilkan and Manigrasso 2012, Townsend, Miller et al. 2012). Estradiol, together with the other sex steroid hormones, such as progesterone and testosterone, provokes the development
and determination of the embryonic reproductive system, masculinizes or feminizes the brain at birth, and controls reproduction and reproductive behavior in the adult, plus the development of secondary sexual characteristics (Angelopoulou, Lavranos et al. 2012, Gilbert 2013). For the purpose of our study, we will focus on two families of receptors: estrogen and androgen receptors.

Hormonal action results from the interaction of ligand/receptor and the consequent effects, which include activation, repression of genes as well as the activation and suppression of various other transcription factors. In the figure below (Figure I1), the general action of transcription factors can be noted, with the most commonly known hormonal interactions, including Estrogen Response Elements (ERE), Glucocorticoid Response Elements (GRE), Progesterone Response Elements, and Androgen Response Elements (ARE).

**Figure I1**: Response Elements: Various response elements exist, where presence of the consensus sequence is generally indicative of receptor DNA interaction resulting in induction or repression of gene expression. Here presented are four hormonally regulated interactions with their consensus sequence.

<table>
<thead>
<tr>
<th>Response Element</th>
<th>Consensus Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERE</td>
<td>AGGTCAnnnTGACCT</td>
</tr>
<tr>
<td>GRE</td>
<td>AGAACAnnnTGTTCT</td>
</tr>
<tr>
<td>Progesterone</td>
<td>GGTACAnnnTGTTCT</td>
</tr>
<tr>
<td>ARE</td>
<td>TGT CCT</td>
</tr>
</tbody>
</table>
For the purposes of this work, we have focused the discussion on estrogen and androgen response elements, as this subject represents the greatest amount of literature and link to sex differences.

**Estrogen Receptors (ER)**

The ER exists in two main forms, ERα and ERβ, which have distinct tissue expression patterns in both humans and rodents (Barkhem, Carlsson et al. 1998, Hayashi, Eguchi et al. 2003, Tsinti, Kassi et al. 2009). Gene-targeted animal models lacking these receptors exhibit distinct phenotypes and provide some of the most definitive experimental models for evaluating the role of the ER in disease and normal physiology (Couse and Korach 1999). ERα and ERβ are encoded by separate genes, *ESR1* and *ESR2*, respectively, found at different chromosomal locations, and numerous mRNA splice variants exist for both receptors in both diseased and normal states (Deroo and Korach 2006, Burns and Korach 2012), although the corresponding protein products have been difficult to confirm (Burns and Korach 2012). Because these splice variants are frequently co-expressed with their wild-type counterparts, the exact function and potential role of these variants in normal physiology and human disease remain to be elucidated. Functions have been described for each receptor α and β in the control of B-cell maturation and selection (Hill, Jeganathan et al. 2011).

The hormone probably enters its target cells by diffusion and activates estrogen receptors (ERs), which are paradigmatic transcription factors in eukaryotes (Barkhem, Carlsson et al. 1998, Gilbert 2013). ERs are members of the steroid hormone receptor family which, in turn, is a subfamily of the nuclear receptor superfamily of proteins (Barkhem, Carlsson et al. 1998, Gilbert 2013). Effects of ERs on the genome are mediated through binding of the hormone–receptor
complex to short, specific DNA sequences, termed estrogen response elements (EREs), located in the vicinity of over 100 estrogen-regulated genes in each cell (Tang, Han et al. 2004, Lam, Lee et al. 2011). The ERE functions as an enhancer and the ERs as enhancer binding factors that are equipped with domains and surfaces responsible for hormone and DNA binding, activation of transcription, and interaction with other nuclear partners.

Selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene are examples of compounds that exhibit tissue-specific estrogenic activity. Tamoxifen, although an ER agonist in bone and uterus, is an antagonist in the breast and has been a safe and effective endocrine therapy for breast cancer for almost 20 years (Hodges, Cook et al. 2003, Musa, Khan et al. 2007, Allred, Anderson et al. 2012). Raloxifene is similar to tamoxifen in its tissue-specific agonist/antagonist profile but it exhibits greater agonist activity in bone and less in the uterus (Dayan, Lupien et al. 2006). Whether a SERM is an ER agonist or antagonist in a particular tissue depends on several factors. Binding of a SERM to the ER causes a specific conformational change in the receptor, and the resulting 3D structure determines which co-activators and/or co-repressors are recruited to the promoter. The ERα/ERβ ratio varies among tissues, and which ER form predominates also affects SERM activity. The physiological response to a particular SERM results from a combination of factors, including its chemical structure and the cellular and promoter context in which the SERM acts (Hodges, Cook et al. 2003, Musa, Khan et al. 2007).

The endocrine system represents one of the largest differences between the sexes. Any difference in the levels of hormone (i.e., estradiol) or receptors (i.e., ERα) leads to differences in response to the signal. In the following section we discuss further existing evidence suggesting differential response to estrogens and the effect exerted on the cell and/or systems.
- **Estrogen Responsive Genes**

As indicated above, estradiol acts primarily as a ligand to transcription factors that can influence genes possessing estrogen response elements. Estradiol regulates the levels of transcripts by interacting directly with DNA, either making the DNA more accessible or functioning as a promoter. In the absence of estrogen, some genes fail to become active; in other cases, addition of estrogen up-regulates or down-regulates levels of gene transcription.

Since estrogens have been linked to many of the sexually dimorphic diseases, it is conceivable that differences in estrogen levels between males and females can lead to the dimorphic expression of genes affecting these diseases. Since many genes respond to estrogen, databases have been created to organize these genes, in efforts to identify similarities among them; as in the case of Dragon Estrogen Responsive Genes Database (ERGDB) (http://datam.i2r.a-star.edu.sg/ergdbV2/). Certain homologies among the genes have been identified, but their mechanism of action is unclear (Tang, Zhang et al. 2007).

Estrogen responsive genes, because of the higher levels of estrogen in females, can be expressed differentially (Penaloza, Estevez et al. 2009, Wang and Stern 2011, Konstandi, Cheng et al. 2013). For some genes such as sex determining genes it is easy to understand why they would be estrogen responsive, but in the case of metabolic enzymes, stress response genes, and others, for which there is no apparent advantage to having higher expression in one sex compared to the other, the differences in their expression are not so easy to understand. There is evidence that in vivo, catecholamine mediated leg free fatty acid release is lower in women than in men (Blaak 2001); in a separate sex-specific genome-wide association study a group showed genome-wide significant differences in beta-estimates for SNPs in the CPS1 locus (carbamoyl-phosphate
synthase 1) for glycine. They showed that the metabolite profiles of males and females are significantly different and, furthermore, that specific genetic variants in metabolism-related genes depict sexual dimorphism; providing insights into sex-specific differences of cell regulatory processes and underscores that studies should consider sex-specific effects in design and interpretation (Mittelstrass, Ried et al. 2011). In this section, we showed possible target regulators of sex differences and a mechanism regulated by dosage differences in estrogens. In the next section, we explore androgen receptors.

**Androgen Receptors (AR)**

Androgen receptor (AR) primarily is responsible for mediating the physiologic effects of androgens by binding to specific DNA sequences that influence transcription of androgen-responsive genes, similar to estrogen receptors. AR’s undergo intermolecular interactions that regulate its interactions with co-activators and influence its activity (Heinlein and Chang 2002, Bodo and Rissman 2007, Migliaccio, Castoria et al. 2011).

Androgen receptors respond to available androgens, which include dehydroepiandosterone (DHEA), androstenedione (Andro), androstenediol, androsterone, dihydrotestosterone (DHT), and for the purposes of this study, testosterone (Chuu, Kokontis et al. 2011, Shelby, Crouch et al. 2011, Sharifi 2013).

Regulation of steroid hormone receptor action occurs, in part, by posttranslational modification, such as phosphorylation (Gelmann 2002, Kumar and McEwan 2012). Phosphorylation of steroid hormone receptors occurs under different conditions and for apparently different reasons depending on the receptor. For example, phosphorylation of progesterone receptor is both hormone-dependent and important for transcriptional activation. Both AR and estrogen receptor
can bind homologous or heterologous ligands in the cytoplasm and exert inhibitory effects on cell death processes. However, these effects are of relatively low magnitude compared with the profound effects on cell growth, survival, and differentiation that occur when ligand-bound steroid hormone receptors enter the nucleus (Gelmann 2002, Kumar and McEwan 2012). Ligand-bound steroid hormone receptors bind to specific DNA sequences and initiate the formation of a transcription-initiating protein complex at the promoter region of hormone-responsive genes. Steroid hormone receptor action is mediated by a family of co-activator proteins that amplify the signal, mediate cAMP-response element-binding protein (CREB-binding protein) recruitment to the receptor complex, and initiate RNA polymerase activity (Gelmann 2002, Kumar and McEwan 2012). Similar to estrogen responsiveness, in the following section, we discuss evidence suggesting differential response to androgens.

- **Androgen Responsive Genes**

Similar to estrogen responsive genes, many androgen responsive genes exist and the list is growing. As a result, the Androgen Responsive Gene Database (ARGDB) (http://argdb.fudan.edu.cn/) has been generated and includes in excess of 1,700 human and 100 mouse genes (Jiang, Ma et al. 2009, Otto-Duessel, He et al. 2012).

Androgen responsive genes, because of the different levels of androgens between the sexes, can be expressed differentially. Sex differences in the levels of certain Ig’s occur only in high androgen responder (HAR) strains, but not in the low androgen responder (LAR) strain. In response to type 3 pneumococcal polysaccharide (SIII), HAR C57L/J males consistently produced lower levels of antibody than C57L/J females; in response to bovine serum albumin (BSA), HAR C57L/J mice showed sex differences in anti-BSA responses both 14 and 21 days
after immunization, whereas HAR A/J mice showed sex differences on day 14 only, before peak responses developed. These results suggested an apparent correlation in male mice between high sensitivity to androgen and poor immune responsiveness (Cohn 1979).

In a separate study, females had higher expression levels of mRNA related to cytoskeleton/contractile apparatus and mitochondrial processes as well as protein, lipid, and amino acid metabolisms. Moreover, DHT treatment induced transcripts which reduce intracellular Ca2+ level at early time points. On the other hand, transcripts of fast/oxidative fiber, oxidative phosphorylation, and ATP production were repressed after DHT administration. These results demonstrate sex differences in DHT actions on skeletal muscle (Yoshioka, Boivin et al. 2007).

Hepatocellular carcinoma (HCC) is sexually dimorphic in rodents and humans, with significantly higher incidence in males. The molecular mechanisms by which estrogens prevent and androgens promote liver cancer remain unclear. However sexually dimorphic HCC is completely reversed in Foxa1- and Foxa2-deficient mice after diethylnitrosamine-induced hepatocarcinogenesis. Coregulation of target genes by Foxa1/a2 and androgen receptor (AR) was increased during hepatocarcinogenesis in normal female or male mice, respectively, but was lost in Foxa1/2-deficient mice. Thus, Foxa factors and their AR targets are central for the sexual dimorphism of HCC (Li, Tuteja et al. 2012).

Since hormonal differences between the sexes can have an impact on gene expression and subsequent biologic processes, it is crucial to explore further gene expression profiles between the sexes. In the next section, we explore potential sources of sex differences including differential gene expression.
Factors that can Influence Disease Manifestation Differently between the Sexes

How have sex differences been examined so far?

In looking for possible factors that can influence sex differences, few non-hormonal factors have been evaluated, and described below.

- Escaping from X inactivation and Dosage Compensation

Dosage compensation mechanisms have evolved in mammals because of the divergence of the sex chromosome complement between males (XY) and females (XX). Chromosome-wide transcriptional silencing of one X in females levels gene expression dosage differences between the sexes (Lyon 1961). X inactivation is a chromosome-wide phenomenon and thus, the majority of genes on the inactive X are silenced. However, some genes escape X inactivation (escape genes), remain expressed from both the active and inactive X alleles (Prothero, Stahl et al. 2009). Genes that escape X inactivation are located throughout the X chromosome, but they predominate in the small regions of homology and pairing that persist on the sex chromosomes called the pseudoautosomal regions (PAR). Genes within the PAR are usually not subject to X inactivation since functional, equivalent alleles are present on the X and Y chromosomes in males and on both X alleles in females. Non-pseudoautosomal genes that retain a Y-linked copy also often escape X inactivation and thus have two expressed alleles in both male and female somatic tissues (DISTeche, Filippova et al. 2002). A systematic survey of human genes has shown that about 15% of X-linked genes consistently escape, based on expression analyses in rodent x human hybrid cell lines that retain a human Xi (Carrel and Willard 2005). In contrast to the situation in human, only 3% of mouse genes escape X inactivation in a somatic cell line
derived from a cross between two mouse species (Yang, Babak et al. 2010). Dosage compensation has evolved to insure equal expression from the X chromosome and autosomes between the sexes. Sexual dimorphisms are largely attributed to the action of sex-specific steroid hormones. Nevertheless, prior to the presence of serum testosterone in rodent embryos, sex differences are already detectable in transcription of specific genes, arguing the involvement of additional factors in sexual differentiation (Burgoyne, Thornhill et al. 1995, Carruth, Reisert et al. 2002, Dewing, Shi et al. 2003, Sanchez and Vilain 2010).

- **Presence of Sex Differences in Biologic Processes**

Some studies have focused attention on sex differences at the cellular level, looking at cell behavior as measured by cell viability, gene expression and gene regulation. While evidence is limited, it is growing rapidly; here we present some of the existing studies.

Various biologic processes are sex dimorphic. Some of these processes allow us to hypothesize potential mediators leading to these differences. In this study, we used information from previous studies to develop a working system whereby we could further elucidate some of the underlying mechanisms that result in sex differences. In this section, we examined various studies that support the idea of sex differences in cellular systems.

We begin by describing work done in our lab, where in studying sex differences at the cellular level we showed that female cells derived from age matched embryonic heart, liver and brain are generally more sensitive to stress induced by ethanol, camptothecin and hydrogen peroxide (Nikezic-Ardolic, Lin et al. 1999). These results suggested that innate processes at the cellular level behaved in a sex-dependent manner in the absence of sex hormones. Reaching the same conclusion, Valentino, Bengasser, et al (2013) showed that β-arrestin 2 linked corticotrophin...
releasing hormone receptor 1 (CRF1) to Gs-independent signaling pathways is sex dimorphic; they proposed that this sex-biased signaling resulted in distinct cellular responses to stress that were translated to different physiologic and behavioral coping mechanisms with different pathologic consequences (Valentino, Bangasser et al. 2013). Because stress has been implicated in many medical and psychiatric diseases, these sex differences in CRF1 signaling could explain sex differences in a multitude of disorders. The possibility that analogous sex differences may occur with other G-protein-coupled receptors underscores the impact of this effect (Valentino, Bangasser et al. 2013). The receptor for corticotropin-releasing factor (CRF), the orchestrator of the stress response that has been implicated in diverse stress-related diseases, shows a female bias. Sex differences exist in the association of the CRF receptor (CRF1) with the Gs protein and β-arrestin 2, rendering females more responsive to acute stress and less able to adapt to chronic stress as a result of compromised CRF1 internalization.

Sex differences have been evaluated at the cellular level in very few instances, and while limited, they suggest ways to continue evaluating sex differences, to better elucidate why male and female disease differences exist. There are many ways to characterize factors that lead to these dimorphisms. We will focus our attention on two cell stressors for which there exists sufficient literature, helping us suggest mechanisms and factors leading to sex dimorphism. We focused our attention to the effect of alcohol (ethanol) on sex differences and next to camptothecin (CPT).

- Alcohol and Sex

Sex differences in alcohol consumption have been discussed for a long time. However, while many anecdotes indicate difference between the sexes, little is documented. In social terms,
women appear to use less alcohol (Nolen-Hoeksema 2004). However, females are more adversely affected as a result of smaller gastric metabolism and lower chi-ADH (the sole alcohol dehydrogenase in mammalian brains) (Beisswenger, Holmquist et al. 1985, Baraona, Abittan et al. 2001). Following this and other sources, we showed that in a cell culture system, male and female cells respond differently to matched toxic conditions (Penaloza, Estevez et al. 2009). Sex hormones have been implicated in the differential response to alcohol between the sexes. However, the evidence is variable, and the systems are questionable (Thomasson 1995, Purohit 2000, Rajasingh, Bord et al. 2007, Penaloza, Estevez et al. 2009, Mackie, Krishnamurthy et al. 2013).

To understand the origin of sex-based differences in response to alcohol, we need to understand its metabolism, its effect on the organism, and on the cell. While alcohol is detrimental, this is only true above a fairly high threshold. Much has been learned about alcohol metabolism, the various enzymes and pathways involved, and how alcohol, directly via its metabolism or indirectly via its solvent-like action affecting cellular membranes, impacts cell function. Yet, despite the tremendous growth in understanding alcohol metabolism and actions, the mechanism by which alcohol causes cell injury is still not clear and even less clear is how male and female response differ. Alcohol induced stress (at toxic levels) is caused by oxidative stress (Cederbaum 2001, Lu and Cederbaum 2008). Many pathways, not necessarily exclusive, may play a key role in how ethanol induces “oxidative stress” (Bondy 1992, Cederbaum 2001, Lu and Cederbaum 2008). Alcohol metabolism produces oxygen radicals, which can result in oxidative stress, lipid peroxidation, and ultimately adducts. Adducts at the cellular level can then pose a detrimental effect to tissues and organs, as in the case of gastric tissues and liver, ultimately affecting the organism.
Administration of antioxidants or iron chelators or GSH-replenishing agents can prevent or ameliorate the toxic action of ethanol (Bondy 1992, French, Morimoto et al. 1997, Lu and Cederbaum 2008, Qi, Miao et al. 2013). The most convincing data that oxidative stress contributes to alcohol-induced liver injury comes from studies using the intragastric infusion model of alcohol administration (Lu and Cederbaum 2008, Lu and Cederbaum 2010). In these studies, alcohol-induced liver injury was associated with enhanced lipid peroxidation, protein carbonyl formation, formation of the 1-hydroxyethyl radical, formation of lipid radicals, and decreases in hepatic antioxidant defense, especially GSH (Lu and Cederbaum 2008, Lu and Cederbaum 2010). Importantly, addition of antioxidants such as vitamin E, ebselen, superoxide dismutase, and GSH precursors prevented the alcohol-induced liver injury (Lu and Cederbaum 2010).

In addition to these in vivo studies, in vitro studies with hepatocytes also showed that ethanol can produce oxidative stress and hepatocyte toxicity. Studies with isolated hepatocytes from control rats or rats chronically fed ethanol indicated that ethanol metabolism via alcohol dehydrogenase results in an increase in ROS (Reactive Oxygen Species) production, hepatocyte injury, and apoptosis, reactions blocked by antioxidants (Kurose, Higuchi et al. 1997, Adachi and Ishii 2002, Bailey and Cunningham 2002).

Because cytochrome P450 2E1 (Cyp2e1) plays a role in ethanol-induced oxidant stress and is a minor pathway of ethanol oxidation, the biochemical and toxicological properties of Cyp2e1 and its expression will form the basis for much of the remainder of this introduction.
Cytochrome P450 proteins, named for the absorption band at 450 nm of their carbon-monoxide-bound form, are one of the largest superfamilies’ of enzyme proteins. The P450 genes (also called CYP) are found in the genomes of virtually all organisms. Their amino-acid sequences are extremely diverse, with levels of identity as low as 16% in some cases, but their structural fold has remained the same throughout evolution (Werck-Reichhart and Feyereisen 2000). P450s are heme-thiolate proteins; their most conserved structural features are related to heme binding and common catalytic properties, the major feature being a completely conserved cysteine serving as fifth (axial) ligand to the heme iron. Canonical P450s use electrons from NAD(P)H to catalyze activation of molecular oxygen, leading to regiospecific and stereospecific oxidative attack of a plethora of substrates (Werck-Reichhart and Feyereisen 2000). The reactions carried out by P450s, though often hydroxylation, can be extremely diverse. They contribute to vital processes such as carbon source assimilation, biosynthesis of hormones and of structural components of living organisms, and also carcinogenesis and degradation of xenobiotics (Werck-Reichhart and Feyereisen 2000). In eukaryotes, they are usually bound to the endoplasmic reticulum or inner mitochondrial membranes. The electron carrier proteins used for conveying reducing equivalents from NAD(P)H differ with subcellular localization (Werck-Reichhart and Feyereisen 2000).

There are 57 active CYP genes in the human genome, which are divided into 18 families. The first three families (CYP1-3) are generally involved in the metabolism of exogenous substances such as drugs, whereas CYP families with higher numbers are usually involved in the metabolism of endogenous substances (Sim and Ingelman-Sundberg 2010). CYP enzymes are responsible for 75-80% of all phase I-dependent metabolism (incorporates an atom of oxygen
into non-activated hydrocarbons, which can result in either the introduction of hydroxyl groups or N’, O’ and S-dealkylation of substrates) and for 65-70% of the clearance of clinically used drugs (Bertz and Granneman 1997, Evans and Relling 1999).

The cytochrome P450 family of enzymes is of great importance for this work, as they have been reported as sex dimorphic in various instances. Cyp1a2 activity was 4 times higher in female than in male minipigs, and of Cyp2e1 was 4 times higher in female than in male minipigs and 2 times higher in female than in male pigs (Skaanild and Friis 1999). Sex differences in the circadian variation of hepatic cytochrome P450 genes and corresponding nuclear receptors in mouse liver have been described (Lu, Jin et al. 2013). Sex-specific changes in certain cytochrome P450 proteins exist in response to insulin-dependent diabetes which cannot be ascribed to sex differences in the severity of diabetes since the degrees of hyperketonaemia and hyperglycaemia were the same in the two sexes. These are likely to reflect sex-specific differences in growth hormone and triglyceride levels in the diabetic animals (Barnett, Rudd et al. 1993). Women show greater hepatic CYP3A activity than men (Hu and Zhao 2010). Taken together, this family of metabolic enzymes, exerting effects in many biologic processes, has been described as sexually dimorphic, and with possible roles in disease manifestation. With Cyp2e1 representing a target for differences in alcohol induced stress, we will evaluate the alcohol metabolism pathway in more detail.

- Alcohol Metabolism
Alcohol is metabolized by two pathways in humans: the ADH (canonical) pathway which accounts for the bulk of the metabolism, and the MEOS (Microsomal Ethanol Oxidizing System) (non-canonical) pathway which, at toxic alcohol levels, contributes to the increased rate of
ethanol elimination (Crabb, Bosron et al. 1987, Lu and Cederbaum 2008, Konstandi, Cheng et al. 2013). The increased rate of elimination that results from chronic alcohol consumption is due to an increase in MEOS activity (Crabb, Bosron et al. 1987, Lu and Cederbaum 2008, Lu and Cederbaum 2010). In addition, individuals inherit different types of ADH isoenzymes that have different kinetic properties (Beisswenger, Holmquist et al. 1985, Crabb, Bosron et al. 1987, Haseba and Ohno 2010). Individuals with different phenotypic variants, e.g. the beta 1 vs. beta 2 isoenzymes, appear to have different rates of ethanol elimination (Crabb, Bosron et al. 1987).

Many of the pathophysiological effects of alcohol ingestion relate to these pathways of ethanol metabolism. However, some of the acute and chronic effects of ethanol use are also attributable to the direct effects of ethanol, e.g. on membrane fluidity. Oxidation of ethanol to acetaldehyde is catalyzed by alcohol dehydrogenase (ADH) (French, Morimoto et al. 1997, Peters and V.R. 1998, Rajasingh, Bord et al. 2007, Ferreira Seiva, Amauchi et al. 2009, Mackie, Krishnamurthy et al. 2013). There are at least six classes of ADH, some of which show inter-individual variation, i.e. genetic polymorphism, that influence the rate of ethanol oxidation (Peters and V.R. 1998, Galter, Carmine et al. 2003, Haseba and Ohno 2010). A consequence of ethanol oxidation is an increase in the NADH/NAD redox potential within the cytosol and mitochondria with subsequent alteration in several tissue metabolites (Peters and V.R. 1998, Haseba and Ohno 2010). Acetaldehyde, the product of ethanol oxidation, is chemically highly reactive, toxic and immunogenic (Peters and V.R. 1998, Guo and Ren 2010). However, the concentrations achieved \textit{in vivo} usually fall short of those used to produce these toxic effects in experimental situations (Peters and V.R. 1998, Guo and Ren 2010, Mackie, Krishnamurthy et al. 2013). Oxidation of acetaldehyde is also coupled to redox changes, although primarily affecting the intra-mitochondrial redox.
A schematic representation of the canonical and non-canonical ethanol metabolism pathways is represented below (Figure I2)

**Cellular Alcohol Metabolism**

![Cellular Alcohol Metabolism Diagram]

**Figure I2 Cellular Alcohol Metabolism**: This cellular representation of alcohol metabolism includes the canonical alcohol metabolism pathway: ethanol enters the cell by diffusion and is metabolized by alcohol dehydrogenase to acetaldehyde. Aldehyde dehydrogenase (ALDH) further metabolizes the acetaldehyde to acetate, which is later used by the cell. The non-canonical alcohol metabolism pathway is similar to the canonical pathway, but the initial metabolic step is performed by Cyp2e1 and results in generation of reactive oxygen species (ROS). Alcohol dehydrogenase is the major enzyme pathway for oxidizing ethanol to acetaldehyde. The morphological observations that chronic ethanol treatment causes proliferation of the liver smooth endoplasmic reticulum suggested that ethanol, similar to certain xenobiotics that are metabolized by cytochrome P450, may also be metabolized by P450 (Qi, Miao et al. 2013). A microsomal ethanol-oxidizing system (MEOS) was characterized by Lieber and Associates and shown to be dependent on P450 (Baraona, Abittan et al. 2001).

Given the substantial implications of cytochrome P450 2e1 in ethanol metabolism and oxidative stress caused by its induction, we dedicate the next section to describing what is known of the interactions of Cyp2e1.
- Cyp2e1 and Alcohol Metabolism

Cyp2e1 metabolizes several small, hydrophobic substrates and drugs (Lu and Cederbaum 2008, Lu and Cederbaum 2010). Cyp2e1 metabolizes and activates many toxicologically important compounds such as ethanol, carbon tetrachloride, acetaminophen, benzene, halothane, and many other halogenated substrates. Toxicity of these compounds is enhanced after induction of Cyp2e1, e.g., by ethanol treatment, and toxicity is reduced by inhibitors of Cyp2e1 or knockout of the gene in mice (Lu and Cederbaum 2008, Lu and Cederbaum 2010).

Cyp2e1 is a minor pathway of ethanol oxidation as it catalyzes the two-electron oxidation of ethanol to acetaldehyde (Kunitoh, Imaoka et al. 1997). Interestingly, acetaldehyde is also a substrate for Cyp2e1 and is oxidized to acetate; thus Cyp2e1 can, at least theoretically, catalyze the oxidation of ethanol to acetate (Lu and Cederbaum 2008, Lu and Cederbaum 2010). Cyp2e1 generates ROS such as O$_2^-$ and H$_2$O$_2$ during its catalytic cycle (Qi, Miao et al. 2013). Because Cyp2e1 can generate ROS during its catalytic cycle, and its levels are elevated by chronic treatment with ethanol, Cyp2e1 has been suggested to be a major contributor to ethanol-induced oxidant stress and to ethanol-induced liver injury (Lu and Cederbaum 2008, Lu and Cederbaum 2010). Initial suggestions of a role for Cyp2e1 in alcoholic liver injury arose from studies with the intragastric model of ethanol feeding in which Cyp2e1 is strongly induced and significant liver injury occurs (Lu and Cederbaum 2010). Experimentally, a decrease in Cyp2e1 induction was associated with a reduction in alcohol-induced liver injury (French, Morimoto et al. 1997). Cyp2e1 inhibitors such as diallyl sulfide (Heber-Katz, Chen et al. 2004), blocked the lipid peroxidation and ameliorated the pathologic changes in ethanol-fed rats (French, Morimoto et al. 1997, Lu and Cederbaum 2010). Ethanol increases CYP2E1, largely by a posttranscriptional
mechanism involving enzyme stabilization against degradation (Gonzalez and Gelboin 1990, Lu and Cederbaum 2008).

Based on this evidence, we hypothesize that the linkage between Cyp2e1-derived oxidative stress, mitochondrial injury, and GSH homeostasis contributes to the toxic actions of ethanol, and that this mechanism is sex dimorphic; in the results section, we explore our findings further corroborating this hypothesis.

Effectively, any differences between the sexes in ethanol metabolism can result in the dimorphic cell response (Penaloza, Estevez et al. 2009). In the next section, we explore sex differences in gene expression and regulation, including cytochrome P450 members, and indicate various mechanisms of gene expression regulation as targets for sex differences.
Sex Differences in Gene Expression and Regulation

Many scientists have found sex differences in gene expression and potentially the regulation of these genes, both *in vivo* and *in vitro*, and in many systems. Here we mention some of the differences and their known impact, and we propose targets for the differential regulation of these genes.

Differences in gene expression resulting in male and female biologic characteristics are well documented, and can explain sex determination and anatomical differences (Morrish and Sinclair 2002, Eggert 2004, Ludbrook and Harley 2004, Manolakou, Lavranos et al. 2006, Bernard and Harley 2007, Karkanaki, Praras et al. 2007, Bull 2008, Barske and Capel 2010, Jacob and Lovell-Badge 2011, Gilbert 2013). However, in tissues not normally thought to be sexually specific, such as liver, adipose tissue, and muscle, half to three-quarters of genes show consistent sex bias in mice (Penaloza, Estevez et al. 2009, Arnold and Lusis 2012). Although the average sex difference in level of expression of genes is modest (~9%), the inequality of expression of so many transcripts suggests that sex is an important variable (Arnold and Lusis 2012). In a genome-wide estimation of sex dimorphic gene expression of human livers, 224 genes out of 2,800 were sex dimorphic in expression, representing 8% of the expressed genes (Shin, Kim et al. 2009). Another study reported large sex differences in aging-related changes in gene expression in specific areas of the human brain, measured globally (Berchtold, Cribbs et al. 2008), suggesting the presence of somatic differences between the sexes.

Studies of gene activity in the mouse brain have indicated at least 50 sex dimorphic genes at a developmental stage at which the fetus produces no sex hormones (10.5 days post coitum)
(Dewing, Shi et al. 2003), which indicates the presence of sex dimorphism in gene expression prior to the onset of hormone production. Members of the Bcl-2 family, as well as caspase-3, behave in a sex dimorphic manner in the brain (Yuan 2009). Deletion of key cell death related genes (Bax) eliminates sex dimorphism in the brain (Forger 2009).

Others have reported sex-related differences in the transcription of candidate autosomal genes such as SLC2A3, HSP70, DNMT3A, DNMT3B, HMT1, ILF3, GSTM3 and PGRMC1 at the blastocyst stage (Bermejo-Alvarez, Rizos et al. 2011). Two global gene expression studies at the blastocyst stage not only enlarged greatly the list of genes known to display transcriptional sexual dimorphism but also allowed an estimation of the extent of this phenomenon and a deeper knowledge of the molecular pathways involved. In the mouse model, 591 transcripts displayed sex specific differences (Eakin and Hadjantonakis 2006). Among the transcripts up-regulated in females, one-fourth were X-linked, with the rest were autosomal (Eakin and Hadjantonakis 2006). In cattle, sexual differences were found in almost one-third of the transcripts expressed (2921 out of 9322), with most of them (2702) autosomally encoded (Bermejo-Alvarez, Rizos et al. 2010). These two studies provide evidence for a large sex chromosome-led transcriptional regulation of autosomal genes.

Gene expression in mouse and human heart is sex dimorphic, including autosomal genes such as Cyp2b10, member of the cytochrome P450 family of genes responsible for drug detoxifying as well as hormone metabolism (Petrick and Klaassen 2007). The cytochrome P450 superfamily is among the largest group of sex dimorphic genes and lately they have been shown to be estrogen responsive as well, as is the case with Cyp7b1, sex dimorphic in MEF (Mouse Embryonic Fibroblasts) (Penaloza, Estevez et al. 2009), Cyp2b10 (Petrick and Klaassen 2007); Cyp1B1 and
Cyp2A6 shown to be dimorphic in minipigs and conventional pigs (Skaanild and Friis 1999). Evidence showing sex differences in cytochrome p450 family members continues to grow, and with the growing roles for this family of enzymes reviewed in (Shu-Feng, Jun-Ping et al. 2009), it has become more important to characterize the upstream factors influencing the dimorphic regulation of these genes. This is the topic discussed in the next section.
Mechanisms Regulating Gene Expression

Many mechanisms affect gene expression, namely: chromosomal composition, transcription factors, epigenetic factors and commonly, a combination of some or all of these. Below, we discuss some of these, and potential sex dimorphic aspects.

- **Chromosomal composition:**

Chromosomal composition represents the presence or absence of a Y chromosome as well as dosage of X-chromosome transcripts, based on different number of copies of the chromosome. While these differences represent a major component of the differences between the sexes, many of the genes reported as sexually dimorphic are autosomal and are localized in non-sex chromosomes (Xu, Burgoyne et al. 2002, Dewing, Shi et al. 2003, Eakin and Hadjantonakis 2006, Berchtold, Cribbs et al. 2008, Bermejo-Alvarez, Rizos et al. 2010, Kolodkin and Auger 2011, Morgan and Bale 2012, Matsumoto, Buemio et al. 2013, Penaloza, Estevez et al. 2013). For a comprehensive review of X-chromosome transcriptional regulation see (Minkovsky, Patel et al. 2012); for a comprehensive review of the Y-chromosome and its regulation see (Ely, Underwood et al. 2010).

Since many of the dimorphically expressed genes are somatic, we must look at other gene regulatory mechanisms. Before gonadal differentiation, male and female embryos only differ in sex chromosome dosage resulting in transcriptional sexual dimorphism that has been detected even before implantation (Zwingman, Erickson et al. 1993, Goto and Monk 1998, Doherty, Mann et al. 2000, Chen, Ueda et al. 2003, Mann, Lee et al. 2004, Kobayashi, Isotani et al. 2006, Hayashi, Erikson et al. 2009, Bermejo-Alvarez, Rizos et al. 2010, Bermejo-Alvarez, Rizos et al. 2011, Gilbert 2013). This phenomenon is responsible for the differences in metabolism, cell

- **Transcription Factors:**

Transcription factors alter the RNA polymerase, making it more or less likely to bind to the promoter of any gene. **Repressors** bind to the operators on coding sequences, preventing RNA polymerase from binding and therefore transcribing. **General Transcription Factors** can position the RNA polymerase at the start of a coding sequence. **Activators** enhance the interaction between RNA polymerase and particular promoters. **Enhancers and Silencers** are sites within the DNA molecule bound to activators of transcription factors resulting in the initiation or silencing of gene expression. These processes describe the roles and actions of nuclear receptors as in the case of the response elicited by estradiol or any other ligand. For a review on transcription factors see review (Hobert 2008). The diagram below represents the different modes of action employed by transcription factors (Figure I3). Depending on the ligand and receptor, the gene modulation can be multifaceted, with induction and repression occurring based on competitive inhibition and or ligand/receptor dosage compensation.
Transcription factor modes of action:

**Figure I.** Transcription Factor Modes of Action: Transcription factors have been described to act in various ways. This figure represents eight (A-H) potential ways in which a simple transcription factor may work. There exist secondary modes of action that depend on other molecules, which can add to the complexity of transcription factor function. A) A constitutively active gene is one that is transcribed in the absence of a transcription factor. B) Receptors specific to consensus sequences on the promoter regions of genes can bind to constitutively active genes and repress the expression, by steric hindrance. C) A constitutively active gene can be repressed by action of a ligand bound receptor complex, which binds to the promoter region, preventing transcriptional machinery from binding and transcribing the gene. D) Ligand binding to its receptor can release the promoter region of the gene and allow its transcription. E) A constitutively repressed gene is inactive, in the absence of a receptor molecule, which activates it. F) Receptor binding to a repressed gene can induce its transcription, similar to G) a ligand-receptor complex bound to the promoter region. H) At times, a ligand binding its receptor can induce the release from the promoter region, resulting in transcription.

There exist other mechanisms of gene regulation including epigenetics, which too can play a role in the differential regulation of sex dimorphic genes.
- **Epigenetics**

The term *epigenetics*, literally meaning ‘above genetics’, refers to modifications made to the genome that can impact gene expression without affecting the underlying DNA sequence. Epigenetic processes are important for coordinating the impact of environmental factors during developmental sensitive periods [reviewed in (Roth and David Sweatt 2011)]. Steroid hormones, components of the internal environment, differ between males and females, and steroid receptors associate directly with DNA and enzymes that mediate some forms of epigenetic changes, making them ideal candidates to exert a lasting effect on the epigenome, or the overall epigenetic state of a cell.

- **Histone Modifications:**

Covalent modifications of the histone proteins forming the core of nucleosomes represent a form of epigenetics. The N-terminal ends of these proteins, especially of histone 3 and 4, undergo modifications such as: methylation, acetylation, phosphorylation, ubiquitination and others. The type of the modification and its localization are responsible for the effect which it exerts on gene expression. Acetylation and deacetylation, catalyzed by histone acetyltransferases (HAT) and histone deactylases (HDAC) are responsible for expression activation and repression, respectively (Portela and Esteller 2010). Histone methylation can have both an activating and an inhibiting effect that depends on the localization of the lysine that is covalently modified. For example, the methylation of lysine 4 and lysine 36 in histone 3 (H3K4, H3K36) was found to be associated with open chromatin structure and activation of gene expression. In contrast, methylation of lysine 9 and 27 in histone 3 is associated with silencing of mRNA transcription. The methylation of histone proteins is catalyzed by histone methyltransferases and histone
demethylases (Lennartsson and Ekwall 2009, Nimura, Ura et al. 2010). Modifications of histone proteins directly influence chromatin structure, for example, acetylation and deacetylation are responsible for chromatin decondensation and compaction, respectively. They are also responsible for the interaction between chromatin and transcription factors and forming of transcriptional complexes (Lennartsson and Ekwall 2009). DNA methylation and histone modifications are not separate mechanisms that have an impact on gene expression, they act together to influence the chromatin structure. The transcriptionally silent chromatin structure is associated with DNA methylation, histone deacetylation and methylation of specific lysines in H3 and H4. In contrast, DNA methylation is not observed when the chromatin structure is open, while the histones are acetylated and methylated on specific positions (Ikegami, Ohgane et al. 2009, Portela and Esteller 2010).

- RNA interference:

This mechanism acts on the RNA level and regulates gene expression post transcriptionally by specific interaction between target RNA and interference RNA. This interaction leads to the recruitment of specific protein complexes that are responsible for the target RNA degradation and decreasing the level of specific proteins (Bicker and Schratt 2008, Bian and Sun 2011, Qureshi and Mehler 2012).

- DNA Methylation:

DNA methylation is regulated by DNA methyltransferase (DNMT) enzymes, which are highly expressed in embryonic stem cells and early developing germ cells (McCarthy, Auger et al. 2009, Roth and David Sweatt 2011, Matsumoto, Buemio et al. 2013). They covalently affix a methyl group to the cytosine residue within a CpG dinucleotide (referred to as a CpG site). CpG sites are commonly methylated throughout the genome. However CpG sites are typically
unmethylated within so-called CpG islands (regions containing large numbers of CpGs) in the 5’ regulatory region, or promoter, of a gene (McCarthy, Auger et al. 2009, Roth and David Sweatt 2011, Absher, Li et al. 2013, Matsumoto, Buemio et al. 2013). Low baseline methylation levels in regulatory regions enable small changes in methylation at specific CpG sites within a gene’s promoter to have a significant effect on gene expression.

The prevailing dogma is that methylation of cytosine residues is permanent, irreversible, and that it consistently results in transcriptional repression. This view has proved true for some biological systems, although large differences in DNA methylation were found in embryonic and fetal cells during cellular differentiation, revealing that cytosine methylation can be a highly dynamic process (Roth and David Sweatt 2011). Maintenance of these patterns is accomplished by the Dnmt family members (Chen, Ueda et al. 2003), DNA methylation suppresses gene transcription by steric hindrance, as methylated cytosines block the transcriptional machinery from binding and transcribing the regulated gene, as shown in the figure below:
Figure I4: DNA methylation regulates expression by means of steric hindrance. In coupling transcription machinery to genomic DNA for transcription it is necessary that the appropriate van der Waals interactions occur. Presence of methyl groups on cytosine nucleotides on DNA can alter these interactions, effectively reducing the chance of the transcriptional machinery interacting with the DNA, thus preventing transcription.

The alterations of epigenetic regulation are frequently observed in case of common diseases like cancer, which affect mainly elderly populations (Miremadi, Oestergaard et al. 2007). However, the defects of epigenetic regulation can also be found in the case of inherited diseases. Epigenetic alterations might be the direct cause of the disease, may also be observed as the effect associated with a specific disease. Many chronic diseases involve deregulation of methylation, which results in abnormal transcription (Chen, Ueda et al. 2003, Roth and David Sweatt 2011, Absher, Li et al. 2013, Dobbs, Rodriguez et al. 2013). In addition to recruiting methyl-binding proteins, DNA methylation can directly block transcriptional machinery from accessing binding sites within a gene’s promoter (Roth and David Sweatt 2011). In genes with multiple promoters, DNA methylation can dictate promoter utilization (Chen, Ueda et al. 2003, Bell 2011, Liang 2011, Matsumoto, Buemio et al. 2013).

Thus, it is likely that differential methylation is at the root of endogenous sex differences, and therefore the mechanisms whereby the differential methylation appears and its impact on disease should be the targets for further study. In the next section, we explore known examples of sex differences in DNA methylation and the potential expansion of this field. From these studies, it is clear that differences between the sexes, whether genetic or hormonally driven, can have a substantial impact in biologic processes, from bioenergetics to cell death and stress response.
Abnormal patterns of DNA methylation are observed in many diseases such as tumors and imprinting disorders. Little is known about inter-individual and sex specific variations. In a study measuring methylation in DNA from total blood in 96 healthy human males and 96 healthy human females, all studied CpGs showed slightly higher methylation in males (El-Maarri, Becker et al. 2007). In a separate study, T-cell demethylation and Lupus flares were compared, with results suggesting that genetic risk and T cell DNA demethylation interact in lupus patients to influence the severity of lupus flares, and that men require a higher genetic risk and/or greater degree of T cell DNA demethylation to achieve a lupus flare equal in severity to women (Sawalha, Wang et al. 2012). Bisulfite sequencing also revealed that CD40LG is unmethylated in men, while women have one methylated and one unmethylated gene (Lu, Wu et al. 2007). 5-Azagytidine, a DNA methyltransferase inhibitor, demethylated CD40LG and doubled its expression on CD4+ T cells from women but not men, while increasing TNFSF7 expression equally between sexes. Similar studies demonstrated that CD40LG demethylates in CD4+ T cells from women with lupus, and that women but not men with lupus over-express CD40LG on CD4+ T cells, while both over-express TNFSF7. These studies demonstrate that regulatory sequences on the inactive X chromosome demethylate in T cells from women with lupus, contributing to CD40LG over-expression uniquely in women. Demethylation of CD40LG and perhaps other genes on the inactive X may contribute to the striking female predilection of this disease (Lu, Wu et al. 2007). While many examples of DNA methylation regulating gene expression exist, few document sex differences. The evidence presented above however, does suggest that there is probable cause to pursue more answers in this area.

Differences in chromosomal biology and gene expression give way to differences in biologic processes as in the case of metabolism, which ultimately have an effect on cell signaling,
behavior and ultimately disease manifestation. Understanding how these gene expression differences arise can be instrumental in the understanding of these diseases. As described earlier, limitations exist in our abilities to study sex differences; overcoming the limitations is the focus of this thesis.

Here we developed a system, whereby we can study sex differences at the cellular level, allowing better control of conditions, and ability to manipulate the system to test more sources of differences. We designed a mouse cell culture system from several developmental stages, including early embryo, late embryo, early mouse and young mouse, affording us the ability to control developmental influences including the onset of responsiveness to endogenous hormones. We were able to assess several cell types, derived from several organs. We were able to further supplement our cell cultures with cell stressors, inducers, inhibitors and other factors to provoke cell response, and measure sex differences.

We explore, in more depth, the role of sex hormones in stress response and gene expression. We identify potential factors dictating the dimorphic cell behavior, rooted in gene regulation mechanisms dictated by differential DNA methylation patterns. The results section is further subdivided into 3 main sections:

- **Cells have sexual identity, and sex dictates cellular behavior and gene expression.**

  Here we explored sex differences in cell viability as well as gene expression profiles between the sexes and the impact of cell stress on gene expression.

  We further explored the potential impact of endogenously produced and exogenously supplemented sex hormones on cell viability and gene expression.
- **Reducing DNA Methylation Differences Ablates Differences in Gene Expression Profiles.** In this section we explored the impact of DNA methylation on gene expression profiles before and after cell stress. We further looked at the impact of DNA methylation inhibition on already established expression patterns.

- **Sex Differences in Ethanol Induced Stress Response can be characterized in patterns of DNA methylation.** Here we looked at ethanol and characterize its sex dependent metabolic process. We evaluated the impact of multiple factors controlling alcohol metabolism and establish a correlation between DNA methylation patterns of **Cyp2e1** and the differential cell sensitivity to ethanol.

We then conclude this thesis by tying all aspects of sex differences together in discussing sex differences with the newly identified factors and their potential for expanding the field of sex biology.
Material and Methods

Mouse mating and Cell Preparation:

CFW Swiss Webster from the Charles River Carworth Colony were purchased and maintained in our animal facility at Queens College.

- Mating:

Male and female age matched (4-9 month) Swiss Webster mice were placed together overnight (16-18hrs), then separated setting the gestational time at day 0.5. Female mice were weighed at this time and checked for vaginal plugs. Females were maintained separately until the desired gestational time was reached.

- Day 10.5 Whole Embryo Isolation and initial Culture:

At embryonic day 10.5, pregnant females were sacrificed by CO₂ and cervical dislocation. Embryos were removed from the mother and placed in chilled sterile 1XPBS (Phosphate Buffered Saline). Each embryo was removed from its embryonic sac and placed in chilled Dulbecco’s Modified Eagle Medium (DMEM). To isolate cells from the embryo, the tissues were triturated by pipetting the tissue until it was completely dissociated. Suspended cells were collected by centrifugation at 3,000 rpm and resuspended in media containing DMEM supplemented with 10% Fetal Bovine Serum (FBS), 100U/mL penicillin, and 100μg/mL streptomycin, and divided into several tissue culture plates and incubated at 37°C in a humidified atmosphere with 5% CO₂. These cells represent a mixed group of cells of different types and populations, predominantly fibroblasts. Cells were grown for approximately 4 days or to 70% confluence before re-plating them. Medium was changed every 3 days. The cells were washed
in PBS before feeding with fresh culture medium. Once confluent at 70%, the culture dishes were trypsinized and cells were counted and plated in equal numbers for treatment at 500,000 cells per 10 mm².

- **Sexing of Day 10.5 Embryo:**

For sexing day 10.5 embryos, we used PCR as testis and ovaries are not visible during this stage. A small piece of tail was placed in a sterile PCR tube. DNA was isolated by digestion with 2μl proteinase K (10mg/ml) and 50μl PCR-D buffer and incubating overnight at 65°C. The enzyme was denatured at 95°C for 10 minutes and 1μl was transferred into a PCR tube, to which was added 21μl dH₂O, 25μlPCR Master Mix (Sigma), 2μl MgCl₂ (20mM), 1μl Primer Mix (25pmol/μl of Zfy and Zfxya, 12.5pmol/μl of Zfx primers). Primers for Zfy were 5’-CTCCTGATGGACAAACTTTACGTCTC and 3’-GCTGAGCCTCTTTGGTATCTCTGAGAAA; primers for Zfxya were 5’-GAGAGCATGGGAGGGCCATG and 3’-GAGTACAGGTGTGCAGCTC; Zfx primers were 5’-CTCTGAAGAAGAGACAAGTT and 3’-CTGTGTAGGATCTTCAATC. PCR was run for 40 total cycles (1 Cycle= 94°C for 45’, 60°C for 25’, 72°C for 1”) in the Eppendorf 2200 thermal cycler. The results of this DNA amplification were visualized on a 12% native polyacrylamide gel in TBE buffer using positive controls (pre-amplified male and female samples) and viewed by UV illumination. Male samples showed 2 bands, a 124bp band for Zfy gene and a 134bp band for gene Zfx and females showed a single band at 134bp for Zfx gene (Inset Figure 1) (Penaloza, Estevez et al. 2009). Each embryo was used as an N=1 in blind experiments, without knowing the sex of the embryo. Cells of each embryo were propagated and seeded at 5x10⁵/mL and exposed to cell death inducers. When the sexes of the blind cultures were determined, the results of each sample were pooled for statistical determination.
- **Organ Harvesting from Day 17.5 Embryos and Initial Culture:**

At embryonic day 17.5, pregnant females were sacrificed by CO$_2$ and cervical dislocation. Embryos were removed from the mother and placed in chilled sterile 1XPBS (Phosphate Buffered Saline). Each embryo was removed from its embryonic sac and placed in chilled Dulbecco’s Modified Eagle Medium (DMEM). Sexing for day 17.5 embryos was done by examining for the presence of immature testis and ovaries. The cells were isolated and cultured as described above for cells from day 10.5 embryos; the cultures however took closer to a week to reach confluency.

- **Organ harvesting from Day 4, Day 17 and Adult mice:**

At day 4, 17 post birth, as well as adult (6-8 months), mice were sacrificed by CO$_2$ and decapitation for day 4 and cervical dislocation for day 17 and adult mice. Organs were collected and placed in 1XPBS (Phosphate Buffered Saline). Organs were then mechanically minced and then triturated by pipetting until homogenous in chilled Dulbecco’s Modified Eagle Medium (DMEM). The cells were then cultured as described above; the cultures however took closer to a week to reach confluency.

- **Cell Culture and Maintenance:**

**Minimum Culture Medium:** Dulbecco’s Modified Eagle medium (DMEM) (catalog no. 12800-017; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (catalog no. LSFB-0500; Equitech-Bio), 1.5 g/liter sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), and 50 U/mL penicillin plus 50 mg/mL streptomycin (PenStrep, catalog no. 15140-122; Gibco) and incubated at 37°C under a humidified 5% CO$_2$ atmosphere.
Cells were passaged upon reaching 70-80% confluence (4 days after dissection for ED10.5, 7 days after dissection for ED17.5 and 10-14 days after dissection for day 4 and 17 postnatal) by aspirating old media, washing twice with 1XPhosphate-Buffered Saline (PBS) (see reagents section for formula) and applying 2.5% Trypsin/EDTA solution (see reagents section). Cells were incubated with trypsin/EDTA solution at 37°C until they began to detach from the bottom of the culture dish. Warmed DMEM was added to neutralize trypsin activity and cells were collected and centrifuged for 5 minutes at 1000 x g. Following centrifugation, the supernatant was aspirated and the cell pellet was resuspended in warmed DMEM.

**Cell Treatments:**

Twenty-four hours before treatment, cells were counted and plated in equal numbers, at 100,000 cells/mL for treatment in pre-culture medium (5% FBS, 1%PS) to synchronize the cells prior to treatment. To assess cell response, cell cultures were exposed to different compounds inducing cell death (cell death inducers) as follows:

*Ethanol*

Ethanol (EtOH): 200 Proof Ethanol (Catalog# 111HPLC20S, Pharmco-AAPER, Brookfield, CT)) was diluted in pre-culture medium to the desired final concentrations. At the time of treatment, old culture medium was aspirated and culture dishes were washed twice in 1X PBS (see solutions list). Medium containing EtOH was then added to the culture dish for the experimental condition, while the controls were given fresh pre-culture medium. This point was considered to be the beginning of the experiment (Time 0). The LD$_{50}$ for ethanol was identified as 50-400 µM for all mixed cell cultures as read at 24 hrs.
Camptothecin
- Camptothecin (CPT): (S)-(+) - Camptothecin (Catalog# C9911-250MG, Sigma-Aldrich, Saint Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) (Catalog# 472301, Sigma-Aldrich, Saint Louis, MO) to a stock concentration of 50 mM. This stock concentration was diluted to final concentration at the time of treatment, in pre-culture medium. The LD_{50} for camptothecin was identified as 5-20 μM for all mixed cell cultures as read at 24 hrs.

17β-estradiol
17β-estradiol (E2): 17β-estradiol-16,16,17-d_{3} (Catalog# 491187, Sigma-Aldrich, St. Louis, MO) was dissolved in ddH_{2}O to a stock concentration of 100 mM and then diluted serially on the date of treatment in pre-culture medium to a final concentration of 5 nM.
- 17β-estradiol (E2) + Ethanol (EtOH) Treatments: combination treatments were done in two ways (with no discernible difference in response):
  - Pretreatment with 17β-estradiol for 24 hrs, followed by co-treatment with EtOH for another 24hrs;
  - No pretreatment, Co-treatment for 24 hrs with both 17β-estradiol and EtOH.

5-Aza-dC
- 5-Aza-2’-deoxycytidine (5-Aza-dC): (Catalog #A3656, Sigma-Aldrich, Saint Louis, MO) was dissolved in DMSO to a stock concentration of 20 mM and further diluted in pre-culture medium to 20 μM. Medium was replenished every 3 days with 5-Aza-dC supplement. Cells were maintained in this culture medium for 5 population doublings.
Calculations of population doubling: Population doubling was calculated as follows: \( DT = (t - t_0)\log 2/(\log N - \log N_0) \), where \( t \) and \( t_0 \) are the times at which the cells were counted, and \( N \) and \( N_0 \) are the cell numbers at times \( t \) and \( t_0 \).

- 5-Aza-dC + EtOH co-treatment: Cells were maintained as described above on 5-Aza-dC for 5 population doublings, then exposed to 400\( \mu \)M EtOH for 24hrs.

**Disulfiram**
Disulfiram (DSF): Tetraethylthiuram disulfide (Catalog #T1132, Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO to a concentration of 0.1 mM, and then further diluted to its final working concentration of 0.5 \( \mu \)M in pre-culture medium. This treatment occurred 1 hr prior to any secondary treatment, which consisted of another chemical that was added directly to the already existing medium.

**N-Acetyl Cysteine**
N-Acetyl Cysteine (NAC): (Catalog #ALX-105-005-G005, Enzo Life Sciences, Farmingdale, NY) was dissolved in ethanol to a concentration of 20 mM and to a final working concentration of 100 \( \mu \)M in pre-culture medium, treatment occurred 1hr prior to supplementing cells with secondary inducer.

- Ethanol: after the 1hr pre-treatment with NAC, medium was supplemented with pretreatment medium supplemented with 400\( \mu \)M EtOH for 2, 4, 12 and 24hrs.
- Pyocyanin: after the 1hr pre-treatment with NAC, medium was supplemented with pretreatment medium supplemented with 50\( \mu \)M Pyocyanin for 2, 4, 12 and 24hrs.
*Pyocyanin*

Pyocyanin: (Catalog #ENZ-53001-C001, Enzo Life Sciences, Farmingdale, NY) was dissolved in DMSO to a stock concentration of 50 mM and to a final concentration of 50 μM in pre-culture medium. Some conditions required co-treatment with NAC as described above.

**Cell Death and Viability Assays:**

- **Trypan blue exclusion assay**

Dead and dying cells experience a loss of plasma membrane integrity, allowing vital dyes such as trypan blue to enter the cell and stain cytoplasmic proteins while the intact plasma membrane of living cells excludes these dyes. Staining with trypan blue leaves living cells clear and dead cells blue under microscopic examination, making this a convenient and reliable method for quantifying the number of living and dead cells in a population (Allison and Ridolpho 1980). After treatment, cells were collected at the desired time and centrifuged at 1000 x g for 5 minutes. The medium was aspirated and the cell pellet was resuspended gently but thoroughly in an appropriate volume of warmed 1x PBS. A 100μL sample of this cell suspension was mixed with an equal volume of 0.4% trypan blue solution (Catalog# T6146-25G, Sigma-Aldrich, Saint Louis, MO) and incubated at room temperature for 3 minutes. Cells were then observed and counted on a hemocytometer under a light microscope. Each assay was run in triplicate. Data were typically expressed as percent cell death and was calculated using the equation: percent cell death = ((number of dead cells)/(total number of dead and living cells)) x (100%).
Dying cells, whether necrotic or apoptotic, become permeable, allowing dyes such as trypan blue to enter the cell (Allison and Ridolpho 1980). Since our results with trypan blue were completely consistent with other assays, such as Live/Dead® and Hoechst 33258 (Hoechst, Frankfurt, Germany) staining (data not shown), we relied primarily on this simple and reliable assay. For each experiment, this number for the experimental sample was normalized by subtracting the basal level of cell death observed in the control (9–20%) for each treatment. Statistical significance of the results was calculated by standard t test; values of P <0.05 were considered significant.

- **MTT assay**

MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Catalog# M2128, Sigma-Aldrich, Saint Louis, MO) is a soluble, bright yellow compound that is reduced to insoluble purple formazan in metabolically active cells by the succinate-tetrazoliumreductase system within the mitochondrial respiratory chain. MTT can also be used to measure changes in mitochondrial activity. To measure the viability of a cell population after treatment, 1.5 x 10^6 cells are seeded into 35mm tissue culture dishes and allowed to attach overnight at 37°C in minimum culture medium (MCM). Cells are treated before incubation at 37°C in the appropriate media until the desired time. Media from each plate was then collected and centrifuged at 1000 x g for 5 minutes to collect and pellet any dead, floating cells. These cells were resuspended in a small amount of media and put back into the cell culture plate. One mL of a 1:10 dilution in CMM of 5mg/mL MTT stock was then applied to each plate, which was then incubated at 37°C for 2 hours to allow formazan formation. One mL of acidic isopropanol (0.04M HCl in absolute isopropanol) was then added to each plate and mixed thoroughly to solubilize the newly formed formazan. All cells and supernatant were then collected from each plate and centrifuged at
14,000rpm for 5 minutes to remove cell debris. The supernatant from each sample was then transferred into a plastic spectrophotometer cuvette and the absorbance of the converted dye was measured at 540 nm. A decrease in absorbance indicates a decrease in mitochondrial activity and/or death (Meerloo, Kaspers et al. 2011).

- **WST-1**

WST-1 (Water Soluble Tetrazolium salts) (Catalog# 630118, Clontech, Mountain View, CA) is cell-impermeable dye and is used to measure cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes and may, under defined conditions, reflect the number of viable cells present. Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferative to resting status) of potential medicinal agents and toxic materials. To measure the viability of a cell population after treatment, 1.5 x 10^6 cells are seeded into 96-well tissue culture dishes and allowed to attach overnight at 37°C in complete maintenance media. Cells are then treated before incubation at 37°C in the appropriate media until the desired time. WST-1 reagent is then added to the medium in dish during the final treatment hour. The dish is then read on a microplate reader at a wavelength of 450 nm with a background subtraction at 690 nm. A decrease in absorbance indicates a decrease in mitochondrial activity and/or death (Ngamwongsatit, Banada et al. 2008).

**Analysis of Gene Expression:**

- **Microarray analysis**

Total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH, USA) and treated with DNase I (Qiagen, Valencia, CA, USA). For the pooled microarray experiments, pools of total RNA from either male or female ED10.5 embryos were arrayed against a control
containing equal amounts of total RNA from the male and female pools. The individual embryo arrays were hybridized as male/female pairs. The RNA samples were reverse transcribed, and the cDNA was labeled with either Cy3 or Cy5 using the amino-allyl method adapted from the University Health Network Microarray Centre (Toronto, ON, Canada; http://www.microarrays.ca). Labeled cDNA was hybridized to mouse M7.4k1 and M15k4 array slides (University Health Network Microarray Centre,) at 37°C for 18 h. The slides were washed and then scanned using a ScanArray 5000 confocal scanner (Packard Bioscience, Waltham, MA, USA). Image files were quantitated using QuantArray v3 (Packard Bioscience) (Walker, Smith et al. 2004, Penaloza, Estevez et al. 2009).

- Microarray data processing and analysis

Raw intensity data from QuantArray were subject to preprocessing in Microsoft Excel (Microsoft, Redmond, WA, USA) using an in-house Excel add-in written for the purpose. Background subtracted spot intensities were logged (base 2), and suspect data were filtered out before normalization. The following criteria were used for filtering: negative intensity values, signal/background =1.5, and intensity values in the lowest fifth percentile for either channel. In addition, spots identified manually as poor quality during quantitation, buffer controls, and positive controls were removed. The remaining data were normalized using methods that correct for intensity-dependent bias using a trimmed running average method and for spatial biases using a Euclidean distance weighted Gaussian function based on the work of (Workman, Jensen et al. 2002) and (Colantuoni, Henry et al. 2002). The logged (base 2) ratio of sample to control signals was then determined for each spot, and spot duplicates were averaged. The data were analyzed in Excel to select for spots that differed between sexes by >1.33-fold with \( P < 0.05 \) (using
either paired or two-sample $t$ test as appropriate). Annotation for spots of interest, identified by their clone ID, was obtained from the annotation database SOURCE (http://source.stanford.edu; Diehn, Sherlock et al. 2003). The Virtual Reality (VR) module of BioMiner, a data mining software package designed and built in-house, was used to generate three-dimensional virtual reality representations of the female vs. male microarrays. The VR module implements a simplified version of DIG (Hybrid Strategies Corp., Ottawa, ON, Canada). It is used for visualizing complex, high-dimensional data by mapping the data to three dimensions with minimal loss of the underlying structure of the original data. Subsets of the normalized intensity data from the female vs. male embryo microarrays were iterated in the VR algorithm until minimum absolute error was achieved. Technical details of the algorithm and its application to gene expression data have been published previously (Walker, Smith et al. 2004).

- RNA Extraction using RNeasy® Columns:
Total RNA was extracted using the Qiagen RNeasy® Mini Kit using manufacturer specifications. Cell pellets are lysed in 600 μL of Buffer RLT (proprietary and contains guanidine salt) and β-ME (β-mercaptoethanol). Homogenized sample is then transferred into a QIAshredder® Column and centrifuged at maximum speed for 2 minutes. Wash through is collected and centrifuged for 3 minutes at maximum speed. Supernatant is then mixed with 1 volume of chilled ethanol. Mixture is loaded into an RNeasy mini column and centrifuged for 15 seconds at 10,000 rpm. The column is washed by adding Buffer RW1 (proprietary and contains guanididine salt) and centrifuged for 15 seconds at 10,000 rpm. The wash is repeated using the RPE Buffer, centrifuged for 2 minutes at 10,000 rpm. The RNA is collected by washing the
column with 30 μL of RNeasy-free water, centrifuged for 1 minute at 10,000 rpm. This extraction represents total RNA isolation (Arshed, Magnuson et al. 2011).

- Reverse Transcriptase – PCR- cDNA Synthesis using RETROscript®:

cDNA synthesis was completed using the Applied Biosystems - Ambion RETROscript® kit using manufacturer specifications. The reverse transcriptase (RT) reaction without heat denaturation of RNA was performed as follows: 1 μg of Total RNA are loaded with 2 μL of Oligo(dT), 2 μL of 10X RT Buffer, 4 μL of dNTP mix, 1 μL of RNase Inhibitor, 1 μL of MMLV-RT and water to 20 μL of final reaction volume. The reaction tube is mixed and microspun, then cycled at 50°C for 60 minutes, 92°C for 10 minutes once and then stored at -20°C. This represents cDNA of active genes, as selected by Poly-A tails (Barrio, Calvo et al. 2009).

- q RT-PCR:

Quantitative Real Time Polymerase Chain Reaction (PCR) - Small PCR products (100–200 base-pairs) were amplified in quadruple on a Roche LightCycler 2.0 real-time PCR machine, using universal PCR conditions (65°C to 59°C touch-down, followed by 40 cycles [15" at 95C, 10" at 59C and 10" at 72C]). 500 μg of cDNA was amplified in 20 μL reactions [0.3X Sybr-green, 3 mM MgCl₂, 200 μM dNTPs, 200 μM primers, 0.5 unit Platinum Taq DNA polymerase (Roche)]. Primer-dimers were assessed by amplifying primers without cDNA. Primers were retained if they produced no primer-dimers or only non-specific signal after 40 cycles. Results were calculated as relative intensity compared to female expression. The last cycle was retained as baseline for comparison with "absent" genes. Data were plotted as “CT” (Cycle Threshold)
values, in which the CT value represents the cycle at which fluorescence is first detected. By this representation, a lower cycle number indicates a higher initial concentration of mRNA, and each decrease of one cycle indicates a doubling of initial concentration (Penaloza, Estevez et al. 2009, Penaloza, Estevez et al. 2013). Below you will find a table with primer sequences used in this thesis together with the gene and product sizes. These represent the final primer sequences retained and reported.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ist</td>
<td><strong>FW</strong> AGCCTTGCTTGTGCTGCTCTA &lt;br&gt; <strong>REV</strong> GTGCTGGCCTTGCTTGAGG</td>
<td>178</td>
</tr>
<tr>
<td>Cyp1a1</td>
<td><strong>FW</strong> GCATCTCATCTGTTACAGC &lt;br&gt; <strong>REV</strong> CTTCTCATCAATCTTTACCC</td>
<td>267</td>
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<tr>
<td>Cyp2e1</td>
<td><strong>FW</strong> ACGTAACGCCCCAAGATTC &lt;br&gt; <strong>REV</strong> AAGTCGTGCTGCTTTATGTG</td>
<td>198</td>
</tr>
<tr>
<td>Cyp7b1</td>
<td><strong>FW</strong> CAAAACCAAGAATGGCATCA &lt;br&gt; <strong>REV</strong> TCTTTGGAAGGGCAGATTG</td>
<td>179</td>
</tr>
<tr>
<td>Gapdh</td>
<td><strong>FW</strong> CTGGCTAGACGAAGACTCAAG &lt;br&gt; <strong>REV</strong> AGGCAGTGCTGCTTGATCAC</td>
<td>199</td>
</tr>
<tr>
<td>SRY</td>
<td><strong>FW</strong> TTTCAACCACCATGCCCTCTTTT &lt;br&gt; <strong>REV</strong> TAATTTGTCGTTGTCATTTTC</td>
<td>201</td>
</tr>
<tr>
<td>SRX</td>
<td><strong>FW</strong> TCAAGGCACAAACGATCTTAGGAACAGAT &lt;br&gt; <strong>REV</strong> CGATCATTTGATCCGATTTTCTG</td>
<td>301</td>
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<tr>
<td>Dnm3tl</td>
<td><strong>FW</strong> CAGAGGCCATTGATATTGCT &lt;br&gt; <strong>REV</strong> CTCTGGCACTCTTCCGTAG</td>
<td>204</td>
</tr>
<tr>
<td>ADH I</td>
<td><strong>FW</strong> TACACCAGTCACCAATAGGAGGTG &lt;br&gt; <strong>REV</strong> CCATGCATTGACACCTTGATATTGCT</td>
<td>321</td>
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<tr>
<td>ADH II</td>
<td><strong>FW</strong> TTCCCAAAAGAGCACATCC</td>
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<tr>
<td>ADH IV</td>
<td><strong>FW</strong> AGAAAATGGCTTGGCCACTA &lt;br&gt; <strong>REV</strong> CAATCCGAGCTTCCATCAA</td>
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<tr>
<td>ADH VII</td>
<td><strong>FW</strong> TCTGCATGAAGGTGACATTTTCTG &lt;br&gt; <strong>REV</strong> AGAAACTGGCTTCCGGCACTA</td>
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<tr>
<td>Erα</td>
<td><strong>FW</strong> CAGAGGCCATTGATATTGCT &lt;br&gt; <strong>REV</strong> CTCTGGCACTCTTCCGTAG</td>
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<tr>
<td>Erβ</td>
<td><strong>FW</strong> AGCCTTGCTTGTGCTGCTCTA &lt;br&gt; <strong>REV</strong> GTGCTGGCCTTGCTTGAGG</td>
<td>189</td>
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</table>
DNA Extraction, Isolation, Treatment and Sequencing:

- **DNA extraction and isolation by isopropanol precipitation:**
  
  Cell pellets containing $5 \times 10^6$ cells were lysed in lysis buffer (0.1M Tris pH 8, 0.2M NaCl, 5mM EDTA, 0.4% SDS) with 0.2 mg/mL Proteinase K. Pellets were incubated overnight at 55°C. Samples were spun, and supernatant was transferred into a new microfuge tube. Isopropanol and NaCl$_2$ were used to precipitate DNA overnight at -20°C (Rake 1972). DNA was cleaned and isolated using the Wizard® DNA Clean-Up System, using manufacturer’s specifications (Engel, Pinnell et al. 2012).

- **Sodium Bisulfite Conversion and Sequencing of Isolated DNA:** Two micrograms of DNA per sample were dissolved into 10μL of H$_2$O. One microliter of 6N NaOH is added to the DNA solution, which was incubated for 15 minutes at 37°C. To this, 120μL of 4.04M NaHSO$_3$, and 1μM hydroquinone was added, and cycled as follows (15 cycles): 30 sec at 95°C, and 15 min at 50°C to allow optimal conversion of unmethylation cytosines to uracils (Zhang, Rohde et al. 2009). Samples are desalted using the Wizard® DNA Clean-Up System as per manufacturer’s suggested procedure. Samples were eluted in 20μL of TE buffer. The purified product was then sequenced by Eurofins MWG Operon automated DNA sequencing service (Eurofins MWG Operon, Huntsville, AL, USA).

- **Specific DNA isolation:** 1μl of bisulfate treated DNA was transferred into a PCR tube, to which was added 21μl dH$_2$O, 25μl PCR Master Mix (Sigma), 2μl MgCl$_2$ (20mM), 1μl Primer Mix (gene specific with sequences outlined above). PCR was run for 40 total cycles (1 Cycle= 94°C for 45’, 60°C for 25’, 72°C for 1”) in the Eppendorf 2200 thermal
cycler. The results of this DNA amplification were visualized on a 1% agarose gel in TBE buffer and viewed by UV illumination (Weisburg, Barns et al. 1991). Specific band sizes were selected based on expected amplification fragment sizes as outlined in the primer sequence table above. Bands were outlined and cut to elute the DNA from the agarose.

**DNA elution from agarose:**
Gel slice containing DNA is loaded into a microfuge tube containing elution buffer (enough to cover the gel slice), sample is heated to 65°C for 5 minutes to melt the gel, then frozen at -80°C for 10 minutes, centrifuged to pellet the agarose for 10 minutes, then more eluting buffer is added to the pellet and again melted at 65°C for 5 minutes, frozen at -80°C. Supernatant is transferred into a new tube, and mixed with butanol. 95% ethanol is added and placed in -80°C, pellet, decant and washed in 80% ethanol. Pellet sample, decant, air dry pellet and finally rehydrate in 0.1x TE buffer (He, Liu et al. 1992).

**Detection of Total ROS:**
Enzo Life Sciences’ Total ROS Detection Kit (Catalog # ENZ-51010, Enzo Life Sciences, Farmingdale, NY) is designed to directly monitor real time production of reactive oxygen and/or nitrogen species (ROS) in live cells using a microplate reader. The kit includes Oxidative Stress Detection Reagent (Green) as the major component. This non-fluorescent, cell-permeable total ROS detection dye reacts directly with a wide range of reactive species, such as hydrogen peroxide, peroxynitrite and hydroxyl radicals, yielding a green fluorescent product indicative of cellular production of different ROS.
Upon staining, the fluorescent product generated can be visualized using a standard green filter (490_{Ex}/525_{Em} nm).

The procedure followed vendor’s specifications. In short, 1hr prior to cell treatment, the ROS dye is added to the culture dish; cell treatment is superimposed, and then readings are taken at specified times (Jambunathan 2010).

**Total Glutathione Assay:**

Total Glutathione (GSSG+GSH) levels were measured using the Sigma-Aldrich Glutathione Assay Kit, using manufacturer specifications. Cell pellets were normalized to total cell number including live and dead cells. Pellets were deproteinized with 3 volumes of 5%-Sulfosalicylic Acid solution (SSA), then centrifuged at 600 x g to remove precipitated proteins. Samples are then freeze thawed twice and left at 4°C for 5 minutes. Samples were centrifuged at 10,000 x g for 10 minutes. Supernatant volumes were measured and retained as the original sample volume. Samples were then serially diluted in 5% SSA; sample in SSA represented 10 μL of the working mixture, the rest presented the working mixture 150 μL (Glutathione Reductase (6 units/mL), DTNB (1.5 mg/mL), in Assay buffer (100mM potassium phosphate buffer, pH 7.0, with 1 nM EDTA)). Reaction started upon adding 50 μL of NADPH (0.16 mg/mL), with a 5 minute, room temperature incubation. Readings were performed in a plate reader at 412 nm with readings every minute for 5 minutes. Absorbance of a blank sample was subtracted as background. The calculation was as follow:

\[
\text{nmoles GSH per mL of sample} = \frac{\Delta A412}{\text{min(samples)}} \times \text{dil} \times \frac{\Delta A412}{\text{min(1 nmol)}} \times \text{vol}
\]
Where:

\[ \Delta A_{412}/\text{min(sample)} = \text{slope generated by sample (after subtracting the values generated by the blank reaction)}. \]

\[ \Delta A_{412}/\text{min(1 nmol)} = \text{slope calculated from standard curve for 1 nmole of GSH} \]

dil = dilution factor of original sample

vol = volume of sample in reaction in mL.

Data was presented as nmoles of GSH per mL of sample (Romagnoli, Marcucci et al. 2013).

**Statistical Analysis:**

- Student T-TEST analysis:

  Using Excel Functions: 
  
  
  "=TTEST(array1, array2, tails, type)". 
  "Array1" is the set of replicates from our first condition, and "array2" is the set of replicated from our second condition. "Tails" is 2 (for a two-tailed test). For a regular t-test, "2" is the "type." The function returns the P-value of the test. 
  P values greater than 0.05 represent no statistical difference between compared samples.

**Reagents:**

- 10x Phosphate Buffered Saline (10x PBS)
  
  Dissolve in 800mL of dH2O: 80g NaCl, 2g KCl, 14.4g Na2HPO4, 2.4g KH2PO4;
  
  Adjust pH to 7.4 w/ HCl; adjust to 1000mL w/ dH2O; Autoclave to sterilize.

- 1x Phosphate Buffered Saline (1x PBS)
  
  100 mL 10x PBS (discussed above); bring to 1000mL with ddH2O. This is equal to
  
  137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4·7H2O and 1.4 mM KH2PO4 in dH2O,
  
  pH 7.3; Autoclave to sterilize
- **2.5% Trypsin EDTA (100mL)**
  
  10mL 25% Trypsin (=10x stock), 10mL 10x PBS, 10mL 10mM EDTA, 0.5mL 5% NaHCO₃, 69.5mL dH₂O. All reagents must be sterile.

- **Dulbecco’s Minimum Essential Media (DMEM)**
  
  Dulbecco’s modified Eagle medium (DMEM) (catalog no. 12800-017; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (catalog no. LSFB-0500; Equitech-Bio), 2 mM L-glutamine (catalog no. 25030-081; Gibco), 1.5 g/liter sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), and 50 U/mL penicillin plus 50 mg/mL streptomycin (catalog no. 15140-122; Gibco)

- **1x TE Buffer**
  
  990mL dH₂O, 10mL 1M Tris-HCl (pH 8), 400 µL 0.25M EDTA

- **30:08 Acrylamide**
  
  30%acrylamide/0.8% bisacrylamide, 150g Acrylamide, 4g N,N'-methylene bisacryl, bring to 500 mL with H₂O

- **10mM Tris, pH 7.6**
  
  1L dH₂O, 0.12g Tris powder, pH to 7.6 using HCl

- **4x Tris/SDS, pH 8.8**
  
  91g Tris base, 20 mL 10% SDS, adjust pH w/ 6N HCl to pH 8.8, bring to 500 mL with dH₂O.

- **0.5M Tris/SDS, pH 6.8**
  
  100mL 0.5M Tris pH 6.8, 4mL 10% SDS
RESULTS
The work presented in this chapter has been published in part in FASEB J. 2009 June; 23(6): 1869–1879 and FASEB J. 2013 October; [Epub ahead of print].

Cells have sex, and sex dictates cellular behavior and gene expression independently of endogenous sex hormones.

Synopsis:

Differences between the sexes are typically attributed to hormonal differences; however, in some cases, the differences cannot be logically linked to sex hormones. In this section, we explore whether sexual dimorphisms can be found at the cellular level, and if so, what factors can contribute to these differences.

To do this, cells derived from different mouse organs at different stages of development were assessed for their response to two known cell death inducers (CDI), namely ethanol and camptothecin. Cell response was measured as the measure of cell viability. We find no sex differences in cell viability in the absence of CDI, but female cells are generally more sensitive than their male counterparts to ethanol or camptothecin.

To further investigate the factors causing this differential response, we looked at profiles of gene expression. We first established a baseline for gene expression in vivo examining whole embryo at ED 10.5 as well as kidney and lung at ED 17.5, and PN days 4 and 17. We find that gene
expression profiles are dependent not only on organ and developmental timing, but that expression is highly regulated by sex. Similarly, using an *in vitro* model of mixed cell cultures at the various developmental stages, we found that differences exist between the sexes for basal gene expression. In this system treatment with CDI’s superimposes further differences in gene expression, indicating sex-specific gene regulatory patterns.

Taken together, we found that sensitivity to CDIs is different between male and female cells and that gene expression patterns as well are sex dependent. Most important, we find that differences exist at ED10.5 when embryos are devoid of endogenous sex hormones, suggesting that sex hormones do not dictate the sex differences at that time, and that the differences precede hormonal modulation. Exogenous estrogen (17β-estradiol) added to cultures exerts an influence on cell viability as well as on gene expression profiles, but this influence is superimposed on already pre-existing differences. Thus, sex differences exist upstream of hormonal influences, and these differences can be mirrored in our cell culture system, where we can better understand underlying differences between the sexes.
Male and Female Cells Respond Differently to Cell Death Inducers

Nikezic-Ardolic et al. developed an in vitro model and showed that under certain conditions male and female cells responded differently (Nikezic-Ardolic, Lin et al. 1999). Since the model was established in this laboratory, we were in position to examine the underlying mechanisms of sex dependent cellular responses.

Male and female cells die at different levels, when exposed to ethanol as a death inducer.

As our laboratory had previously demonstrated, male and female ED17.5 mouse embryonic fibroblast (MEF) respond in a sex dependent manner to several cell death inducers (CDI) (Nikezic-Ardolic, Lin et al. 1999, Penaloza, Estevez et al. 2009). To further evaluate the existence of sex differences under this situation, while controlling for endogenously produced sex hormones, cells from male and female ED10.5 whole embryos, ED17.5, postnatal Day 4 (PN4) and Day17 (PN17) kidney, lung and liver were isolated, as described in Material and Methods. While differences in confluence (cell growth and division) were noted among developmental stages and different organs, no differences in cell viability were noted between the sexes (Figure 1). Kidney, lung and liver are easily isolated in late embryo and postnatal stages; at ED10.5 the organs are not easy to isolate, and therefore whole embryo homogenates were used. Kidney, lung and liver also represent organs commonly reported as sex dimorphic in disease (Mollerup, Ryberg et al. 1999, Bain, Feskanich et al. 2004, Reyes, Lew et al. 2005, Wisnivesky and Halm 2007, Wang, Wang et al. 2009, Essack, MacPherson et al. 2012, Townsend, Miller et al. 2012) and therefore, represent good models to investigate for sex differences.
Working *in vitro* presents the advantage of controlling more variables, and thereby effectively identifying factors responsible for sex differences. However, first we must verify whether differences exist prior to stressing the cells. Cell viability was measured using the trypan blue exclusion assay, as described in Material and Methods, taking advantage of compromised cell membrane in dying cells (Allison and Ridolpho 1980). We found that ED10.5 whole embryo cell culture basal death was consistently around 10%, independent of sex (*Figure 1*). Similarly, for ED17.5 we found basal death for kidney, liver and lung between 17%-21%, with no differences between the sexes. For postnatal days 4 and 17 kidney, liver and lung cells, we found similar trends with cell death (*Figure 1*). The absence of differences in basal viability allows us to measure sex differences superimposed by factors independent of already existing differences.

To assess the effect of development and endogenous hormones on cell response to ethanol (EtOH), we used cells from ED10.5 whole-embryo and mixed kidney cells of ED17.5, PN4, and PN17 mice and used the viability of the cells as measurement of cell response. Toxicity of ethanol to cells, documented in the introduction, has been linked to cell membrane fluidity as well as oxidative stress under high levels of exposure (Beisswenger, Holmquist et al. 1985, Kunitoh, Imaoka et al. 1997, Lu and Cederbaum 2008, Mukhopadhyay, Rezzoug et al. 2013).

The concentration of ethanol used was determined by the LD$_{50}$, defined as the concentration at which ~50% of cells die in at least one sex, in our case females. The LD$_{50}$ was determined for EtOH to be 400 μM for 24 hrs of exposure (*Figure 2*). The percentages reported here represent total cell death, *i.e.*, that caused by EtOH as well as the basal level. At day 10.5, the embryos are not yet producing their own sex hormones. At this developmental stage, more female cells die than male cells. These were obtained from at least 3 independent experiments, with error bars
representing standard deviations, and significance indicated by asterisks (*) representing p-values of 0.05 or less, as measured by the standard Student t-test, described in Material and Methods.

Similarly embryonic day 17.5 kidney cells, from animals producing some endogenous hormones, responded in a sex-dependent manner with cells from females dying more (Figure 3), with p<0.05. Other organs at ED17.5 did not show any sex differences (Figure 3), although we found liver and lung cells to be significantly more sensitive to EtOH, in comparison to kidney cells (Figure 3).

On the other hand, looking at the same organs, but at an early postnatal developmental stage, 4 day post-natal, no differences between the sexes in cell sensitivity to EtOH were seen for any of the organs. The sensitivities however differed by organ, with liver and lung being most sensitive (Figure 3).

Day 17 post-natal cells were more sensitive to EtOH, in comparison to the other developmental times. Kidney cells were the only ones, as in ED 17.5, to have a sex dimorphic response, with females exhibiting higher sensitivity. For liver and lung, we found no differences in death levels between the sexes (Figure 3).

These results suggest that male and female cells respond differently to ethanol in a developmental time- and organ-specific manner, suggesting a response mechanism regulated by differentiation or hormones. These results confirmed that male and female differences exist, and that these occur whether or not endogenous sex hormones are produced. Since differences precede gonad development and subsequent production of sex hormones, as noted with
sensitivity of cells from ED10.5 whole embryos, other factors may be responsible for the differences, upstream of hormonal influences, contrary to common assumptions.

**Male and female cells are differentially sensitive to CPT.**

To establish whether the differences observed are ethanol-specific, we used camptothecin (CPT) as another cell death inducer. Knowing that CPT inhibits topoisomerase, hence blocking the cell cycle and resulting in cell death (Beretta GL 2013), we asked whether this cell death response was sex dependent or independent.

Similar to EtOH, we determined the LD$_{50}$ for camptothecin (CPT) to be 20 μM for 24 hrs in at least one sex (female), while resulting in a measurable level of induced death on the other sex (Figure 4).

For ED10.5 cells, we found a substantial sex difference in cell death between male and female cells exposed to 20 μM CPT for 24 hrs, with females being more sensitive (Figure 5). However the response to CPT of embryonic day 17.5 cells is indifferent to sex compared to EtOH exposed cells. Additionally lung cells are more susceptible to the effect of CPT than are kidney and liver cells (Figure 5).

Similar to the ED17.5 cells, postnatal day 4 cells displayed no sex differences in response to CPT, with lung being most sensitive (Figure 5).

As was the case for ethanol, postnatal day 17 cells regained sex difference in response to CPT, similar to ED10.5 cells, with female kidney cells dying more. Liver and lung cells did not show any significant differences between the sexes (Figure 5).
Camptothecin causes a response specific for sex, organ and developmental time that was different from what we found with ethanol. This suggested that cell response to stress was dependent not only on cell type, but also its sex and the stimulant. Based on CPT’s mechanism of induction of stress (Beretta GL 2013), differences can exist between the sexes in the DNA replication, transcription and or repair mechanisms, suggesting targets for further evaluation.

**Cell response to EtOH and CPT as measured by mitochondrial activity assays is sex dimorphic.**

In the previous sections, we analyzed cell response, by measuring cell viability using trypan blue, as described in the Material and Methods. Here we used the WST-1 assay, which takes advantage of the mitochondrial membrane potentials, to measure cell viability. As cells destabilize as a result of stress induction, the membrane potential of the mitochondria becomes compromised, allowing several molecules in and out of the mitochondria (Ngamwongsatit, Banada et al. 2008). The WST-1 product (formazan) is metabolized in the mitochondria, changing in color, which is measurable at 450 nm. Higher conversion of formazan in the mitochondria indicates higher activity and viability (Ngamwongsatit, Banada et al. 2008). We further confirmed our results using the more widely accepted MTT assay, which measures formazan reduction, eliciting a measurable color change that can be read at 540 nm (Meerloo, Kaspers et al. 2011). For MTT as well, higher formazan conversion indicates better mitochondrial integrity and impermeability (Meerloo, Kaspers et al. 2011). These techniques were used to confirm the previously reported trypan blue data, and are widely accepted cell viability assays (Ngamwongsatit, Banada et al. 2008, Meerloo, Kaspers et al. 2011).
Cells exposed to either EtOH or CPT were incubated with the WST-1 mixture for the last hour of the experiment, as described in Materials and Methods. The samples were then read at 450 nm and compared for cell viability. These values represent 5 replicates per reading, with at least 3 independent experiments, resulting in 15 or more replications for each condition that have been normalized to background optical density. The error bars represent standard deviations, and asterisks represent p <0.05. Experiments were limited to ED10.5 whole embryo cells and ED17.5 kidney cells, which consistently displayed sex differences in viability, in response to CDIs.

For ED10.5 cells exposed to EtOH or CPT, male cells had higher formazan conversion than female cells, suggesting healthier or more active mitochondria as measured by the Wst-1 assay (Figure 6). For ED17.5 kidney cells, males have higher formazan conversion in response to EtOH, and though high variability undermines statistical significance for CPT treated ED 17.5 cells, they may also differ (Figure 6). This data was consistent with cell death (Figs. 3 and 5).

Using the MTT assay with ED10.5 cells, we found that in comparison to the sex indifferent controls, ethanol and camptothecin lowered formazan reduction in both sexes, but more in cells from females, suggesting that cells from females responded more to CDIs when compared to the male counterparts (Figure 7).

Similarly, for ED17.5, female kidney cells were more sensitive to EtOH, while CPT did not elicit a sex dimorphic response in ED 17.5 (Figure 7), confirming our previously reported dimorphic ED10.5 and sex indifferent ED17.5 kidney cell viability in response to CPT.
The reduction in formazan conversion seen in these experiments is consistent with the cell death results reported previously (Figs. 3 & 5). Since EtOH in ED10.5 and kidney ED17.5 cells gave us consistent results and since ED10.5 cells are devoid of endogenous sex hormones, we decided to focus on ED10.5 for the remainder of our experiments.

We confirmed cell death measurement of cell response, by two other viability assays. The confirmation of differences between the sexes in response of cells prompted us to evaluate differential gene expression between the sexes, to further investigate the underlying regulator of sex-differential behavior of cells.

Having established that stress induces sex differences in cell behavior, we asked whether these differences are reflected by differential gene expression.

Male and Female Cells Have Different Gene Expression Profiles

**Gene array shows sex dimorphic gene expression.**

Here, whole ED10.5 embryos, as well as ED17.5 kidney, cerebrum, and cerebellum were used, as these tissues have been described to be sex dimorphic (Schwarz and Bilbo, Dewing, Shi et al. 2003, Berchtold, Cribbs et al. 2008, Forger 2009, McCarthy, Auger et al. 2009, Bourke, Harrell et al. 2012). These tissues were dissected and pulverized for RNA extraction, as indicated in Materials and Methods. This RNA was then hybridized with gene-array chips containing 7,000 genes (Peñaloza et al. 2009). We found that the samples profiled according to their sex, whereby male samples grouped with male samples and female samples grouped with female samples,
independent of the organ. Each sphere represented an individual embryo assessed; the sexes created their own plane, and did not cross planes (Peñaloza et al. 2009 Figure 4A).

ED10.5 embryos were tested further with a second gene-array chip with 15,000 genes for a total of 22,000 assayed genes. Of these, conservatively 51 genes were significantly dimorphic, of which 50 were autosomal, with X–ist being the only dimorphically expressed gene in a sex chromosome, namely the X-Chromosome (Peñaloza et al. 2009 Table 1).

We isolated the organs for these arrays, while the actual gene arrays were performed by our collaborators from the National Research Council of Canada.

Establishing sex differences in gene expression using gene arrays provided us with a substantial amount of information that required further testing to confirm, using more sensitive techniques, as will be discussed in the next section.

Our collaborators performing the gene arrays verified various differentially expressed genes, including X-ist, validating the gene arrays (Data not shown). In the next section, we look at some of these genes, as well as others, further confirming and validating these results.

**Differences in gene expression profiles exist in our model.**

We verified some of the genes originally reported to be sex dimorphic by the gene array using qRT-PCR and found that the genes were not only sex dimorphic, they were dimorphic at much greater levels than the gene array reported.

Results were calculated as intensity relative to female expression. As the PCR cycles continued to incorporate modified nucleotides to each amplified product, the intensity of the fluorescence was compared between the sexes, with females retained as the baseline for comparison. The last
cycle was retained as baseline for comparison with “absent” genes. The data was plotted as cycle threshold (CT) values in which the CT value represents the cycle at which fluorescence is first detected and our gene expression data is reported in CT averages. By this representation, a lower cycle number indicates a higher initial concentration of mRNA and each decrease of one cycle indicates a doubling of the initial concentration.

We used X-ist as our positive control for sex differences, as females are known to transcribe X-ist at much higher levels than males, leading the X-chromosome inactivation; and Gapdh, considered a housekeeping gene for its role in glycolysis, was used as our negative control,. For the experiment we examined the in vivo expression of various members of the Cytochrome P450 family.

Members of the Cytochrome P450 family have been of interest to us, as we showed Cyp7b1 to be sex dimorphic (Penaloza, Estevez et al. 2009), and other groups have also described other Cyp450’s to be sexually dimorphic as described in the introduction (French, Morimoto et al. 1997, Skaanild and Friis 1999, Bailey and Cunningham 2002, Coon 2005). Furthermore, Cyp2e1, shown to have functions redundant to that of alcohol dehydrogenase (French, Morimoto et al. 1997, Nolen-Hoeksema 2004, Lu and Cederbaum 2008), and Cyp1a1, known for its xenobiotic and drug metabolizing roles (Mollerup, Ryberg et al. 1999, Salnikova, Belopolskaya et al. 2013), can help explain the sex differences observed during stress response.

The gene expression profiles for ED10.5 whole embryo homogenates revealed that X-ist, Cyp1a1, Cyp7b1, and Cyp2e1 are sex dimorphic, with females expressing higher levels (Figure 8). Gapdh showed minimal to no sex differences in gene expression, thus verifying our
We further looked at the gene expression profiles of samples derived from ED17.5, PN17 Day and adult kidneys and lungs. For X-ist, independent of tissue and developmental stage, expression was significantly higher in females when compared to their male counterparts (Figure 9), as expected.

Similarly, Cyp1a1 manifested differences in expression between the sexes with little variation between organs and developmental age. Female Cyp1a1 expression was higher in all samples and developmental timings (Figure 10), with the greatest difference in adult kidney with 256 fold more Cyp1a1 in females.

Cyp2e1 on the other hand was not dimorphic in all assessed samples. Female ED17.5 and adult kidney homogenates expressed higher Cyp2e1 (Figure 11); while PN17 day tissues did not express any differences. Female ED17.5 lung cells expressed higher levels of Cyp2e1 than their male counterparts, while no differences are seen for PN 17 and adult lung (Figure 11).

Similarly, females express more Cyp7b1 than males for kidney tissues at ED17.5 (Figure 12); however, for kidney tissues at PN17, and for adult kidney, there is little to no difference in the expression of Cyp7b1. Cyp7b1 is expressed higher in female ED17.5 and PN17 day lung tissue (Figure 12). Adult lung expressed Cyp7b1 similarly between the sexes.

Finally, Gapdh was highly expressed in both sexes, with female lung PN17 tissues expressing more Gapdh than males (Figure 13). While in most cases expression of Gapdh was independent of sex, it is not 100% sex-independent, and thus, it is important to note that we use it as a control but do not normalize to it.
Taken together, our findings indicate that ED10.5 whole embryos and ED17.5 organs demonstrate *in vivo* sex dimorphic expression of X-ist, Cyp1a1, Cyp2e1 and Cyp7b1. It is important to note that, while differences in gene expression were identified, these differences in many instances and without a clear pattern were not seen across the board. Establishing sex differences in gene expression using qRT-PCR provided us with substantial information, however, it became important to test whether the differences observed *in vivo* are maintained in our *in vitro* system. If differences are maintained in cell cultures, it will allow us to better manipulate our system to characterize factors involved in the dimorphisms.

**Dimorphic gene expression is maintained in vitro.**

We evaluated whether the dimorphic gene expression is sustained in our *in vitro* cell culture system. Cells were isolated from ED10.5 whole embryo and ED17.5 and day 17 kidney cells, cultured to confluence, lysed for RNA, as described in Material and Methods. This RNA was then assessed for *in vitro* expression of X-ist, Cyp1a1, Cyp7b1, Cyp2e1 and Gapdh genes.

In ED10.5 cells, females expressed higher levels of X-ist, Cyp1a1, Cyp7b1, and Cyp2e1 as shown in **Figure 14**. These differences reflected the *in vivo* model previously described, demonstrating consistency in the two systems and validating our *in vitro* system.

For ED 17.5 kidney cells, the differences in expression of X-ist, Cyp1a1, and Cyp2e1 were maintained wherein cells from females expressed X-ist, Cyp1a1, and Cyp2e1 6 to 125 fold higher than cells from males. However for Cyp7b1 and Gapdh, there were no differences in expression between the sexes (**Figure 15**).
In cultures from PN 17 kidney, females expressed significantly more (5-355 fold) X-ist, Cyp1a1, Cyp2e1, and Cyp7b1, with no differences in the expression of Gapdh (Figure 16).

Thus, with the possible exception of Cyp7b1, our cell culture system closely reflects in vivo expression, confirming sex dimorphic gene expression profiles in vitro. We can therefore use cell culture to explore the origin of the sex differences.

Now that we have validated that our cell culture system maintains identity found in vivo, we are now in a position to look at sex differences in our cell cultures.

**Gene expression profiles are affected by EtOH exposure in a sex-specific manner.**

Having demonstrated consistency of differential gene expression between the in vitro system compared to that found in vivo, we moved to examine the alteration, if any, in gene expression after stress as a function of the sex of the cell. Here, ED10.5 cells were treated with 400 μM EtOH for 24 hours and were then homogenized for RNA extraction, as described in Material and Methods.

Cells exposed to EtOH not only have sex different viability as shown previously (Figure 3), but also have altered gene expression profiles for X-ist, Cyp1a1, Cyp2e1, and Cyp7b1 (Figure 17). Ethanol exposure increased the dimorphism of X-ist and Cyp2e1, by increasing female expression, while not altering male expression (Figure 17). On the other hand, ethanol exposure reduced the differences for Cyp1a1 and Cyp7b1, by increasing male expression only, while leaving female expression unchanged (Figure 17).

These results suggested that male and female differences exist prior to onset of endogenous hormone production and that these differences were maintained in our cell culture model and
influenced by the addition of cell death inducer (EtOH). Since, in the absence of endogenous sex hormones, differences exist, and depending on development these differences vary, we tested whether exogenous hormones influence this response, thus testing by an additional method the role of hormones on sex differences.

**Exogenous 17β-estradiol further modulates sex dimorphic cell sensitivity.**

In the previous section we looked at sex differences in cell sensitivity as well as gene expression profiles, in a hormonally controlled system, where we are able to account for endogenous exposure to hormones using early and late embryo as well as early and late postnatal development. Here we assess the effect of exogenously supplemented estrogen on male and female cell survival. ED10.5 mixed cell cultures were exposed to 17β-estradiol with and without ethanol supplementation. Cells were treated and cell death was measured by trypan blue exclusion (as described in Materials and Methods). Embryonic day 10.5 cells were used, since as previously described embryos at this stage do not as yet produce endogenous hormones.

To assess the optimal treatment concentration of 17β-estradiol, male and female ED 10.5 mixed cell cultures were exposed to various concentrations of 17β-estradiol for 24hrs. Exposure of cells to 17β-estradiol for 24 hrs at 5 nM to 100 nM did not have a major impact in cell viability (**Data not shown, as no change from basal was seen in cell viability**). Ethanol at 400 μM killed 20% of cells from males and 39% from females (**Figure 18A**). However the combination of EtOH and 17β-estradiol (E2) protected both male and female cells, abolishing the sex differences with 2% and 1% cell death in males and females respectively (**Figure 18A**). This reduction in cell death originally caused by EtOH can be as a result of delayed response, cell proliferation or a combination of these. Cell proliferation is not significantly increased, as measured by cell
recovery. Male and female control vs. 17β-estradiol treated cell recovery is the same as measured by trypan blue (Figure 18B).

We confirmed these results with the two other viability assays discussed previously (Ngamwongsatit, Banada et al. 2008, Meerloo, Kaspers et al. 2011), namely the MTT and Wst-1 assays. Both assays confirmed our trypan blue result in that addition of 17β-estradiol abolishes cell death induced by EtOH (Figures 19A-19B).

Having established that exogenous 17β-estradiol can superimpose an effect on an already sex dimorphic system, we sought to further characterize the effect by looking at gene expression and determining whether hormonal influences impact the expression profiles of the Cytochrome p450 family of genes.

**Hormones Affect Male and Female Gene Expression Profiles**

We previously showed that male and female gene expression profiles are different in vitro, and furthermore that cell stress can influence the gene expression profiles differently for various members of the cytochrome P450 family.

Here we report the effect that exogenous estrogen has on gene expression profiles of cells with different sex.

Male and female ED10.5 mixed cells were exposed to 5 nM 17β-estradiol for 24 hrs; RNA was extracted and assessed by PCR for level of gene expression, as described in Material and Methods. We examined Cyp450 members 1a1, 2e1 and 7b1 as well as X-ist and Gadph, the latter two examined as our controls. For all the genes examined 17β-estradiol alone increased gene expression and abolished the normal differential expression between the sexes.
Exposure to 17β-estradiol alone resulted in a marked reduction of sex differences in our positive control gene, X-ist (Figure 20). In the absence of exogenously added 17β-estradiol, females expressed 16 fold more X-ist RNA than their male counterparts. After exposure to 17β-estradiol male and female cells lose their differences in expression, resulting from an increase in the expression of both male and female cells, but more so in male cells (Figure 20).

Similarly, for cytochrome P450 family member Cyp1a1 and Cyp2e1, in the absence of exogenous 17β-estradiol, female cells express 128 and 13 fold more Cyp1a1 and Cyp2e1 respectively, than their male counterparts. 17β-estradiol exposure resulted in a substantial reduction in sex differences, while the cells maintained a 4-fold sexual dimorphism for Cyp1a1 and 2-fold for Cyp2e1 (Figure 20); as a result of increased gene expression in both sexes.

Cyp7b1 prior to 17β-estradiol supplementation was sex dimorphic, with female cells expressing 16 fold more Cyp7b1 than their male counterparts. 17β-estradiol increased expression in male cells only, while no discernible effect was detected for female cells. This resulted in a loss of significant differences between the sexes, in the amount of Cyp7b1, now with a slightly male predominant expression (Figure 20).

Finally, for our loading control, we found no differences in its expression before and after 17β-estradiol supplementation (Figure 20).

These results suggest that while differences exist prior to gonad development and onset of production of hormones, 17β-estradiol changes the gene expression profile, such that in most instances differences in gene expression are lost or reduced. X-ist, Cyp1a1, Cyp2e1 and Cyp7b1 are directly or indirectly responsive to estrogen, as expression increased in response to estrogen
supplementation in at least one sex. Cyp7b1 is estrogen responsive in only one sex suggesting that another level of gene expression regulation may be at play, at the very least for ED10.5 whole embryo cell cultures.

Response to 17β-estradiol suggests that these genes either have an estrogen response element (ERE) on their promoter region, described in Figure 11 (See Introduction); or that alternatively, these genes are regulated by a transcription factor that is itself regulated by an ERE described in Figure 13 (see Introduction). EREs are not always activators of gene expression; they can at times act as repressors. For these reasons, in the next section we decided to look at modulation of gene expression and regulation.
**Discussion:**


To study the factors in play we established a cell culture system, where we can control for different variables, while attempting to characterize the underlying causes resulting in sex dimorphisms at the cellular level (Nikezic-Ardolic, Lin et al. 1999, Penaloza, Estevez et al. 2009, Penaloza, Estevez et al. 2013).

We have demonstrated that male and female mouse cells, derived from different tissues and developmental stages, behave in a sex-dependent manner when induced to die and that this dimorphism does not derive from the embryo's hormones. We see these differences in cells from
10.5 day embryos, where there is no sexual development. Male and female cells derived from whole embryo, kidney, lung, and liver do not differ by sex in either their growth in culture or basal frequency of cell death. Thus we can discriminate between differential stress response caused by the CDI and that caused by common culture conditions. When we expose these cells to stressors such as EtOH and camptothecin, we identify male and female specific responses, with the greatest sex differences existing in cells from whole ED10.5 embryos, ED17.5 kidneys, and postnatal day 17 kidneys; both stressors gave consistent sex dimorphic response.

These initial experiments were designed to strengthen our model system, so that we could then alter and test different variables, to elucidate causes of sex differences at the cell level. Since we were able to develop a model where we can control for most conditions, including hormonal influences, we were able to go one step further and ask: do male and female cells have differential gene expression profiles? We found that male and female organs and cells express many genes differentially by sex, as determined by gene array analysis as well as quantitative real time PCR analysis.

We further established that sex differences in gene expression vary depending on the conditions of cell culture. Male and female expression of three members of the cytochrome P450 family, as well as X-ist, known to be sex dimorphic, under cell stress shifted expression one of four ways:

- Increase sex differences in gene expression;
- Reduce sex differences in gene expression;
- Eliminate sex differences in gene expression;
- Undergo no detectable change in gene expression profile.
Male and female cells exposed to 17β-estradiol vary in cell viability and gene expression profiles, but this variability is superimposed on already existing differences. More specifically, estradiol protects cells so that initial differences in EtOH induced stress response as measured by trypan blue assays are altered in both male and female cells. This change was mirrored by changes in the gene expression patterns, where initially different expression profiles in X-ist, Cyp1a1, Cyp2e1, and Cyp7b1 are dramatically reduced, as a result of increase in transcription. While this effect was pronounced for the Cytochrome P450 members assessed, the profiles were not unidirectional, suggesting an underlying regulatory mechanism at play.

These differences suggest that factors acting upstream of gene expression regulate the gene profiles, and that these upstream factors can affect the behavior of the cells. Factors that can contribute to dimorphic regulation of gene expression include regulation of transcription factors, as in the case of response elements and hormones, as well as other genetic control mechanisms like DNA methylation and histone modifications. Differences in any of these mechanisms can result in the observed sex differences, and these are the areas addressed in the subsequent section of this thesis.
Effect of DNA methylation on dimorphic gene expression

Synopsis:
As suggested in the introduction and previous section, sex differences in manifestation of disease are attributed to sex hormones. While sex hormones certainly account for some of the observed differences, non-hormonal factors, such as differential gene regulation, remain major role players. We have shown that cells have sex and that in the absence of sex hormones cells behave in a sex dependent manner when stressed.

The differential sensitivity to ethanol and camptothecin exists prior to the appearance of sex-specific hormones. When cell cultures are supplemented with 17β-estradiol, cell sensitivity is affected, but sex differences persist, and the effects are also sex specific.

Thus, sex hormones do not account for all the sex-based differences in stress response and cell survival. Such differences could create or arise from differential gene activity.

In this section, we asked: what other factors can influence gene expression differently between the sexes? And allude to how these differences can have consequences resulting in differences in cell behavior.

To understand the underlying elements leading to sex differences in cell behavior, we asked if the state of methylation found in the cells plays a role.

As explained in the introduction and can be noted in I4 (see introduction), differential methylation can result in repressed or delayed transcription, based on accessibility of the
transcriptional machinery to DNA. These differences can affect gene expression, potentially explaining the differences observed in the previous section.

To address this question, we looked at members of the cytochrome P450 superfamily, a family responsible for metabolizing drugs and hormones that display sex-dependent gene regulatory patterns in mouse. We mapped the methylation of genes known to be sex-dimorphic and tested whether inhibiting that methylation would eliminate the dimorphism.

We found that the sex-dimorphic stress-induced genes Cyp1a1, Cyp2e1 and Cyp7b1, are differentially methylated. Reducing the differences in methylation decreases or eliminates the sex dimorphism in gene expression, while factors such as ethanol and estradiol, which are known to decrease the sex differential, also alter methylation patterns. Thus it is likely that differential methylation is at the root of endogenous sex differences; therefore, the origin of the differential methylation and its impact on disease are targets for further study.

**Promoter regions of X-ist, Cyp1a1, Cyp2e1 and Cyp7b1 contain several response elements and CpG islands**

The male/female difference in expression, presented in the previous section, suggests sex specific targets of transcriptional regulation, such as those typically found in the promoter regions (I1, I3 in introduction). Therefore we searched for consensus sequences in the genome of X-ist and several members of the CYP450 family. We found CREB, ERE, GRE and ARE response elements in the promoter region of Cyp1a1, Cyp2e1, Cyp3A4, Cyp4A11, Cyp5A1, Cyp7b1 and Cyp8A1 (Table 1) and selected these genes for further study. Cyp1a1, -5A1 and -7b1 contain estrogen response elements (ERE); Cyp1a1, -3A4, -5A1 and -8A1 contain Glucocorticoid Response Elements (GRE); none contain cAMP response elements (CREB); one
contained Androgen Response Element (ARE) but five of the seven assessed sequences possess CpG islands greater than 200bp, potential sites of methylation (Table 1).

Since the Cyp450 genes show sex-specific regulation and consistently possess large CpG islands, we evaluated methylation as a potential regulator. Dnmt31, a DNA methyltransferase gene, is expressed dimorphically, and X-ist is subject to epigenetic regulation (McMahon, Fosten et al. 1981, Chitnis, Monteiro et al. 2000, Huynh and Lee 2005, Okamoto, Arnaud et al. 2005, Dobbs, Rodriguez et al. 2013). We therefore asked whether DNA methylation explained the dimorphic expression of X-ist. We identified more than 3 CpG islands spanning the promoter region of X-ist that were greater than 100 nucleotides (Table 2). We focused on the region spanning from -2393 to -2011 on the promoter region of X-ist, which contained the largest GC rich region, as well as several promoter regions from Cyp genes.

We selected single CpG islands spanning more than 200 bases. We identified positions -873 to -566 for Cyp1a1, -1231 to -982 for Cyp2e1 and -667 to -402 for Cyp7b1 representing CpG islands of 307, 249 and 265 bases respectively (Table 2). The presence of CpG islands suggested regulation of methylation.

**Expression of Dnmt3l, a subunit of methyltransferase, is sex dimorphic**

In the previous section we described sex differences in gene expression, using homogenates from ED10.5 embryos, whole kidney and lung from ED 17.5, PN 17, and adult mice (Figures 8-13). We also showed that these differences persisted in vitro in cultures derived from these organs (Figures 14-16). We also showed that Dnmt3l (cytosine-5)-methyltransferase 3-like, known to interact with HDAC1 (histone deacetylase 1) was sex dimorphic in our system (Peñaloza 2009,
Table 1. Here we show that in vivo expression of Dnmt3l is 4-97 fold greater in males (Figure 21). Furthermore, in our cell culture system, the differences persist albeit at lower levels (12-32 fold) (Figure 22).

Differential expression of Dnmt3l in vivo and in vitro, as well as sex specific gene expression regulation for several genes, prompted us to further evaluate DNA methylation as a potential target for sex dimorphic gene regulation. In the next section, we evaluate further the viability of this idea.

Methylation of promoters of X-ist, Cyp1a1, Cyp2e1, and Cyp7b1 are dimorphic.

Since we identified CpG islands within the promoter region of these genes as well as differences in the expression of Dnmt3l, we tested whether the DNA methylation of these genes is sex specific.

We used sodium bisulfate, which converts all unmethylated cytosines to uracils, allowing us to identify site-specific methylation. Subsequently, we purified the treated DNA and amplified the region spanning the CpG island on the promoter of X-ist, Cyp1a1, Cyp2e1, and Cyp7b1. The amplicons were again purified, repurified, and submitted for sequencing (Eurofins MWG Operon, Huntsville, Alabama 35805, US). In Figure 23, the diagrams presented are the parental DNA sequences, with notations on variations between the sexes. Solid circles indicate methylated sites, and shaded circles indicate unmethylated sites. 5 individual rounds of amplification and sequencing are averaged into the representation, with each site resulting from at least 4 of 5 replicates. We describe below the methylation status of these specific promoter regions as seen in Figure 23:
X-ist: There were five sites of methylation in males and three in females, one common to both sexes. Two were unique to females and four to males. This difference has not been previously reported.

Cyp1a1: There were three sites of methylation in males, and four in females; of these, only one was common. Three were unique for females and two were unique for males.

Cyp2e1: Males and females each had five methylated sites. Of these, three were common. Two sites were female specific and two sites were specific to males.

Cyp7b1: Males had four methylation sites and females two, of which one is common. One site was unique to females and three to males.

Dnmt3l and Gapdh were not analyzed, as these sequences do not possess detectable CpG islands.

Thus, the promoters from differentially expressed genes are methylated differently. These differences suggest an explanation for sex dimorphic gene expression, though regulation of Dnmt3l must be by other mechanisms. We next tested this hypothesis by inhibiting methylation.

Inhibition of DNA methylation reduces sex differences in methylation of X-ist, and Cyp450 family members and gene expression of X-ist, CYP450 family members, and Dnmt3l.

To examine the relationship between expression and methylation, we blocked the de novo transfer of methyl groups, using 20 μM 5-aza-2'-deoxycytidine (5-Aza-dC), which prevents the faithful copy of methylated sites to the daughter strand from the parental strand. This inhibition was maintained for 5 rounds of replication as measured by population doublings, which should reduce the methylated strands to 3% of the cell population. The 5-Aza-dC was not toxic and, in fact, the cells did better and delayed or evaded senescence when exposed to chronic 5-Aza-dC
Inhibition of methylation reduces differential methylation of Cyp P450 members, and eliminates or reduces sex differences, as described below and illustrated in **Figure 25**:

**X-ist:** Inhibition of DNA methylation almost eliminates the sex differences in both methylation and expression of X-ist. Blocking *de novo* DNA methylation resulted in a partial loss of DNA methylation in the promoter region of male and female X-ist. Males lose three sites of methylation (open circles) but maintain two sites. Females lose one site of methylation but maintain two. 5-Aza-dC does not block all *de novo* methylation. However, the reduction of DNA methylation resulted in fewer differences in DNA methylation between the sexes, from six in control conditions to two in 5-Aza-dC treated cells, with one male-specific and one female-specific site remaining. The expression of X-ist was increased by reduction of methylation in both sexes, but more in males so that the ratio dropped from 8:1 (female to male ratio) to near unity in cells exposed to 5-Aza-dC

**Cyp1a1:** The promoter for Cyp1a1 contains two male-specific sites of methylation, while in females there are three, and one of which is common. When methylation is inhibited, two sites survive in males and females, of which one is unique to each sex (**Figure 25**). This reduction of differences in DNA methylation sites (5 to 2) resulted in a drop from 23 fold to 6 fold difference in the expression of Cyp1a1 (**Figure 26**).

**Cyp2e1:** The promoter for Cyp2e1 has five methylated sites in males and five in females. Of these, two are unique to males and two to females. When methylation is blocked, the number of methylated sites is reduced to three in males and four in females, with only one unique site in females (**Figure 25**). This reduction of methylation differences resulted in a reduction in differential Cyp2e1 expression from 8 fold to 2 fold (**Figure 26**).
**Cyp7b1:** The promoter for Cyp7b1 has four sites methylated in males and two in females; three of the male sites are unique, with only one being common. After methylation is blocked, only one site, the male specific site, persists in males and none in females (Figure 25). Similar to the other CYP members, loss of DNA methylation sites results in a reduction of sex differences in Cyp7b1 expression, dropping from 16 to 6 fold (Figure 26).

**Dnmt3l:** The promoter for Dnmt3l did not possess a CpG island, and therefore differences in methylation status were not identified; gene expression varied minimally as a result of 5-Aza-dC exposure, indicating that it is not regulated by DNA methylation (Figure 26). Possible explanations for changes to Dnmt3l expression levels are discussed in this sections discussion.

**Gapdh:** Methylation status for Gapdh was not analyzed, as Gapdh did not have any long CpG islands. Expression of Gapdh (negative control) did not vary significantly as a result of loss of de novo DNA methylation (Figure 26).

In all cases, blocking methylation reduced both the number of sites methylated as well as the number of sex-unique sites. Expression of X-ist, Cyp1a1, Cyp2e1, and Cyp7b1 increased significantly more in males than females. These results suggest that methylation patterns establish the transcriptional regulation of Cyp1a1, Cyp2e1 and Cyp7b1 (Figure 26). Normally these patterns—presumptively imprinted—likely generate the sex-differential expression. Blocking methylation destroys or reduces these differences and results in the loss of sex differences in expression for these genes (Figures 25-26).
Ethanol-induced stress produces differences in gene expression for X-ist, Dnmt3l and Cyp450 family members, and methylation differences for X-ist, and Cyp450 family members.

When male and female cells are exposed to stressors such as ethanol or camptothecin, female cells are more sensitive to the stress and the sex dependence of gene expression likewise responds to stress (Penaloza, Estevez et al. 2009). Gene expression in response to stress is not unidirectional; stress can induce expression in one sex, and suppress it in the other sex (Penaloza, Estevez et al. 2009). To examine the relationship between differential DNA methylation and expression in response to stress, we exposed cells to 400 μM ethanol (EtOH), representing LD₅₀. The cells were maintained for 24 h and assessed for gene expression and DNA methylation. After 24 h exposure to EtOH, gene expression and DNA methylation differences were (as illustrated in Figures 27 and 28):

**X-ist:** In the short promoter region assessed for X-ist, females lost two sites (Figure 27, open circles). These changes resulted in six sites differing, five unique male sites and one unique female site, compared to six differences in controls. These changes to the sites of methylation resulted in an increase of sex differences in X-ist expression from 16 fold to 32 fold, with females having higher expression after exposure to ethanol (Figure 28). Although the difference between sexes remains significant, the doubling of the difference is not a significant change.

**Cyp1a1:** One site is lost in males (open circle) and two sites in females, leaving two sites in each sex, all unique. This reduction of differences in DNA methylation, from five to four unique sites, resulted in a marked reduction of sex differences in the expression of Cyp1a1, from 128 to 3 fold after ethanol exposure.
**Cyp2e1:** The number of methylated sites is reduced to three in males by loss of two sites (open circle) and increased to eight in females by gain of three sites (filled circle), resulting in nine unique sites and one common. This increase of methylation differences, in response to ethanol exposure, results in an increase from 13 fold to 32 fold differences between the sexes in expression of Cyp2e1.

**Cyp7b1:** Only two male-specific sites persist by loss of two sites in males (open circle); females lost two sites and retained another site, resulting in two male specific sites. As above, loss of DNA methylation sites results in a reduction of sex differences in Cyp7b1 dropping from 16 to 4 fold after ethanol exposure.

**Dnmt3l:** Expression of Dnmt3l varied in response to ethanol, since Dnmt3l did not have a measurable CpG island, it suggests that a regulator of Dnmt3l is itself regulation by DNA methylation.

Exposure of cells to ethanol, a form of stress, alters methylation and results in both a reduction of sex differences in methylation and in expression of Cyp1a1 and Cyp7b1 genes, whereas for Cyp2e1, an increase in difference in methylation sites is reflected in an increased difference between the sexes. In the case of Dnmt3l and Gapdh, we found no measurable CpG island, and therefore presumptively these are not under the regulation of DNA methylation. Variations in the expression levels of these genes suggest that they may be under the regulation of a transcription factor that is itself regulated by methylation.
Exposure of cells to 17β-estradiol results in differences in gene expression and methylation for X-ist and Cyp450 family members.

Similar to ethanol, 17β-estradiol modulates sex differences in gene expression. When male and female cells are exposed to estradiol cell survival is sex dimorphic, and gene expression is sex dependent. 17β-estradiol can induce genes in one sex and suppress the genes in the other sex (Penaloza, Estevez et al. 2009). To examine the relationship between differential DNA methylation and expression in response to sex hormones, we exposed ED10.5 cells to 5 nM 17β-estradiol and maintained them for a further 24 h. DNA methylation profiles (Figure 29), and gene expression (Figure 30) were assessed, with the following results:

**X-ist:** Compared to control after exposure to 17β-estradiol, the short promoter region lost four sites of methylation (open circle) and gained (filled circle) one site in males, whereas females lost one site. These changes resulted in four sex-unique sites, two male and two female. These changes to the sites of methylation, from six to four unique sites, resulted in a reduction of differences in X-ist expression from 16 fold higher in female to 1 fold

**Cyp1a1:** In comparison to control, one site survives in each sex, as a result of a loss of two male and three female sites (open circle). This reduction from five to two unique sites results in a reduction from 128 to 4 fold differences in the expression of Cyp1a1.

**Cyp2e1:** The number of methylated sites is reduced from five to three in males and four in females, with only one unique, as a result of two male and one female site losses (open circle). This reduction of differences in methylation results in a reduction from 13 fold to 2 fold higher in females for Cyp2e1.
Cyp7b1: The promoter for Cyp7b1 has four sites methylated in males and two in females; three of the male sites are unique, and one is common. After exposure to estradiol, only one male site persists and three in females, resulting from three sites lost in males (open circle) and a site gained in females (filled circle). Similar to the other CYP members, restructuring of DNA methylation sites as a result of estrogen results in a reduction of sex differences in Cyp7b1 between the sexes, dropping from 16 fold higher in females to 4 fold higher in males after blockage of DNA methylation induced by estrogen supplementation.

Dnmt3l: the promoter for Dnmt3l does not possess a large CpG island; therefore we did not evaluate methylation status. However, estrogen exposure of these cells resulted in a loss of initial differences in Dnmt3l levels between the sexes. This loss of differences from 8 to 1 fold, resulted from a marketed increase in the expression of Dnmt3l in females; suggesting an estrogen responsiveness in only females. Since other assessed genes had restructured DNA methylation patterns, it is possible that a regulator of Dnmt3l is differentially regulated by estrogen, in relation to its DNA methylation patterns.

Exposure of cells to 17β-estradiol alters methylation status and results in reduction of sex differences in both methylation and gene expression. These results suggest that DNA methylation selectively regulates autosomal genes such as CYP in a sex-dependent manner, which can in part explain the many instances where gene expression is sexually skewed, presumptively resulting in differences at the physiological level. In the case of Dnmt3l and Gapdh, lacking known CpG islands, any changes in their expression are likely a result of modifications on another gene in trans, influencing their expression indirectly. Such differences
could persist throughout life and are relevant to evaluation of normal physiology and pathology in men and women.
**Discussion:**

We have previously shown that cells differing only in chromosomal sex respond differently to various stressors. Here we address the mechanism of this dimorphism.

A previously unrecognized sex-differential gene regulation pattern persists in isolated primary cells. Male and female cells from similar organs have sex-distinct patterns of DNA methylation, with the magnitude of the difference somewhat proportional to the differential in expression. This conclusion is valid for cytochrome P450 family members 1a1, 2e1 and 7b1, as well as X-ist but not Dnmt3l, which shows differential expression but no known difference in methylation; nor for Gapdh, which shows no difference in methylation and almost no sex differential in expression. Inhibition of DNA methylation or exposing cells to ethanol or 17β-estradiol (both of which reduce differences in methylation) reduces or eliminates the differential expression. Thus reduction or elimination of sex differences in gene expression can largely be attributed to a loss of differences in DNA methylation.

The most consistent regulatory element in the promoters of Cyp P450 family members and X-ist was CpG islands, which were present in most sex dimorphic Cyp P450 family members. Of these, cytochromes 1a1, 2e1 and 7b1 are expressed in a sexually dimorphic manner and have CpG islands of at least 200bp. We previously reported that Dnmt3l was sex dimorphic in the absence of stress (Penaloza, Estevez et al. 2009).

Sex differences in the expression of the Cyp P450 genes in vivo exist regardless of tissue type or developmental time. These differences in gene expression persist or are enhanced in culture. Likewise, under normal conditions, the inherited DNA methylation patterns for cytochrome P450 family members 1a1, 2e1 and 7b1 are sex dimorphic. We therefore first attempted to disrupt this pattern using 5-Aza-dC, which was not toxic, even though both sexes lost methylation sites, resulting in fewer differences in DNA methylation. Thus we would expect reduced sex dimorphism in expression.

5-Aza-dC increased all gene expression and reduced or eliminated sex differences, even with X-ist. Ethanol changed methylation patterns within 24 hours, modestly impacting gene expression, most characteristically increasing male expression to sharply decrease sex differential (CYP 1a1 and 7b1). Prolonged exposure to ethanol was lethal, which prevented us from assessing the long-term effect of ethanol on DNA methylation. However, 24 hour exposure to ethanol elicited differential gene expression and modified the methylation profile of dimorphically expressed genes. Likewise, estradiol modulated DNA methylation, influencing the expression profiles of these genes.

The female sex hormone 17-β estradiol had markedly different effects on male and female cells. When ED 10.5 cells were cultured for 3 days without exogenous estradiol and the last 24 hours
in the presence of the hormone, both male and female methylation patterns were changed. Typically, expression was increased in both sexes, but substantially more in males, thus reducing sex-based differences.

Thus, all our experiments lead us to the conclusion that differences in patterns of methylation can at least partially explain the sex-based differences in expression of CYP family members and X-ist. Whether we block methylation by 5-Aza-dC, stress cells with ethanol, or expose them to a female sex hormone, changes in methylation differences coincide with changes in expression and difference in expression, approximately proportionate to the number of methylation differences. In the case of our negative control Gapdh, lacking CpG islands, and still varying in expression, this is possibly due to regulation by another factor that is itself differentially regulated by DNA methylation. Similarly for Dnmt3l, its expression varied in response to 17β-estradiol, in a sex dependent manner, presumptively from trans regulation, by a factor or factors regulated by estrogen and or DNA methylation. While much remains to be learned about the origins of these differences and the manner in which additives and other factors affect each site of methylation, the evidence points to the argument that inborn differences between males and females and their different responses to chronic and acute changes in their lives could derive from these differences in methylation. The medical impact of differential methylation will become clearer as we learn more.
Characterization of the mechanisms leading to the sex dimorphic response to CDI ethanol.

Synopsis:
In the metabolism of alcohol, Cyp2e1 becomes activated when alcohol levels are toxic, allowing EtOH to enter microsomes, where it is metabolized into acetaldehyde by Cyp2e1, and generating ROS as a byproduct (French, Morimoto et al. 1997, Lu and Cederbaum 2008, Lu and Cederbaum 2010, Qi, Miao et al. 2013). With this knowledge we can postulate that higher Cyp2e1 in females (Figure 8), results in higher ROS generation in females, ultimately accounting for the higher toxicity experienced in females as a result of EtOH exposure (Figures 2, 3, 6 and 7). In this section we test this possibility. We used male and female cells and modulated the ethanol (EtOH) metabolic pathway, by chemically inhibiting an integral enzyme in the ethanol metabolic pathway, aldehyde dehydrogenase (ALDH); blocking DNA methylation to eliminate differences in Cyp2e1 (an ADH-like enzyme) expression; and blocking reactive oxygen species (ROS) using scavengers to establish the role of ROS generation in EtOH induced cell death.

In previous sections, we established a model where we could test sex differences at the cell level and tested various procedures to characterize the dimorphisms. We established that female cells have higher sensitivity to ethanol (Figure 3), and that gene expression profiles are dimorphic prior to and after exposure to ethanol (Figure 17). We noted a sex-specific modulation of gene profile induced by ethanol (Figure 17); and characterized DNA methylation as the potential factor, upstream of gene expression, perhaps causing differences resulting in the dimorphic cell sensitivity (Figures 27 and 28). Here we report that blocking aldehyde dehydrogenase pharmacologically using disulfiram, an inhibitor of aldehyde dehydrogenase (Lipsky, Shen et al. 2001), eliminated the differences observed in cell sensitivity. We had previously reported too
that Cyp2e1 responds to EtOH (Figure 17), and that as described in the introduction, it performs a role redundant to alcohol dehydrogenase, namely to metabolize ethanol to acetaldehyde and in doing so generating ROS, via the non-canonical alcohol metabolizing pathway (French, Morimoto et al. 1997, Lu and Cederbaum 2008). We found a strong correlation between cell sensitivity to ethanol and Cyp2e1 expression. Modulating cells to express equal levels of Cyp2e1 by blocking de novo DNA methylation eliminates the sex dimorphism in cell viability. We also found, in correlation, that blocking aldehyde dehydrogenase using disulfiram resulted in increased Cyp2e1 expression in both males and females, eliminating the previously reported differences.

Cyp2e1 increases cellular ROS generation (Gonzalez and Gelboin 1990, French, Morimoto et al. 1997, Lu and Cederbaum 2010, Qi, Miao et al. 2013). The ROS scavenger N-Acetyl Cysteine reduced EtOH-provoked cell death, reducing the existing sex differences in cell viability, further corroborating the argument that induction of death by EtOH is modulated by ROS.

Here we report a mechanism characterizing how EtOH induces stress acting on the same metabolic pathway in males and females, but, since Cyp2e1 is higher in females, EtOH is more toxic to female cells.
Inhibition of aldehyde dehydrogenase abolishes the sex dimorphic sensitivity to ethanol.

We previously described male and female differences in cellular sensitivity to ethanol. Aldehyde dehydrogenase is one of the enzymes of the ethanol metabolism pathway, as indicated in the introduction. It converts acetaldehyde to Acetyl-CoA (Lipsky, Shen et al. 2001). Using cells from ED 10.5 embryos we blocked aldehyde dehydrogenase using 0.5μM disulfiram (DSF) (as suggested in (Lipsky, Shen et al. 2001)); starting 1 hr prior to and during the exposure to 400 μM EtOH for 4, 12 and 24 hrs. DSF alone is not toxic (Figure 31). At 24 hrs post EtOH exposure, significantly more male and female cells die when compared to control, supplemented with DMSO only (Figure 31) but more importantly the sex dimorphic cellular sensitivity to EtOH is abolished when aldehyde dehydrogenase (ALDH) is inhibited with DSF at 24hrs (Figure 31). The abolishment of sex dimorphic cellular sensitivity is not due to a reduction of cell death in female cells but more so because of an increase in the amount of cell death in the males, suggesting that blocking aldehyde dehydrogenase activity alone was sufficient to eliminate our originally described differences in cell sensitivity. In effect, the differences in ALDH originally present help male cells cope with alcohol induced stress, and that in blocking ALDH using DSF, this preferential protection is eliminated.

Since we had found sex differences in the gene expression profile for several genes, which were further modulated by EtOH (Figure 17), we asked whether other genes in the alcohol metabolism pathway are dimorphically expressed.

In the introduction we discussed two possible paths for the breakdown of EtOH: the canonical pathway where ADH oxidizes alcohol to acetaldehyde; and the non-canonical pathway where cytochrome P450 2e1 performs the same function. Because of this redundancy, it was important
for us to look at the expression of Cyp2e1 in the presence of ethanol exposure, and to evaluate the role of Cyp2e1 in the dimorphism observed. As illustrated in Figures 8, 11 and 17, Cyp2e1 is expressed in a dimorphic manner whereby in all instances females express more. Cyp2e1 becomes activated when alcohol levels are toxic, allowing EtOH to enter microsomes, where it is converted into acetaldehyde generating ROS as a byproduct (French, Morimoto et al. 1997, Adachi and Ishii 2002, Bailey and Cunningham 2002, Lu and Cederbaum 2008). We can postulate that higher Cyp2e1 in females results in higher ROS in females, ultimately accounting for the higher toxicity experienced in females as a result of EtOH.

There are no pre-existing differences in the expression of alcohol dehydrogenases class I-V (Figure 32 A-E). For Class I ADH 1B, Class III ADH 5 and Class V ADH 6, not only are these transcripts sex independent, they are also not responsive to EtOH (Figure 32A, C and E). Class II ADH 4 and Class IV ADH 7 are responsive to EtOH exposure, but there are no differences between the sexes (Figure 32B, D), suggesting that the differences in cell sensitivity are not established by differences alcohol dehydrogenase.

Downstream of the alcohol metabolic pathway, aldehyde dehydrogenases (ALDH) convert acetaldehyde to Acetyl-CoA. We looked at the expression of ALDH representatives of Class I and Class II isoforms. In the case of ALDH 1 and ALDH 2, no differences in transcription are seen, and furthermore these isoforms are not induced by EtOH, or by the inhibition of ALDH using disulfiram (Figure 33A-B).

The lack of differences in ADH or ALDH suggested that the non-canonical pathway induced by EtOH is activated differently between the sexes.
Sex-based differences in non-canonical, alcohol metabolizing cytochrome P450 family member Cyp2e1 provides an explanation for EtOH induced dimorphism in cell viability

In the previous section, we showed that inhibiting the ethanol metabolism pathway reduces sex differences observed in cell survival, but lack of difference in ADH and ALDH suggested that a non-canonical metabolic pathway operates. We also demonstrated that three Cyp450 family members are expressed in a sex specific manner and suggested that these could be important in detoxification. Cyp2e1 is an enzyme that acts redundantly with alcohol dehydrogenase, converting ethanol to acetaldehyde when EtOH levels are toxic, in our case 200 μM, achieving a measurable cell death response over controls (French, Morimoto et al. 1997, Adachi and Ishii 2002, Bailey and Cunningham 2002, Lu and Cederbaum 2008). Even in the absence of EtOH, Cyp2e1 is expressed more in females, and after exposure to EtOH female expression is induced further, increasing the differences between the sexes (Figure 17). We therefore asked whether Cyp2e1 could affect the previously described sex differences in cell viability in response to EtOH.

To address this question, male and female ED10.5 cells were cultured as previously described, and exposed to DSF, followed by EtOH exposure as described in Material and Methods. As shown in Figure 31 disulfiram was toxic only in the presence of EtOH at 24 hrs, at which time male cell death increases, to that seen in females, effectively reducing the described sex differences.

As previously shown, female Cyp2e1 transcription is 13 fold higher than males, and ethanol increase this difference to 32 fold, by selectively increasing the expression in females over males (Figure 34). Inhibiting Aldehyde Dehydrogenase by DSF alone resulted in an up-regulation of
Cyp2e1 for both male and female cells; eliminating differences in expression of Cyp2e1 (Figure 34). Exposing cells to both DSF and EtOH resulted in no differences between the sexes, as well as an increase of Cyp2e1, making Cyp2e1 EtOH responsive (Figure 34).

These results suggested a correlation between differences in expression of Cyp2e1 and cell viability in response to EtOH. We are now in a position to ask: how does the difference in Cyp2e1 result in differences in cell viability? when its role is redundant to that of alcohol dehydrogenase.

**Sex-differential DNA methylation results in differential Cyp2e1 transcription and EtOH induced death.**

In section II we showed that blocking de novo DNA methylation effectively reduced differences in expression of several genes that showed sex dimorphisms, including Cyp2e1 (Figure 26). Since 5-aza-2-deoxycytidine, a DNA methyltransferase inhibitor, reduced the difference in the expression of Cyp2e1, we asked whether this reduction could affect cell viability.

Here we used ED10.5 cells exposed to 5-Aza-dC for 5 population doublings as described in Materials and Methods, followed by EtOH for 24 hrs, and assessed cell viability.

When cells pre-treated with 5-Aza-dC are exposed to EtOH, the differences in viability are eliminated, when compared to cells exposed only to EtOH (Figure 35). The death level differences are leveled, as a result of an increase in cell death in males and females, compared to cells exposed only to EtOH (Figure 35).

When cells pre-treated with 5-Aza-dC are exposed to EtOH the cells are more sensitive, 29% more male cells and ~35 % more female cells when exposed only to EtOH, so that ~60-65% die,
reducing the differences between the sexes (Figure 35). Although cell death increases in both sexes there is more in the male cells, eliminating the dimorphic cell death seen with ethanol alone (Figure 35). Thus there may be a correlation between Cyp2e1 and cell death induced by EtOH.

**Pre-treatment with ROS Scavenger, N-acetyl cysteine, reduces EtOH induced cell death**

In the introduction, we indicated that at concentrations of EtOH higher than 100 μM, Cyp2e1 as well as alcohol dehydrogenase oxidize EtOH, but that Cyp2e1 generates Reactive Oxygen Species (ROS) (Gonzalez and Gelboin 1990, French, Morimoto et al. 1997, Petrick and Klaassen 2007, Lu and Cederbaum 2010, Qi, Miao et al. 2013). Thus, if females have more Cyp2e1, and are generally more sensitive to EtOH, it is possible that differential generation of ROS can account for the difference.

We initially looked at the effect of ROS generation in response to EtOH exposure by supplementing ED10.5 cells with a ROS scavenger, N-Acetyl Cysteine (NAC) and examining cell death. ED10.5 cells were exposed to ethanol and NAC alone or in combination. NAC exposure does not affect the basal levels of death caused by normal cell culture conditions (Figure 36). However, when cells are pretreated with NAC and then exposed to toxic levels of EtOH (400 μM) for 24 hrs the difference between the sexes was abolished due to a decrease in cell death in the females (Figure 36), when compared with cells treated with ethanol alone. This suggesting a correlation between cell sensitivity in females and ROS, even though NAC only partially rescues cells from EtOH induced toxicity.

NAC, while protecting female cells, does not affect expression of Cyp2e1, ADH or ALDH, suggesting that NAC acts downstream of ethanol breakdown (Data not shown).
The fact that NAC does not eliminate cell death suggests that EtOH kills by other means as well or that the half-life of NAC is less than that for EtOH. Importantly, modulating ROS scavenging alone eliminates sex differences, but without completely eliminating the toxicity.

Since we were able to indirectly link ROS to the toxicity elicited by EtOH, we were in a position to further evaluate ROS and its role in sex differences.

**Endogenous ROS scavenging mechanisms are higher in males**

Glutathione (GSH) is an endogenous scavenger of free radicals, quenching them by redox reactions. Endogenous GSH influences the cell’s ability to cope with ROS generation. High GSH levels are linked to high ROS generation, as GSH is responsive to intracellular free radical. GSH levels before and after generation of ROS can suggest where differences between the sexes exist.

Male and female ED10.5 cells were used to determine total GSH. ED10.5 male and female cells exposed to EtOH, NAC, and a combination of both, as described in Materials and Methods, were suspended and lysed to extract intracellular GSH. The extract was then normalized for equal loading and assayed for NADPH conversion, an indirect method of measuring total GSH. We found that under normal culture conditions, male cells had more total GSH, and that EtOH causes increase in GSH in both, while still maintaining the sex differences (Figure 37). EtOH increases GSH in both sexes.

Blocking ROS generation using NAC with EtOH exposed cells reduces the toxicity of EtOH. In all assessed conditions male cells have higher GSH, suggesting that male cells are better able to cope with EtOH induced stress perhaps by differential inactivation of ROS.
**ROS levels are sex dimorphic only after exposure to EtOH**

We have indirectly shown that EtOH elicits ROS generation in male and female mouse cells. Here we show, using the Total ROS/Superoxide Detection Kit (Enzo Technologies), that EtOH induces the generation of ROS. The ROS detection dye reacts directly with a wide range of reactive species (i.e. hydrogen peroxide, peroxynitrite, and hydroxyl radicals) yielding a green fluorescent product, visible at (490\text{Ex}/525\text{Em}).

The positive control for ROS is pyocyanin, a toxin capable of oxidizing and reducing other molecules, therefore a generator of radicals. ED 10.5 cells were cultured and plated in 96-well dishes as previously described and exposed to 50 μM pyocyanin for 30 minutes, at which time the wells were washed and immersed in the ROS Detection Mix and incubated for the last hour of treatment, as described in Materials and Methods. The plates were read without removing the detection mix using a microplate reader at 490\text{Ex}/525\text{Em}. The output values were normalized for background, by subtracting the signal of wells with only the detection mix. The normalized values were averaged over 6 repeated conditions, and divided by the control cells. The resultant values represent ratios of ROS over controls.

Male and female cells have similar basal levels of ROS, and in the presence of pyocyanin, there is a substantial increase of ROS signal in both sexes, suggesting that the technique has worked (Figure 38). Pre-treatment with NAC 1hr prior to Pyocyanin treatment resulted in a substantial reduction of ROS signal, but not a complete depletion. NAC exposure alone resulted in a significant reduction of ROS in control cells, suggesting that our basal conditions still result in ROS generation (Figure 38).
Having established the use of the Total ROS detection assay, we now asked whether EtOH alone would induce ROS generation, and whether this ROS generation was sexually dimorphic. To this end, we seeded equal numbers of ED10.5 cells into 96-well microplates, and select cells were pretreated 1hr prior to EtOH exposure with NAC, to see if NAC could counteract the ROS generation induced by EtOH. Cells were then treated with EtOH for a period of 2, 4, 12 and 24 hrs. ROS generation is highest at 4 hours post treatment. Female cells produce significantly more ROS than their male counterparts; and pretreatment with NAC reduced the ROS generated as a result of EtOH exposure (Figure 39), significantly reducing but not totally eliminating the sex differences. Since ROS were not persistent, we evaluated ROS for EtOH exposed cells at 12 and 24 hrs. At these times, ROS is reduced. Beyond the fact that ROS are very unstable, we have no explanation for the transience (Figure 39). While at 24 hrs we do not see sex differences in ROS generation, the substantial difference at 4 hrs correlates directly to the dimorphic response between the sexes, measured at 24hrs (Figure 31).

These results suggest that ethanol induces ROS generation, and that female cells produce more ROS in response to EtOH, confirming our results from the previous sections. ROS generation is induced within 2 hrs of EtOH treatment, peaks at 4 hrs and slowly drops by 24hrs post treatment (Figure 39); cell death on the other hand peaks at 24hrs (Figure 31), suggesting that death is a product downstream of the ROS generation. Even though there is a reduction in total ROS at 24hrs, the levels are still high, indicating a continuing toxic microenvironment in the dying cells.
Discussion:

In previous sections we discussed sex differences in cell response as measured by cell viability as well as gene expression profile and other differentiating factors. We found that male cells are less sensitive to ethanol. Anecdotally, many suggest that men are better able to metabolize alcohol, and therefore are more tolerant (Thomasson 1995, Baraona, Abittan et al. 2001, Nolen-Hoeksema 2004). There is little evidence in the literature suggesting this. Most efforts have gone into understanding how different populations with different isoforms of alcohol dehydrogenase (ADH) respond to or metabolize alcohol (Baraona, Abittan et al. 2001, Bailey and Cunningham 2002, Adachi, Takeuchi et al. 2009, Morgan, Synold et al. 2010).

Our cell culture system allowed us to work on sex differences in alcohol response, while controlling many other variables. In establishing mechanisms leading to these differences, we examined the metabolic pathways of alcohol, initiated by alcohol dehydrogenase (ADH’s), followed by aldehyde dehydrogenase (ALDH’s), and then entering other cellular pathways, including glycolysis (Peters and V.R. 1998, Cederbaum 2001, Adachi and Ishii 2002, Lu and Cederbaum 2008). We evaluated various representative isoforms of ADH, and ALDH and find no differences in the expression of these genes.

Other groups identified Cyp2e1 as another target for alcohol metabolism, as it has a redundant function to alcohol dehydrogenase, when alcohol reaches toxic levels, in our case 200 μM (French, Morimoto et al. 1997, Lu and Cederbaum 2008, Lu and Cederbaum 2010, Penaloza, Estevez et al. 2013, Qi, Miao et al. 2013). Additionally, Cyp2e1 leads to the generation of free radicals in the cells, in the process of metabolizing alcohol (Lu and Cederbaum 2008, Penaloza, Estevez et al. 2013, Qi, Miao et al. 2013).
Having established the potential role of Cyp2e1, we manipulated the cell milieu, to characterize the role of Cyp2e1 in the sex dimorphic response to ethanol previously demonstrated.

All of the evidence that we have accumulated suggests that ethanol induced sex differences are linked to Cyp2e1 levels, which are regulated by DNA methylation. We demonstrated that blocking ALDH activity resulted in an increase of Cyp2e1 expression, and induction of cell death in both sexes. We previously showed that blocking DNA methylation results in an increase of Cyp2e1 levels, yielding a similar outcome to that produced by blocking ALDH. These experiments suggest that the differential death between the sexes, in response to alcohol, result from variations in Cyp2e1, modulated by DNA methylation and alcohol responsiveness.

Increasing differences in Cyp2e1 levels between the sexes by EtOH supplementation results in greater female susceptibility and higher ROS generation. Supplementing ROS scavengers eliminates the differences. Eliminating differences in expression of Cyp2e1 reduces sex differences in cell viability.

This is a first glimpse at differences upstream of transcription, resulting in differences in cell behavior, devoid of sex hormones. Understanding further what factors are leading to these already existing differences, may help in characterizing other similar unknowns.
OVERALL DISCUSSION

There are many diseases that show sex bias in their frequency of occurrence. Understanding the how and why of this bias may help in the development of appropriate treatments.

Work by various groups suggests that sex hormones represent one major factor leading to the observable sex differences in disease manifestation (Kalin and Zumoff 1990, Marceglia and Priori 2007, Crabtree-Hartman 2010, Pérez-López, Larrad-Mur et al. 2010, Dorak and Karpuzoglu 2012, Fairweather, Petri et al. 2012, Townsend, Miller et al. 2012, Fairweather, Cooper Jr et al. 2013); however, there is growing evidence suggesting that other factors may also be at play.

The uncertainty derives from limitations in the systems in which one can evaluate sex differences. At the organismal level, underlying difference differences can be hidden by a myriad of factors. Additional limitations exist since most biomedical studies are performed almost exclusively on male subjects (Polit and Beck 2009, Beery and Zucker 2011, Alspach 2012). It is rationalized that the female mammal hormonal milieu are intrinsically more variable than males and too troublesome for routine inclusion in research protocols (Beery and Zucker 2011).

Sex hormones, in addition to their developmental functions, influence various other systems; including immune function with females having higher immunoglobulin levels and mounting stronger immune responses following immunization or infection (Verthelyi 2001). Studies in normal mice show that estrogen treatment induces polyclonal B cell activation with increased expression of autoantibodies characteristic of autoimmune diseases (Bynoe, Grimaldi et al. 2000). In addition, sex hormone levels in both humans and experimental models correlated with
the activity of their cytokine-secreting cells, suggesting that altered sex hormonal levels in autoimmune patients contribute to the skewed cytokine milieu characteristic of systemic lupus erythematosus (SLE), again suggesting a sex hormone driven difference (Smith-Bouvier, Divekar et al. 2008). Persistence of these differences throughout life however, with varying hormonal stages, and studies on genetically modified mice casts doubts on the direct relationship of dimorphism with individual hormonal status.

Non-hormonal factors have increasingly important consequences. Males are less prone to develop Type 1 Helper T Cell (Th1)-mediated autoimmunity because they have higher T cell expression of PPARalpha (Peroxisome proliferator-activated receptor alpha), as well as increased cytokine production leading to increased production of inflammatory markers (CRP, ESR and neutrophil counts) in females (Dunn, Ousman et al. 2007). Suggesting differences in autoimmunity dictated by differences in the expression of one gene. An early report by our laboratory also suggested that male and female cells respond differently to camptothecin and hydrogen peroxide, with a female biased sensitivity (Nikezic-Ardolic, Lin et al. 1999). In a separate study, using two immunologically distinct disease models, experimental autoimmune encephalomyelitis (EAE) and pristane-induced lupus, suggested that mice of the XX sex chromosome complement, as compared with XY, demonstrated greater susceptibility to both EAE and lupus (Smith-Bouvier, Divekar et al. 2008). Again suggesting an intrinsic chromosomal difference, and not a hormonally driven difference.

Since we needed a good model system to study the influence of sex on cells and its response to other factors, we sought to:

- Establish a model in which we can characterize sex differences (Section I);
- Identify factors that can result in observable sex differences (Section II);
- Test whether we can modulate existing differences, and eliminate the differences (Section III).

As part of this thesis, in section I, we developed a mouse model that allowed us to control for endogenous hormonal influences by using samples from mouse embryos too young to produce their own sex hormones (at embryonic day (ED) 10.5 of gestation); as well as late embryonic samples (ED 17.5) when embryos are already producing their own sex hormones and gonads have differentiated. We were able to expand our model to include animals at early post birth (Day 4) and adolescent stages (Day 17) of development, to assess the impact of endogenous hormones on sex differences. Using this model provided tighter control, as all animals used were inbred for more than 50 generations, suggesting almost identical genetic makeup with the exception of the presence or absence of a Y chromosome.

The dimorphisms we were able to identify included cell viability as well as gene expression profiles. We found that in general, female cells are more sensitive to cell death inducers, ethanol and camptothecin (Figs. 3 and 5). We further find that in the absence of cell stress, there are no differences in cell behavior (Fig. 1). At the gene expression level however, differences were noted prior to and after stress induction.

We looked at gene expression profiles, by gene arrays as well as qRT-PCR to show sex differences in gene expression profiles at the organismal level (Figs. 8-13) as well as in vitro before and after induction of stress (Figs. 14-17), as other groups have also documented in vivo (Xu, Burgoyne et al. 2002, Dewing, Shi et al. 2003, Eakin and Hadjantonakis 2006, Berchtold, Cribbs et al. 2008, Penaloza, Estevez et al. 2009, Bermejo-Alvarez, Rizos et al. 2010, Wang and
Few have documented differences at the cellular level (Nikezic-Ardolic, Lin et al. 1999, Dunn, Ousman et al. 2007, Maselli, Matarrese et al. 2009, Penaloza, Estevez et al. 2009, Regitz-Zagrosek, Straface et al. 2012, Penaloza, Estevez et al. 2013, Pollitzer 2013). We did not find a clear gene expression pattern between the sexes, but, even in the absence of sex hormones, many genes are expressed differentially between the sexes, including members of the cytochrome P450 family of metabolizing enzyme, as others have also documented (Pan, Hong et al. 1993, Skaanild and Friis 1999, Shu-Feng, Jun-Ping et al. 2009, Penaloza, Estevez et al. 2013). This differential in expression was modulated by alcohol as well as exogenous estrogen, which others have reported while not considering cell sex. Since sex hormones influenced gene expression in our system, and others have reported an effect by sex hormones on sex differences, we decided to look at the effect of estrogen in our system and assessed cell response by viability and gene expression.

Our data indicates that in the absence of embryonic production of sex hormones (ED10.5), sex differences as far as cellular response to stress and gene expression profile exist, suggesting that hormones do not dictate the sex differences. However, we noted that our results varied when we used cells from other stages of the mouse development, when sex hormones are produced, suggesting that hormonal production can superimpose an effect on the existing differences.

Interestingly, we found that at late embryonic development (ED17.5) and early post natal development (4 day post natal) most sex differences are reduced or abolished (Figs. 3 and 5). This loss of differences is possibly due to the very variable hormonal milieu that occurs close to birth. At day 17 post birth, we again find major sex differences in response to ethanol and
camptothecin as seen prior to hormonal onset (ED10.5) (Figs. 3 and 5). While similar effects have been documented, developmental approaches have not been used to study sex differences. Most work does suggest however, that other mechanisms of regulation must be at play, regulating sex differences upstream of hormonal influences (Okamoto, Otte et al. 2004, McCarthy, Auger et al. 2009, Lockshin 2010, Angelopoulou, Lavranos et al. 2012, Absher, Li et al. 2013, Dobbs, Rodriguez et al. 2013, Matsumoto, Buemio et al. 2013). Our system provided optimal conditions to address the effect of endogenous hormones on existing sex differences.

To better characterize factors that can influence gene expression and therefore cell sensitivity, we looked at regulatory systems for gene expression, including regulation of transcription factors and epigenetics.

The approach we initially took was to take the genes we had described as sex dimorphic (mainly the cytochrome P450 genes), and look for potential targets or existing gene regulatory elements within the genomic sequence. We found that individually these genes possessed: Estrogen response elements; Androgen response elements; Glucocorticoid Response Elements; and cAMP Response Elements.

While these regulatory elements existed in a few of these genes, there was no consensus; however, most of the dimorphically expressed and regulated genes possessed CpG islands, suggesting that these genes could be regulated by DNA methylation (Table 1). Further support came from our initial gene array, in which we found Dnmt3l, a DNA methyltransferase subunit, to be expressed dimorphically in unstressed cells (Peñaloza 2009 Table 1). Similar findings have been reported for DNA Methyltransferase 3a in the rat amygdala during development, expressed in a sex dependent manner (Bermejo-Alvarez, Rizos et al. 2011, Kolodkin and Auger
2011). Thus we need an alternative hypothesis such as the influence of X chromosome gene products escaping X inactivation in females or potential epigenetic regulation to further characterize the cause of these differences (Dunn, Ousman et al. 2007, McCarthy, Auger et al. 2009, Bermejo-Alvarez, Rizos et al. 2011, Dobbs, Rodriguez et al. 2013, Penaloza, Estevez et al. 2013). We hypothesized that these genes were under the regulation of DNA methylation.

To address whether these genes were under the regulation of DNA methylation, we looked at the DNA methylation patterns on the promoter region of select genes from the cytochrome P450 family, as well as X-ist (Fig. 23), known to be regulated by DNA methylation as our positive control (Goto and Monk 1998). In the absence of cell stress, the DNA methylation patterns are sex dimorphic. When we inhibit DNA methylation, thus reducing the differences, we reduce the differences in expression of the corresponding genes as well (Figure 25-26). Even at the pre-implantation stage of development, sex differences in gene expression are maintained by patterns of DNA methylation, as reported by other groups (McCarthy, Auger et al. 2009, Bermejo-Alvarez, Rizos et al. 2011, Dobbs, Rodriguez et al. 2013).

Ethanol can modulate DNA methylation patterns, resulting in changes in gene expression (Figs. 27-28), similar to an effect reported recently, in which global DNA methylation was reduced ~5% in MEF’s as a result of alcohol exposure (Mukhopadhyay, Rezzoug et al. 2013); ethanol was likewise reported to affect DNA methylation patterning during neurulation (Liu, Balaraman et al. 2009).

When we exposed cells to exogenous estrogen, both DNA methylation patterns and gene expression profiles were modulated, further correlating the DNA methylation patterns to the
expression of the same genes (Figure 29-30). Estradiol can also repress large chromosomal regions by DNA looping and acquisition of DNA methylation (Hsu, Hsu et al. 2010).

Finding differences in the DNA methylation patterns of these genes as well as modulation of their expression does not confirm that methylation is responsible, as this regulation can be in Trans, with the modulation still occurring on a different gene that then regulates the genes we assess, a process documented to occur frequently (van Eijk, de Jong et al. 2012). More work is necessary to further establish the link between DNA methylation and the direct regulation of these genes, as we can only confirm an indirect regulation. Individually regulated genes must be mutated in specific regions, to assess how methylation modulates expression; this can be further corroborated by identifying transcriptional factors directly interacting with the regions of interest. Looking at these factors can further suggest more upstream regulation resulting in these sex differences.

Since we were able to see differences in gene expression and then reduce these differences by blocking DNA methylation, we asked whether reduction of differences in methylation could reduce the sex-based differences in sensitivity to toxins. In section III of this thesis, we explored this idea, and further characterized the ethanol induced cell death mechanism.

As we had initially documented, blocking de novo DNA methylation resulted in a loss of gene expression differences, including that of Cyp2e1 (Figs. 25-26). As we had documented in the introduction and section III of this thesis, alcohol metabolism is initiated by alcohol dehydrogenase, and when alcohol reaches toxic levels, Cyp2e1 performs a redundant function to that of alcohol dehydrogenase (French, Morimoto et al. 1997, Lu and Cederbaum 2008). Cyp2e1 is expressed more in females (Figs. 8, 11, 14-17); similar differences are reported in humans.
(Chen, Han et al. 2002). The Cyp2e1 difference persists in the presence of alcohol, and can be eliminated by blocking DNA methylation (Figs. 25-26). Vieira et al had previously described regulation of Cyp2e1 by methylation, but they did not control for sex. They concluded that other regulatory factors were involved (Vieira, Pasanen et al. 1998). We therefore decided to look at what this modulation of gene expression would do to the sex dimorphic cell viability shown in section I. Blocking DNA methylation not only resulted in a reduction of differences in gene expression, but also in a loss of sex differences in cell viability (Fig. 35). Interestingly however, the loss of cell death differences resulted from increased death in males and not a reduction of death in either sex. We therefore looked at what could lead to the increased death and loss of sex differences.

Since Cyp2e1 differences were eliminated in response to blocked DNA methylation, we decided to look again at the alcohol metabolism pathway. When male and female ED10.5 cells are maintained in 5-Aza-2-Deoxycytidine (5-Aza-dC) for 5 population doublings, de novo DNA methylation patterning is reduced as measured by loss of DNA methylation sites (Section II of this thesis), and expression of several genes increases. This effect has been described by other groups as well, where p16 (a cyclin-dependent kinase (CDK) inhibitor that decelerates the cell cycle by inactivating the CDKs that phosphorylate retinoblastoma (Rb) protein) was modulated to influence tumorogenic cell line growth, as a result of DNA methylation patterning (Bender, Pao et al. 1998, Lavelle, Saunthararajah et al. 2008). Other factors involved in the metabolism of alcohol (alcohol dehydrogenase and aldehyde dehydrogenase) are not sex dimorphic and while their expression can be modulated by alcohol, this modulation is not sex specific (Fig. 32-33). Again we are left with Cyp2e1, performing a redundant role to that alcohol dehydrogenase (French, Morimoto et al. 1997, Lu and Cederbaum 2008). Loss of differences in Cyp2e1 levels,
when combined with exposure to alcohol, resulted in increased death in males, effectively eliminating differences between the sexes in cell viability.

This reduction or elimination of differences can be a consequence of Cyp2e1’s generation of radicals under toxic alcohol levels as reported by others (Lu and Cederbaum 2008, Lu and Cederbaum 2010, Qi, Miao et al. 2013). Under basal and ethanol induced conditions female cells express more Cyp2e1, potentially resulting in higher ROS generation. ROS scavenger N-Acetyl-Cysteine (NAC) reduces ethanol induced death, and so are the differences between the sexes (a previously reported observation-(Ferreira Seiva, Amauchi et al. 2009)). Finally DSF (Disulfiram), which blocks ALDH, increases death in both sexes as well, possibly as a result of increased concentration of acetaldehyde, which can be metabolized by Cyp2e1 as previously documented (Kunitoh, Imaoka et al. 1997). We further correlated that the death induced by ethanol was mediated by ROS generation, we measured ROS generation. Ethanol alone induces ROS generation, and NAC partially rescues cell from the ROS generation, two findings that have been previously documented in systems not measuring for sex differences (Ferreira Seiva, Amauchi et al. 2009).

In section III of this thesis we showed that male and female differences in cell response to ethanol are mediated by ROS and Cyp2e1; and that modulating DNA methylation can itself lower the observed male and female differences.

The work presented in this thesis proved that we can characterize sex differences at the cellular level; that differences observed in response to ethanol as a stimulant are likely due to differences in cytochrome p450 member 2e1; and the ROS generation mediated by Cyp2e1. Importantly, we find that blocking DNA methylation alone reduces the observed differences in cell viability, gene
expression and ROS generation induced by alcohol. This is the first instance where sex differences in alcohol metabolism are described molecularly, and suggesting that DNA methylation is one of the initial mediators of this response.

The mechanism detailed in this thesis whereby differential DNA methylation has consequences in cellular processes, as integral as cellular metabolism, suggests that other processes too can be influenced by differential methylation.

While this work provides evidence for sex differences modulated by DNA methylation, it is important to still identify modulators that trigger these differences further upstream, getting closer to the core of these differences.

Since we found differences in DNA methylation patterns, and other have reported this as early as the pre-implantation stage, it is important to search for modulators of DNA methylation upstream of differences in methyltransferase levels. This can be accomplished by looking at transcriptional regulators for dimorphically expressed DNA methyltransferases, possible response elements within their promoters and altering the promoters of the DNA methyltransferases, to further evaluate what is necessary within the promoters for the differences in expression. This can provide more information on the source of the differences; and while currently these differences are somatic, it is probable that further upstream, an X-chromosome dosage compensation mechanism may exist, ultimately regulating methylation differently between the sexes.

Having DNA methylation as a target up stream of sex differences in gene expression, which can then alter cellular behavior can be used to better elucidate mechanisms by which differences in
disease manifestation are occurring. Here we provided a model system, where we can test for single differences resulting in sex dimorphisms at the cellular level. It is critical to continue to expand our knowledge in this area, as this work can be extrapolated and applied to other instances where differences are being measured between the sexes, potentially providing more tools and evidence necessary to better characterize those conditions, specifically those outlined in the introduction of this thesis.
FIGURES
Figure 1: Cell viability is sex indifferent under normal culture conditions

Cell death measured by trypan blue exclusion. Blue bars, males; red bars, females. Amount of cell death in primary mixed cell cultures from ED10.5 whole embryos, ED17.5, Day 4 and Day 17 whole kidney, liver and lung organs exposed to culture medium (DMEM supplemented with 10% FBS and 1% penicillin/streptomycin) only for 4-7 days. Cells show no sex differences in basal viability. Data represent percent total cell death for all conditions. Data points are in triplicates. Inset: native polyacrylamide gel with PCR amplification (see Materials and Methods) of Zfy and Zfx, indicative of X- and Y-chromosome-specific genes. Males (right) possess both bands; females (left) have only a single band.
**Figure 2: Ethanol LD$_{50}$ for Embryonic Day 10.5**

Cells were from primary mixed cell cultures from ED10.5 whole embryo. Cell death measured by trypan blue exclusion. Blue bars, males; red bars, females. Cell viability after exposure to 400 μM EtOH for 24 hrs resulted in the best measurable conditions for ethanol induced stress, with females reaching close to 50% death. These experiments were done in triplicates. Asterisk (*) indicates p<0.05.
Figure 3: Cell viability after ethanol exposure is sex dimorphic in ED10.5; ED17.5 kidney and day 17 postnatal kidney cells

Cell death measured by trypan blue exclusion. Blue bars, male; red bars, females. Cultured cells exposed to 400 μM ethanol. Higher female cell sensitivity to ethanol is seen in cells from ED10.5 whole-embryo, ED17.5 kidney, and PN17 kidney mixed cell cultures, but not in mixed cells cultures from PN4 kidney. Data represent percentage total cell death for all conditions. These experiments were done in triplicates. Asterisks (*) indicate p<0.05.
Cell death measured by trypan blue exclusion. Blue bars, male; red bars, females. Cell viability after exposure to 20 μM CPT for 24 hrs resulted in the best measurable conditions for CPT induced stress, with males and females reaching close to 50% death. These experiments were done in triplicates. Asterisks (*) indicate p<0.05.
Figure 5: Cell viability is sex dimorphic after cell CPT exposure in ED10.5 and Day 17 kidney only

Cell death measured by trypan blue exclusion. Blue bars, males; red bars, females; 10.5, cells from whole embryos, ED17.5, day 4 and day 17 kidney, liver and lung cells. Cultured cells exposed to 20 μM CPT for 24 hrs show female-biased sensitivity at ED10.5 and PN17, but no differences are seen for ED17.5 and PN4 kidney mixed cell cultures. Data represent percentage total cell death for all conditions. These experiments were done in triplicates, with asterisk (*) representing p<0.05.
Figure 6: Cell viability is sex dimorphic after cell stress induction in ED10.5 and ED17.5 kidney cells as measured by WST-1.

Measure of mitochondrial membrane integrity by WST-1. Blue bars, male; red bars, female. Cell mitochondrial integrity after exposure to either 400 μM EtOH or 20 μM CPT. Cells were seeded and incubated in 96-well dishes for the final hour of CDI treatment with the Wst-1 compound and read at 450 nm with background subtracted at 690 nm. Male cells have higher WST-1 activity in the presence of EtOH for both ED10.5 and ED17.5 kidney cells, as well as ED10.5 after exposure to CPT. These experiments were done in triplicates, with asterisks (*) representing p<0.05.
Figure 7: Cell viability is sex dimorphic after cell stress induction in ED10.5 and ED17.5 kidney cells as measured by the MTT assay

Confirmation of the WST-1 assay was done using the MTT assay. Blue bars, male; red bars, female. Cell mitochondrial integrity after exposure to either 400 μM EtOH or 20 μM CPT. Cells were supplemented with MTT stock for 2 h, and then solubilized. Supernatants were then read at 540 nm. Differences in activity are noted for ED 10.5 cells exposed to EtOH and CPT as well as ED17.5 kidney cells exposed to EtOH only. These experiments were done in triplicates, with asterisks (*) representing p<0.05.
Figure 8: Gene expression in homogenates of ED10.5 whole tissue

Expression was measured by qRT-PCR using specific primers to evaluate baseline expression of several genes. Blue bars, males; red bars, females. Female tissues express more X-ist, Cyp1a1, Cyp7b1 and Cyp2e1 than their male counterparts. No significant difference is observed for Gapdh, an internal housekeeping gene. Samples were normalized at the RNA and cDNA levels for equal cDNA loading. Experiments were done in triplicate. CT mean values are shown. *$P < 0.05$; Student’s $t$ test. Ordinate is average CT of fluorescence detection for real-time PCR. Numbers below bars indicate fold differences by which female is higher.

![In Vivo Gene Expression Profile of ED10.5 Whole Embryo Homogenates](image-url)
Figure 9: Expression of X-ist in ED17.5, PN17 day and adult lung and kidney whole organ homogenate

Expression was measured by qRT-PCR using specific primers to evaluate baseline expression. Blue bars, males; red bars, females. Female organs express more X-ist, in all instances. Experiments were done in triplicate. Individual CT points are shown together with mean values. *$P < 0.05$; Student’s $t$ test. Ordinate is average CT of fluorescence detection for real-time PCR. Numbers below bars indicate fold differences by which female is higher.
Figure 10: Expression of Cyp1a1 in ED17.5, PN17 day and adult lung and kidney whole organ homogenates

Expression was measured by qRT-PCR using specific primers to evaluate baseline. Blue bars, male; red bars, females. Female tissues express more Cyp1a1 in all instances. Experiments were done in triplicate. CT mean values are shown. *$P < 0.05$; Student’s $t$ test. Ordinate is average CT of fluorescence detection for real-time PCR. Numbers below bars indicate fold differences by which female is higher.
Figure 11: Expression of Cyp2e1 in ED17.5, PN17 day and adult lung and kidney whole organ homogenates.

Expression was measured by qRT-PCR using specific primers to evaluate baseline. Blue bars, male; red bars, females. Female ED17.5 tissues as well as Adult kidney express more Cyp2e1, with no significant differences identified in other instances. Experiments were done in triplicate. CT mean values are shown. *P < 0.05; Student’s t test. Ordinate is average CT of fluorescence detection for real-time PCR. Numbers below bars indicate fold differences by which female is higher.
Figure 12: Expression of Cyp7b1 in ED17.5, PN17 day and adult lung and kidney whole organ homogenates

Expression was measured by qRT-PCR using specific primers to evaluate baseline expression. Blue bars, male; red bars, females. Female ED17.5 tissues as well as PN17 lung tissue express more Cyp7b1, with no significant differences identified in other instances. Experiments were done in triplicate. CT mean values are shown. *P < 0.05; Student’s t test. Ordinate is average CT of fluorescence detection for real-time PCR. Numbers below bars indicate fold differences by which female is higher.

![Cyp7b1 Expression in vivo](image-url)

<table>
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<tr>
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<td>Adult</td>
<td>30</td>
<td>28</td>
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* Asterisk indicates significant difference.
Figure 13: Expression of Gapdh in ED17.5, PN17 day and adult lung and kidney whole organ homogenates

Expression was measured by qRT-PCR using specific primers to evaluate baseline. Blue bars, male; red bars, females. Female PN17 lung tissues express higher levels of Gapdh, with no significant differences identified in other instances. Experiments were done in triplicate. CT mean values are shown. *$P < 0.05$; Student’s $t$ test. Ordinate is average CT of fluorescence detection for real-time PCR. Numbers below bars indicate fold differences by which female is higher.
Figure 14: Expression of cytochrome P450 family members is sex dependent in vitro, in early embryo

This figure represents male (blue bars) and female (red bars) embryonic day 10.5 cells. Expression of X-ist, Cyp1a1, Cyp2e1, Cyp7b1, and Gapdh are shown in that order, with the fold differences written on the abscissa, the ordinate axis representing Cycle Threshold, of PCR cycle at which signal was first detected. A reduction of one CT unit represents doubling of expression. Mixed cells from ED10.5 females consistently expressed higher levels of X-ist, Cyp1a1, Cyp2e1, and Cyp7b1 with Gapdh not differing between the sexes. The samples were normalized at the RNA and cDNA levels for equal cDNA loading. Experiments were done in triplicate, and statistical significance was declared at p values <0.05. Standard deviations are shown.
Figure 15: Cytochrome P450 family members are expressed in a sex dependent manner *in vitro*, in late embryo

This figure represents cells from embryonic day 17.5 males (blue bars) and females (red bars). Expression of X-ist, Cyp1a1, Cyp2e1, Cyp7b1 and Gapdh are shown in that order, with the fold differences written on the abscissa, the ordinate axis representing Cycle Threshold, of PCR cycle at which signal was first detected. In ED17.5 Kidney Cells, females consistently expressed higher levels of X-ist, Cyp1a1, and Cyp2e1 with Cyp7b1 and Gapdh not different between the sexes. The samples were normalized at the RNA and cDNA levels for equal cDNA loading. Experiments were done in triplicate, statistical significance was declared at p values <0.05. Standard deviations are shown.
Figure 16: Cytochrome P450 family members are expressed in a sex dependent manner in vitro, in postnatal cells

This figure represents cells from postnatal day 17 males (blue bars) and females (red bars). Expression of X-ist, Cyp1a1, Cyp2e1, Cyp7b1 and Gapdh are shown in that order, with the fold differences written on the abscissa, the ordinate axis representing Cycle Threshold, of PCR cycle at which signal was first detected. In PN 17 Day Kidney Cells, females consistently expressed higher levels of X-ist, Cyp1a1, Cyp2e1 and Cyp7b1 with Gapdh not different between the sexes. The samples were normalized at the RNA and cDNA levels for equal cDNA loading. Experiments were done in triplicate, statistical significance was declared at p values <0.05. Standard deviations are shown.
Figure 17: Expression of cytochrome P450 family members is altered by exposure of cells to EtOH

This figure represents cells from male (blue bars) and female (red bars) embryonic day 10.5 cells under basal (no differences), or 400 μM ethanol treatment. For every transcript there are two sets of bars, the first representing gene expression under normal culture conditions, the second set representing exposure to EtOH. The ordinate represents Cycle Threshold, of PCR cycle at which signal was first detected. Lower CT value represents higher expression. Cyp1a1, Cyp2e1 and Cyp7b1 respond significantly to ethanol, with a reduction, increase and reduction of sex differences respectively, while ethanol does not affect X-ist or Gapdh. The samples were normalized at the RNA and cDNA levels for equal cDNA loading. Experiments were done in triplicate, statistical significance was declared at p values <0.05. Standard deviations are shown.
Figure 18: Cell viability is sex dimorphic after exposure to estrogen at ED10.5, cell
recovery is sex independent after estrogen exposure

18A) Cell death measured by trypan blue exclusion. Blue bars, males; red bars, females. Amount
of cell death in primary mixed cell cultures from ED10.5 whole embryo. Cell viability after
exposure to 5 nM E2 (17β-estradiol) or 400 μM EtOH (Ethanol) for 24 hrs. Female EtOH
sensitivity is reduced dramatically by exposure of cells to estrogen. Male cells are also affected,
but to a much lesser degree. These experiments were done in triplicates, with p<0.05. 18B)
Number of recovered cells, including live and dead cells were consistent for male and female
samples, as well as control and E2 treated samples, suggesting no differences in proliferation.
These experiments were done in triplicates, with p<0.05
Estrogen Influences Ethanol Induced Cell Death

A

% Cell Death over Control

0 10 20 30 40 50 60 70 80 90 100

Estrogen | EtOH | Estrogen + EtOH

ED10.5

Male | Female

B

Cells Recovered Live + Dead

0 100000 200000 300000 400000 500000 600000

Control | E2 | EtOH | E2+EtOH

Male | Female

*
Figure 19: MTT and WST-1 activity levels correlates with cell viability

19A) Measure of mitochondrial membrane integrity by MTT. Blue bars, male; red bars, female. Cell mitochondrial integrity after exposure to either 5nM E2, 400 μM EtOH or a combination of both. Cells were supplemented with MTT stock for 2 h, and then solubilized. Supernatants were then read at 540 nm. Ethanol induces sex dimorphic MTT conversion, as a result of reduced activity in females when compared to males but estradiol protects against ethanol. 19B) Blue bars, male; red bars, female. Cell mitochondrial integrity after exposure to either 5 nM E2, 400 μM EtOH or a combination of both. Cells were seeded and incubated in 96-well dishes for the final hour of CDI treatment with the Wst-1 compound and read at 450 nm with background subtracted at 690 nm. Ethanol induces sex dimorphic WST-1 conversion, as a result of reduced activity in females when compared to males.
MTT Activity Correlates with Cell Viability

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<thead>
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</tr>
<tr>
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Wst-1 Relates to Cell Death and MTT Results

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<td>EtOH+E2</td>
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Figure 20: Estrogen up regulates the expression of three members of the cytochrome P450 family

This figure represents male (blue bars) and female (red bars) embryonic day 10.5 cells under basal (no differences), or 5 nM estrogen treatment. For every transcript there are two sets of bars, the first representing gene expression under normal culture conditions, the second set representing estrogen exposure. The ordinate represents Cycle Threshold, of PCR cycle at which signal was first detected. Lower CT value represents higher expression. Estrogen supplementation increases X-ist, Cyp 1a1/2e1/7b1 levels in at least one sex. Induction did not always occur in both sexes. For each set the experimental is paired to the control. The samples were normalized at the RNA and cDNA levels for equal cDNA loading. Experiments were done in triplicate, statistical significance was declared at p values <0.05. Standard deviations are shown.
Table 1: Regulatory consensus across CYP P450 members

Regulatory consensus sequences were searched within the promoter region of various cytochrome p450 family members. Response elements included in this search were Estrogen response elements (ERE), cAMP response elements (CREB), glucocorticoid response elements (GRE) and androgen response elements (ARE). ERE response elements were found in the promoter region of Cyp1a1, Cyp5A1, Cyp7b1; no CREB response elements were found in the promoter region; GRE response elements were found in the promoter region of Cyp1a1, Cyp3A4, Cyp5A1, and Cyp8A1; ARE response elements were found in the promoter of Cyp5a1 and five (Cyp1a1, Cyp2E1, Cyp3A4, Cyp5A1, and Cyp7b1) of the seven assessed sequences possess CpG islands greater than 200bp.

<table>
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<th>Gene</th>
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<th>GRE</th>
<th>ARE</th>
<th>CpG &gt; 200bp</th>
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<td>+</td>
<td>-</td>
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<td>Cyp7b1</td>
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<td>-</td>
<td>+</td>
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Table 2: CpG island identification and selection for select Cytochrome P450 members

We identified 6, 12, 3, over 3, and 0 CpG islands spanning the promoter regions of Cyp1a1 (positions -873 to -566), Cyp2E1 (positions 1231 to -982), Cyp7b1 (positions 667 to -402), X-ist (positions -2393 to -2011) and Gapdh, respectively. Below are the chromosomal locations of each CpG within their respective promoter regions.

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<th>Gene</th>
<th>Gene Location</th>
<th>Number of CpGs</th>
<th>Number of CpGs &gt;200bp</th>
<th>CpG Islands Analyzed</th>
<th>CpG Island Position</th>
<th>Size of Region</th>
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<td>Cyp7b1</td>
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<td>-667- (-402)</td>
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<td>&gt;3</td>
<td>1</td>
<td>-2393- (-2011)</td>
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<td>GAPDH</td>
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Figure 21: Dnmt3l expression is sex dimorphic in vivo

This figure represents male (blue bars) and female (red bars) ED10.5 whole embryo, ED17.5, Day 4 and Day 17 kidney and lung homogenates. The ordinate represents Cycle Threshold, of PCR cycle at which signal was first detected. Lower CT value represents higher expression. Dnmt3l levels are significantly higher in all male samples. The samples were normalized at the RNA and cDNA levels for equal cDNA loading. Experiments were done in triplicate, statistical significance was declared at p values <0.05. Standard deviations are shown.
**Figure 22: Dnmt3l expression is sex dimorphic *in vitro***

This figure represents male (blue bars) and female (red bars) ED10.5 whole embryo, ED17.5, and Day 17 kidney cell culture. The ordinate represents Cycle Threshold, of PCR cycle at which signal was first detected. Lower CT value represents higher expression. Dnmt3l levels are significantly higher in male samples, confirming the *in vivo* results. The samples were normalized at the RNA and cDNA levels for equal cDNA loading. Experiments were done in triplicate, statistical significance was declared at p values <0.05. Standard deviations are shown.
Figure 23: Sex dimorphic DNA methylation patterns under normal conditions

Genomic DNA extracted from male and female cells was treated with sodium bisulfite, and specific promoter regions were amplified and sequenced. Presented are the parental DNA sequences, with notations on variations between the sexes. 5 individual rounds of amplification and sequencing are averaged into the representation, with each site resulting from at least 4 of 5 replicates. DNA methylation status is sex dimorphic under normal conditions, as observed with X-ist (five methylation sites in males and three in females), Cyp1a1 (three methylation sites in males and four in females), Cyp2e1 (5 methylation sites in males and females, 3 of which are common), and Cyp7b1 (four methylation sites in males and two in females). Shaded circles represent unmethylated, filed circles represent methylated CpG.
Figure 24: ED10.5 recovery of male and female cells after exposure to 5-Aza-dC

Male and female cells exposed to 5-Aza-dC over 4 cell passages. Inhibition of DNA methylation by 5-Aza-dC results in increased cell recovery after each round of passage for ED 10.5 cells, when compared to controls.
Figure 25: 5-Aza-dC reduces sex differences in methylation

Extracts of genomic DNA from male and female cells were treated with sodium bisulfite, and specific promoter regions were amplified and sequenced. Presented are the parental DNA sequences, with notations on variations between the sexes. 5 rounds of amplification and sequencing are averaged into the representation, with each site resulting from at least 4 of 5 replicates. 5-Aza-dC treatment of ED10.5 cells over 5 population doublings reduces differential methylation as observed with X-ist, cytochrome P450 family members’Cyp1a1, Cyp2e1, and Cyp7b1, as noted by loss of methylated CpG’s (clear circles). Shaded circles represent unmethylated, filed circles represent methylated, and clear circles represent loss of methylated CpG.

- Not Methylated
- Methylated
- Loss of Methylation
Figure 26: 5-Aza-dC reduces sex differences in expression

The sex dimorphism of X-ist decreases from 8 to 1; Cyp1a1, from 23 to 6; Cyp2e1, from 8 to 2; Cyp7b1, from 16 to 6; Dnmt3l, from 8 to 3; and Gapdh, from 4 to 1. Expression was measured by qRT-PCR using specific primers to evaluate their baseline expression. Blue bars, male; red bars, females; ED10.5 mixed cells. Samples were normalized at the RNA and cDNA levels for equal cDNA loading. Asterisks (*) represent p<0.05; standard deviations shown.
**Figure 27: Ethanol alters methylation patterns for X-ist, Cyp1a1, Cyp2e1, and Cyp7b1**

Extracts of genomic DNA from male and female cells were treated with sodium bisulfite, and specific promoter regions were amplified and sequenced. Presented are the parental DNA sequences, with notations on variations between the sexes. 5 rounds of amplification and sequencing are averaged into the representation, with each site resulting from at least 4 of 5 replicates. 400 μM treatment of ED10.5 cells over 24hrs resulted in variations of DNA methylation patterns for X-ist, Cyp1a1, Cyp2e1 and Cyp7b1 as indicated by clear circles (loss of site) and outlined grey circles (gained site).
Figure 28: Ethanol alters dimorphism of expression

Blue bars, male; red bars, females; ED10.5 mixed cells exposed to 400 μM EtOH for 24 h. The dimorphism of X-ist increases from 16 to 32; Cyp1a1 decreases from 128 to 3; Cyp2e1 increases from 13 to 32; Cyp7b1 decreases from 16 to 4; Dnmt3l, increases from 8 to 16; and Gapdh, decreases from 2 to 1. Expression was measured by qRT-PCR using specific primers to evaluate their baseline expression. Samples were normalized at the RNA and cDNA levels for equal cDNA loading. Experiments were done in triplicate. Average CT values are shown. Ordinate is average CT of fluorescence detection for real-time PCR. Asterisks (*) represent P<0.05, standard deviations shown.
Figure 29: Estradiol alters methylation sites of X-ist, Cyp1a1, Cyp2e1, and Cyp7b1

Extracts of genomic DNA from male and female cells were treated with sodium bisulfite, and specific promoter regions were amplified and sequenced. Presented are the parental DNA sequences, with notations on variations between the sexes. 5 individual rounds of amplification and sequencing are averaged into the representation, with each site resulting from at least 4 of 5 replicates. 5 nM E2 treatment of ED10.5 cells over 24hrs resulted in variations of DNA methylation patterns for X-ist, Cyp1a1, Cyp2e1 and Cyp7b1 as indicated by clear circles (loss of site) and outlined grey circles (gained site).
Figure 30: Estradiol alters expression levels of X-ist, Cyp1a1, Cyp2e1, Cyp7b1 and Dnmt3l

In the presence of 17-β estradiol, the sex dimorphic ratio of X-ist is reduced from 16 to 1; of Cyp1a1, from 128 to 4; Cyp2e1, from 13 to 2; Cyp7b1, from 16 to 4; and Dnmt3l, from 8 to 1, with no change for Gapdh. Expression was measured by qRT-PCR using specific primers to evaluate their baseline expression. Blue bars, male; red bars, females; ED10.5 mixed cells. Samples were normalized at the RNA and cDNA levels for equal cDNA loading. Experiments were done in triplicate. Average CT values are shown. Ordinate is average CT of fluorescence detection for real-time PCR.
Figure 31: Cell viability is sex dimorphic after exposure to ethanol in ED10.5, and inhibition of ALDH eliminates this difference

Cell death measured by trypan blue exclusion. Blue bars, male; red bars, females. Cultured cells exposed to 400 μM ethanol, DSF or a combination. Higher female cell sensitivity to ethanol is seen in cells from ED10.5 whole-embryo, but not in mixed cells cultures co-treated with DSF. Data represent percentage total cell death for each condition. These experiments were done in triplicates. Asterisks (*) represent P<0.05, standard deviation shown.
Figure 32: Expression Profile for Alcohol Dehydrogenase Isoforms are not sex specific

Expression was measured by qRT-PCR using specific primers to evaluate their baseline expression. Blue bars, male; red bars, females; ED10.5 mixed cells. Samples were normalized at the RNA and cDNA levels for equal cDNA loading. Experiments were done in triplicate. Average CT values are shown. Ordinate is average CT of fluorescence detection for real-time PCR. ED10.5 cells exposed to 400 μM EtOH for 24hrs or DSF and in combination of both.  

A) ADH 1B is neither EtOH responsive nor sex dimorphic; 
B) ADH 4 is EtOH responsive but not sex dimorphic; 
C) ADH 5 is neither EtOH responsive nor sex dimorphic; 
D) ADH 7 is EtOH responsive, but not sex dimorphic; 
E) ADH 6 is neither EtOH responsive nor sex dimorphic.
Expression Profile for Alcohol Dehydrogenase Class II is Sex Independent

Expression Profile for Alcohol Dehydrogenase Class III is Sex Independent

Expression Profile for Alcohol Dehydrogenase Class IV is Sex Independent

Expression Profile for Alcohol Dehydrogenase Class V is Sex Independent

Figure 32
Figure 33: Expression Profile for Aldehyde Dehydrogenase isoforms are not sex specific

Expression was measured by qRT-PCR using specific primers to evaluate their baseline expression. Blue bars, male; red bars, females; ED10.5 mixed cells. Samples were normalized at the RNA and cDNA levels for equal cDNA loading. Experiments were done in triplicate. Average CT values are shown. Ordinate is average CT of fluorescence detection for real-time PCR. ED10.5 cells exposed to 400 μM EtOH for 24hrs or DSF and in combination of both. A) AHDH 1 is neither EtOH responsive nor sex dimorphic; B) ALDH 2 is neither EtOH responsive nor sex dimorphic
Figure 33

**A**

ALDH Class I Levels are Sex Independent and not EtOH or DSF Responsive

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<th>EtOH + DSF</th>
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</table>

**B**

ALDH Class I Levels are Sex Independent and not EtOH or DSF Responsive

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<th>DSF</th>
<th>EtOH + DSF</th>
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<td>ALDH 2</td>
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</table>
Figure 34: Expression Profile for Cyp2e1 is DSF and EtOH responsive

Expression was measured by qRT-PCR using specific primers to evaluate their baseline expression. Blue bars, male; red bars, females; ED10.5 mixed cells. Samples were normalized at the RNA and cDNA levels for equal cDNA loading. Experiments were done in triplicate. Average CT values are shown. Ordinate is average CT of fluorescence detection for real-time PCR. ED10.5 cells exposed to 400 µM EtOH for 24hrs or DSF and in combination of both. Cyp2e1 levels are increased in male samples in response to EtOH, increasing already existing sex differences. Aldehyde Dehydrogenase Inhibition, results in an increase of Cyp2e1 expression in both sexes, ablating sex differences. Asterisks (*) represented P<0.05, standard deviation shown.
Figure 35: Cell viability is sex dimorphic after cell Ethanol Exposure in ED10.5, DNA methylation inhibition ablates this difference

Cell death measured by trypan blue exclusion. Blue bars, male; red bars, females. Cultured cells exposed to 400 μM ethanol, 5-Aza-dC or a combination. Higher female cell sensitivity to ethanol is seen in cells from ED10.5 whole-embryo, but not in mixed cells cultures co-treated with 5-Aza-dC. Data represent percentage total cell death for all conditions. Asterisk (*) represents p<0.05, standard deviation shown.
Figure 36: Cell viability is sex dimorphic after cell Ethanol Exposure in ED10.5, ROS inhibition ablates this difference

Cell death measured by trypan blue exclusion. Blue bars, male; red bars, females. Cultured cells exposed to 400 μM ethanol, N-Acetyl Cysteine or a combination. Higher female cell sensitivity to ethanol is seen in cells from ED10.5 whole-embryo, but not in mixed cells cultures co-treated with NAC. Data represent percentage total cell death for all conditions. Asterisk (*) represents p<0.05, standard deviations shown.
Figure 37: Cell Glutathione Levels are higher in males

Total GSH levels were assessed between the sexes, normalized. Male (blue bars) and female (red bars) ED10.5 cells were treated with EtOH 400 μM for 24hrs, or pre-treated with NAC for 1 h prior to EtOH treatment, followed by co-treatment with EtOH, or NAC alone for 24hrs. Higher male GSH is found in all conditions, GSH levels are spiked in EtOH treated cells. Data represent percentage total GSH in cultures. Asterisks (*) represent p<0.05, standard deviations shown.
Figure 38: ROS generation is induced by pyocyanin and partially rescued by N-acetyl cysteine

Total ROS generation was detected for all conditions. Data represents 6 replicates. ED10.5 male (blue bars) and female (red bars) were treated with ROS inducer pyocyanin for 24 h or NAC for 24 h individually, or with a 1 h pretreatment of NAC followed by co treatment with pyocyanin and NAC for another 24 h period. Average OD values are shown. ROS generation is sex independent in normal cell culture conditions, heavily induced by Pyocyanin, and partially rescued by pre-treatment with NAC. Standard deviations are shown.
Figure 39: ROS generation is induced by EtOH and partially rescued by N-acetyl cysteine

Total ROS generation was detected for all conditions. Data represent 6 replicates. ED10.5 male (blue bars) and female (red bars) were treated with EtOH for 24 h or NAC for 24 h individually, or with a 1 h pretreatment of NAC followed by co treatment with EtOH and NAC for another 24 h period. Average OD values are shown. ROS generation is sex independent in normal cell culture conditions, heavily induced by ethanol, and partially rescued by pre-treatment with NAC. Asterisks (*) represent p<0.05, standard deviations are shown.
REFERENCES


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topotecan in a phase I trial of topotecan, tamoxifen, and carboplatin, in the treatment of recurrent or refractory brain or spinal cord tumors." Cancer Chemother Pharmacol 66(5): 927 - 933.


