DIMORPHIC GENE EXPRESSION BEFORE AND AFTER THE ONSET OF SEXUAL DIFFERENTIATION

By

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The process of sexual development encompasses two continuous phases: a hormone independent sexual determination phase, followed by a hormone dependent sexual differentiation phase. It is well known that androgen plays a central role in masculinizing the genitalia from its indifferent stage to its final form as the penis or clitoris. During this second phase, 5alpha-dihydrotestosterone acts on the androgen receptor (AR) to masculinize the genital tubercle. AR mRNA is detected at E12.5 and onward, AR protein is detected at E14 and embryo hormone production does not being until E13 Therefore, it can be deduced that E13.5 signifies the onset of the hormone dependent phase.

The molecular mechanisms of sexual differentiation are largely unknown. The targets of the androgen receptor, and how these genes interact and react, and consequently resulting in the final form of the external genitalia, remain a mystery. To explore this subject we used a microarray and compared the transcriptomes of males and females at E13.5, the onset of sexual differentiation, and at E15.5, after the “androgen surge”, to elucidate the key players responsible for the developmental fate of the genital tubercle.
CHAPTER 1
INTRODUCTION

Introduction

The external genitalia begin as a genital tubercle, which is an embryonic precursor to the penis or clitoris in the fully developed organism. The developmental mechanisms that lead the formation of the genital tubercle are beginning to be understood (1), however the downstream targets of androgens, which lead to the virilization of this organ, remain unknown. The overarching aim of this study is to identify the genes that mediate sexual differentiation of the external genitalia.

Sexual Development

The central paradigm of sexual development, cultivated by Jost (5), is that chromosomal sex leads to gonadal sex, which leads to phenotypic sex.

Sexual Determination

Sex determination is a genetic (and hormone-independent) event, which determines the sexual characteristics of an organism. Humans and other mammals have a XY sex determination system, in which females are homogametic, having a XX pair of sex chromosomes and males are heterogametic, having a XY pair of sex chromosomes (2). The Y chromosome carries the SRY gene, which encodes the SRY protein. A mutation or translocation of part of the SRY gene underlies some of the earliest of disorders of sexual development (see below) including Swyer Syndrome (XY females with gonadal dysgenesis) and XX male syndrome (3). The expression of SRY is a determinant of the male phenotype and allows the proliferation of Sertoli cells, which will release anti-mullerian hormone (AMH) (4). AMH in turn causes the regression of the female urogenital primordium, otherwise known as the Mullerian ducts, and plays a role in
development of Leydig cells, which will produce androgens and lead into the second hormonal dependent phase of development of external genitalia in males (4).

**Genetic Control of GT Development**

Development of the genital tubercle is under genetic control, which involves tight coordination of proximodistal outgrowth, patterning, and urethral tubulogenesis. Several developmental regulators, such as *homeobox-containing* Hox genes, Wnt/β-catenin signaling, *bone morphogenic proteins* (BMPs), *sonic hedgehog* (Shh) and *fibroblast growth factors* (Fgf) have been suggested to play an orchestrating role in the patterning and outgrowth of the genital tubercle before masculination (13-20). These genetically-controlled morphogenetic processes are followed by androgen-mediated virilization of or estrogen-mediated feminization of the genital tubercle to form the male and female external genitalia.

**Sexual Differentiation**

Androgens play a vital role in the second phase of sexual development, sexual differentiation, which determines the phenotypic sex of the organism. Jost (5) performed several experiments, which determined that testicular hormones ultimately induce the male phenotype. Through the castration of male rabbits prior to phenotypic differentiation of the external genitalia, he found all resulting phenotypes to be female, concluding that the male form was the induced phenotype that resulted from testicular hormones. He also deduced that there were two embryonic testicular hormones, AMH and androgens.

A series of separate endocrinologic and embryologic experiments proved testosterone to be the hormone ultimately involved in masculinizing the male urogenital tract. Administration of anti-androgen pharmacological agents during embryogenesis was
shown to inhibit male development (6, 7). In another experiment, testosterone was shown to immediately precede virilization in many species, exhibiting a cause and effect relationship (8). In female embryos, administration of a testosterone analogue was shown to induce male development of internal and external genitalia (9). Although much is known about the role of sex hormones in sexual differentiation, the genetic mechanisms by which they ultimately induce the differentiation of the sexually indifferent genitalia remains unresolved.

**Testosterone, DHT and the Androgen Receptor**

Testosterone is produced by the Leydig cells in the gonads beginning in the embryo at embryonic day (E) 13, and acts locally to promote maturation of spermatagonia. Testosterone also is secreted into the circulatory system where it acts to masculinize the external genitalia (4). Testosterone can be converted to a more active form, dihydrotestosterone (DHT), by 5-alpha reductase in the cell. This metabolite proves to be a much more potent agonist for androgen receptor activation, as it does not have the ability to be converted to estrodiol through the enzyme aromatase, as testosterone can. Thus, DHT it is purely androgenic. Either of these androgenic hormones can bind to the androgen receptor (AR) in the cytoplasm of the cell. After binding, this hormone-receptor complex translocates into the nucleus and binds to androgen responsive elements (AREs) on DNA, where it functions as a DNA-binding transcription factor that regulates gene expression of downstream targets. Endocrine disruptors, like the pharmacological agent flutamide, can bind to the AR and inactivate signaling, thereby preventing the expression of downstream targets and therefore organogenesis due to androgenic factors (21).

Testosterone is mainly responsible for development of the male primary sexual characteristics (e.g., epididymis, vas deferens, and seminal vesicle), whereas DHT
induces the development of secondary sexual characteristics (e.g. the prostate and penis). If there is an impairment of testosterone or the androgen receptor during development, the abnormalities can involve both the internal and external genitalia. If the impairment is only within the formation of DHT, only the external genitalia will be affected, resulting in female external genitalia with male internal reproductive organs (22). Mutation of the AR gene, which lies on the X chromosome, will result in testicular feminization mutation (TFM) in mice and androgen insensitivity syndrome (AIS) in humans. This syndrome results in 46, XY karyotype individuals, which are phenotypic females with male gonads that result as a function of the SRY gene. As the Wolffian structures are hormone-dependent, they will generally be absent in such mutants, the severity of which depends on the mutation. This is yet another description of how impairments in any step of the path of sexual development can lead to a disorder of sexual development.

**Disorders of Sexual Development**

Disorders of sexual development (DSD) refer to an extensive list of anomalies that result from deviations of the normal sexual development. These deviations can be broken down into anomalies of the sex chromosomes, the gonads, the reproductive ducts, and the genitalia (10). The defects focused mainly on the abnormalities in the development of external genitalia and can include micropenis, clitoromegaly and hypospadias.

**Current Research**

Research in the field of sexual differentiation and organogenesis of the genital tubercle has focused mainly on the genetic mechanisms of development. Little is known about the molecular mechanisms that mediate the masculinization process induced by androgens. Recently, Miyagawa et alii performed a DNA microarray analysis to compare gene expression between male and female genital tubercles at E15.5, an early stage of
genital tubercle differentiation. The complete results of this study are unpublished, but a few genes were extracted and analyzed rtPCR and *in situ* hybridization using tissue from the ventral bilateral mesenchyme and urethral epithelium (11). Li et al. (2006) previously performed microarray analysis of genital tubercle development; however, the data presented only a list of genes that increased in E16 and E17 GT versus E14. In addition, these data were collected from cDNA arrays, which have been shown to have lower reproducibility and concordance among platforms (12), and not Affymetrix. Hence, a comparison cannot be made with these data (23). Our aim is to explore the dimorphically expressed genes at the onset of sexual development (E13.5), as well after the onset (E15.5)

The genetic targets of the androgen receptor, and how these genes interact to ultimately result in the male external genitalia, are unknown. To explore this subject we used a microarray and compared the transcriptomes of males and females at E13.5, when sexual differentiation is initiated, and at E15.5, after androgenic action has begun to virilize the male genitalia, to elucidate the key players responsible for the development of the external genitalia. To identify the downstream targets of the androgen receptor, we administered the anti-androgen flutamide to the pregnant dams beginning at E12.5 through 14.5, a window during which a phenotypic and feminizing effect can be induced (21). Total RNA was taken at E13.5 and E15.5 to identify the androgen targets before and after sexual differentiation. To identify which genes are involved in the maturation of the genital tubercle, we compared the two time points within each sex. Identification of the candidate genes for embryonic genital tubercle maturation and sexual differentiation will facilitate further molecular and developmental studies of sexual differentiation.
CHAPTER 2
METHODS

Animals

Timed-pregnant CD1 mice (Harlan Laboratory) were sacrificed via by cervical dislocation. Embryos were collected at embryonic day (E) E13.5 and E15.5 (stage was determined initially by the day of an appearance of vaginal plug as 0.5 days post conception). The stage of development was confirmed by morphological examination using a dissecting microscope. Embryos were sacrificed by decapitation immediately after removal from the yolk sac. The sex of the pups was confirmed by determining the presence of ovaries or testes, and also by PCR of embryonic tail DNA for SRY and ZFY as described (24). Fetal GTs were examined to confirm normal morphology and then microdissected and stored in RNALater® (Cat. No. 76104, QIAGEN®, Valencia, CA) at 4°C for total RNA isolation.

RNA Collection

Total RNA from the frozen samples was extracted using an RNeasy Plus Micro Kit according to the manufacturer’s instructions (RNeasy® kit; Cat. No. 74034, QIAGEN®, Valencia, CA). RNA concentration was determined on a Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA integrity was evaluated by microfluidic analysis using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA).

Microarray

Samples for microarray analysis were prepared by isolation of RNA from genital tubercles of E13.5 and E15.5 male and female mice from the following treatment groups; untreated and corn oil treated females and untreated, corn oil treated, and flutamide-
treated males. Four litters were harvested for each treatment group, and 3 male and 3 female embryos were collected from each litter. The flutamide treated and vehicle treated timed-pregnant CD 1 dams were gavaged with 150mg/kg/day of flutamide dissolved in 10% ethanol and vehicle (tocopherol-stripped corn oil), or 3ul/g/day of the vehicle dissolved in 10% ethanol, respectively, once daily at noon from E12.5 through E14.5. For embryos sacrificed at E13.5, the timed-pregnant CD 1 dams were gavaged once on E12.5 with either flutamide or vehicle. For the embryos sacrificed at E15.5, the timed-pregnant CD 1 dams were gavaged on E12.5, E13.5 and E14.5 with either a succession of flutamide or vehicle.

cDNA synthesis, cRNA synthesis, amplification, biotin labeling and chip hybridization was performed as described in the IVT Expression Kit (Ca. No. 901229; Affymetrix) protocol using 200ng of total RNA for the reverse transcription to synthesize first-strand cDNA. An in vitro transcription incubation time of 16hrs was used to synthesize biotin-modified cRNA from double stranded cDNA templates. Biotin-labeled product was hybridized onto a GeneChip Mouse Genome 430 2.0 Array, which contains 42102 probe sets. Staining and washing followed the protocol (EukGEWSv4; Affymetrix) with GeneChip Fluidics Station 450.

**Analysis**

GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix) were used for the expression analysis. Four independent samples were collected for each combination of sex, stage and condition, for a total of 40 gene chips and 120 independent samples. Each independent replicate is a pool of RNA from three mice. Library files and cell files were quantified using Affymetrix Expression Console. MAS 5 signal values were extracted.
Control probe sets and those where signal was “absent” on all arrays were removed before analysis. The remaining 31,109 probe sets were analyzed.

To identify sexual dimorphisms in gene expression before and after the onset of sexual differentiation, and genes involved in maturation of each sex during sexual differentiation we examined multiple contrasts between untreated samples

The following ANOVA model was fit: \( Y_{ij} = \mu + \tau_i + \varepsilon_{ij} \), where \( Y_{ij} \) is natural log transformed signal for condition \( i = (\text{female at E13.5, female at E15.5, male at E13.5 and male at E15.5}) \), and replicate \( j \) for all the untreated samples (n=16). Where \( \mu \) is the overall mean, \( \tau \) is condition, and \( \varepsilon \) is the error. In order to identify the genes involved in sexual differentiation the following contrasts were performed: female at E13.5 versus female at E15.5, female at E13.5 versus male at E13.5, male at E13.5 versus male at E15.5, and male at E15.5 versus female at E15.5. A False Discovery Rate (FDR) was used to correct for multiple tests (25). Since this analysis is an initial screen for genes involved in sexual differentiation between sexes or temporally within a sex, we are most concerned with type II error (false negatives). Accordingly, all probes significant at a false discovery rate (FDR) of \( P < 0.05 \) were retained for further analysis (25).

Probe sets differentially expressed among sexes (at least 1 of the contrasts above significant n=5691) are then candidates for regulation mediated by the androgen receptor. For the treated samples (n=24), the following ANOVA model was fit: \( Y_{ij} = \mu + \tau_i + \varepsilon_{ij} \), where \( Y_{ij} \) is natural log transformed signal for treatment \( i = (\text{vehicle treated female at E13.5, vehicle treated female at E15.5, vehicle treated male at E13.5 and vehicle treated male at E15.5, flutamide treated male at E13.5, and flutamide treated male at E15.5}) \), and replicate \( j \). Where \( \mu \) is the overall mean, \( \tau \) is the treatment, and \( \varepsilon \) is the error. In order to
identify direct or indirect targets of the androgen receptor the following contrasts were performed: vehicle treated female at E13 versus vehicle treated male at E13, vehicle treated female at E13 versus flutamide treated male at E13, vehicle treated female at E15 versus vehicle treated male at E15, vehicle treated female at E15 versus flutamide treated male at E15, vehicle treated male at E13 versus flutamide treated male at E13, vehicle treated male at E15 versus flutamide treated male at E15. A False Discovery Rate (FDR) was used to correct for multiple tests. Accordingly, all probes significant at a false discovery rate (FDR) of P < 0.05 were considered to be significantly targets of the androgen receptor. Probe sets differentially expressed for at least 1 of these contrasts were selected for further analysis (n=3027).
CHAPTER 3
RESULTS

Dimorphic Genes Before and After the Onset of Sexual Differentiation

Dimorphic Genes Before the Onset of Sexual Differentiation

There are 45102 probes sets available on the Affymetrix 430 2.0 Mouse Chip. After the absent calls were removed, 31100 probe sets were detected on at least one of the 40 arrays. The expression of 5691 of those probe sets was significantly different in the comparisons between untreated males and females at E13.5, untreated males and females at E15.5, untreated males at E13.5 and E15.5, and untreated females at E13.5 and E15.5. These 5691 probe sets represent those with significant differences in expression during sexual differentiation and maturation between sexes at each stage, as shown with the contrasts: untreated males versus untreated females at E13.5 and E15.5, and temporally within a sex, as shown with the contrasts: untreated males at E13.5 versus untreated males at E15.5, and untreated females at E13.5 versus untreated females at E15.5.

From the 5691 significantly different probe sets expressed within the pair wise male (E13.5, E15.5), female (E13.5, E15.5) comparisons, contrasting within sexes and between sexes, 265 distinct genes, excluding duplicates, were significant between males and female genital tubercles at E13.5. In analyzing the probe sets comparatively between males and females at this stage, the expression of 163 genes was significantly higher in males than in females, while the expression of the remaining 103 genes were significantly higher in females than in males (Object 3-1). Thus, at E13.5 in the genital tubercle, 58% more genes were expressed at a level significantly higher in males than in females. These developmentally interesting genes included Cybr3, Ctnl1, Mib2, Daam1, Activinr1,
Pak3, Fap, Egr2, Egfr, Slit3, Mib2, and Foxc1. In females, up regulated genes included Senp2 and Dab2.

When clustered together by similarity in expression level using the LSmeans value, the hierarchical cluster analysis produced 10 clusters (Object 3-2). The first two clusters as well as clusters 4, 5, 6 and 7, represent genes in which the expression levels in males were comparatively higher than that in females, and clusters 3, 8, 9 and 10, represent those in which the expression levels in females was comparatively higher than that in males. In the clusters up-regulated in males, Col17a1, Cyb5r3, and Mib2 presented in cluster 1, Pak3, Egfr, Egr2, Foxc1, and Bmp pathway associated gene, Acvr1, presented in cluster 2. Within the female clusters, Senp2 presented in cluster 3. There were no genes known to be important in the development of other organ systems in clusters 4 through 10.

The annotated genes were sorted into their biological function as guided by Affymetrix Mouse 430 2.0 annotation Gene Ontology terms. The most dimorphic biological process included “enzyme/amino acid and protein metabolism,” which constituted 10% of the genes shown to be more highly expressed in males (Figure 3-1), while only 2% of genes more highly expressed genes in females had this biological function (Figure 3-2). The remaining 28% of the genes upregulated in the females at E13.5, and 36% of the genes upregulated in the males at E13.5, had no known function.

After the Onset of Sexual Differentiation

During mouse embryonic development, hormone production begins at E13 (27, 29), and AR protein is detected at E14 (26), and so it is that by E14.5 the embryo is within the hormone dependent phase of sexual development. By E15.5, AR and its ligand DHT have been diffusing through the blood for a time that is sufficient to activate
downstream targets that are responsible for the virilization of the genital tubercle (30, 31). I investigated which genes are differentially expressed at this stage of development in order to determine which targets of the AR are responsible for the developmental fate of the genital tubercle.

From the 5691 significantly different probe sets expressed within the pair wise male (E13.5, E15.5) and female (E13.5, E15.5) comparisons, contrasting within sexes and between sexes, 28 of those, excluding duplicates, were significant between males and females at E15.5 (Object 3-3). In analyzing the probe sets comparatively between males and females at this stage, the expression of 20 genes was significantly higher in females than in males, while the expression of the remaining 8 genes was significantly higher in males than in females. In males at this stage, 8 genes were up regulated, and in females, 20 genes were up regulated in males. Thus, at E15.5 in the genital tubercle, 60% more genes were expressed at a level significantly higher in females than in males. These genes that were up regulated in females at E15.5 included Abcf3, Vamp3, Fras1, Eif2s3x. The genes that were up regulated in this comparison in males included mainly Y linked genes like Ddx3y.

When clustered together by similarity in expression level using the LSmeans value, the hierarchical cluster analysis produced 10 clusters (Object 3-4). The first 5 clusters consist of genes that are comparatively higher in males than in females. In the first cluster there were 6 genes, which consisted of Cox4i2, Eif2s3x, Zscan12, Abcf3, Kdm6a. In the second cluster, the genes presented were Ssbp2, Tmem213, Vamp3, and Fras1. The third, fourth and fifth clusters consisted of Ncapd3, Tsc22d1, and Xist. The
fifth through tenth clusters consisted of genes that were comparatively higher in females than in males at E15.5 and they were Ddx3y, Uty, and Gca.

The annotated genes were sorted into their biological function as guided by Affymetrix Mouse 430 2.0 annotation Gene Ontology terms. The most dimorphic biological process included “chromatin modification and regulation”, which constituted 20% of the genes shown to be more highly expressed in males (Figure 3-3), while only 9% of genes more highly expressed genes in females had this biological function (Figure 3-4). There were also genes present in males that had biological function in the “enzyme/amino acid and protein metabolism”, “transport protein,” and “transcription factors” categories, of which no genes were present in these categories in females. The remaining 64% and 56% genes in the female / male comparison at E15.5, respectively had no known function.

**Temporal Change in Gene Expression**

To understand the molecular events that occur during sexual differentiation, we examined the gene expression profiles of each gender at two time points, E13.5, before the onset of differentiation, and E15.5, when sexual differentiation is underway. We were particularly interested in identifying the genes that control maturation of the male and female genitalia.

**Gene Expression in Females Before and After the Onset of Sexual Differentiation**

Pairwise comparisons of male (E13.5, E15.5) and female (E13.5, E15.5) genital tubercles, which were contrasted within sexes and between sexes, identified 5691 probe sets with significantly different expression profiles. Pairwise comparisons of females at E13.5 versus females at E15.5 revealed 3968 distinct genes that are significantly different (Object 3-5). In analyzing the probe sets comparatively between females at each stage,
the expression of 2262 genes were significantly higher at E13.5 than at E15.5, while the expression of the remaining 1706 genes were significantly higher at E15.5 than at E13.5. Thus, at E13.5 in the genital tubercle, 25% more genes were expressed at a level significantly higher at E13.5 than at E15.5. Among the genes upregulated at E13.5 were collagens (Col1a1, Col1a2, Col3a1, Col5a1, Col5a2 Col6a1, Col8a1, Col8a2, Col23a1), keratins (krt6a, krt13, krt42, krt77, krt84), forkhead genes (Foxc1, Foxf1a, Foxh1, Foxj2, Foxk1, Foxk2, Foxn1, Foxn2, Foxn3, Foxo3, Foxp1, Foxq1), DNA binding transcription factors, particularly homeobox genes (Hoxa5, Hoxa9, Hoxa10, Hoxd13, Msx1, Meox2, Shox2, Prx1, Cux1, Hopx, Lhx5, Mkx), fibronectin III domain encoding genes (Fn1, Ephal, Epha3, Epha7, Ephx1), members of the TGF Beta superfamily of ligands (Bmp2, Bmpr2, Bmp2k, Acvr1), Wnt signaling pathway genes (Wnt2, Wnt5a, Wnt6, Wnt7a, Wnt9a, Wnt16, Frz1, Frz2, Frz3), members of the protocadherin gene family, which is a subset of the cadherin family (Pcdh7, Pcdh9 Pcdhb9, Pcdh10, Pcdh12, Pcdh17), and other developmentally interesting genes (Senp2, Senp3, Sumo3, Rhoa, Notch3, Jun, Dkk3, Hey2, Igf1, Fras1).

When clustered together by similarity in expression level using the LSmeans value, the hierarchical cluster analysis produced 10 clusters (Object 3-6). The first 4 clusters consist of genes that are comparatively higher at E15.5 than at E13.5. In the first cluster, some developmentally interesting genes exhibited were Ephal, Jag1, Ctnna1, Egfr, Efr3, Klf6, Acvr1, Ror1, Dkk3, Lrp10, Jak2, Fkbp7, Fkbp9, Abcd3, Cyb5, and Notch3. In the second cluster, some interesting genes presented were Foxc1, Foxj2, Foxk1, Foxo1, Foxq1, Ephal, Epha3, Epha7, Col1a2, Col3a1, Col5a1, Col8a1, Col23a1, Wnt5a, Fkbp14, Sox2, Ar, Pak3, Jun, Shox2, Hey2, Igf1, Mkx, and Rhoq. The third cluster
contains Tgfbr2, Ephx1, Wnt9a, Fkbp10 Colla1, Col5a2, and Col6a1. Cluster four contained many keratins (e.g., krt6a, krt13, krt84), along with Meox2 and Cadm3. The last cluster whose expression was comparatively higher at E15.5 than E13.5 was cluster five which contained a Wnt ligand, Rspo1, and genes involved in epidermal differentiation and cornification, Hrnr, Dpt and Lor. Clusters six through ten contain genes that were more highly expressed at E13.5, before the onset of sexual development in females. In cluster six included Foxk2, Sox12, Hoxa9, Hoxd13, Fkbp4, Ryk, Msx2, Senp2, Senp3, and Sumo3. Examples of genes in cluster seven include Rac3, Fzd3, Hoxa5, Msx1, Fkbp3, Wnt6 and Actb. Examples of genes contained in cluster eight are, Wnt5a, Wnt7a, Senp6 and Foxf1a. The genes expressed in cluster nine included Rhoa and Hoxa10. In the final cluster, ten, examples are Fras1, Hoxa10, and Tbx3.

The annotated genes were sorted into their biological function as guided by Affymetrix Mouse 430 2.0 annotation Gene Ontology terms. Analysis of the genes that are significantly higher E13.5 than E15.5 females, the most dimorphic ontology group included “cell proliferation, differentiation, and apoptosis” (46%) (Figure 3-5), whereas only 9% of the genes more highly expressed at E15.5 belonged to this class (Figure 3-6). Comparisons of female genitalia at E13.5 and E15.5 also showed that 9% of the genes upregulated in E13.5 females and 27% of the genes upregulated at E15.5 had no known function.

**Gene Expression in Males Before and After the Onset of Sexual Differentiation**

From the 5691 significantly different probe sets expressed within the pair wise male (E13.5, E15.5), female (E13.5, E15.5) comparisons, which were contrasted within sexes and between sexes, 885 of those, excluding duplicates, were significant between males at E13.5 and E15.5 (Object 3-7). In analyzing the probe sets comparatively in males
between E13.5 and E15.5, the expression of 614 genes were significantly higher at E13.5, while the expression of the remaining 271 genes were significantly higher at E15.5 than at E13.5. Thus, 56% more genes were expressed at a level significantly higher at E13.5 than at E15.5. These genes include \( Fkbp1b, Fkpb7, Fkbp9, Foxc1, Foxk2, Bmpk2, Jak2, Ptg1l, Igf1, Fap, Mkx, TGFB2, Sox7, Sox9, Meis1, Meis2, Frem2, Fras1, Senp6, Lix1, Dlx1, Dlx2, Notch1, Hoxa9, Wnt6, Wnt7a, Pitx2, \) and \( Mexx1. \)

When clustered together by similarity in expression level using the LSmeans value, the hierarchical cluster analysis produced 10 clusters (Object 3-8). The first five clusters consist of genes that are comparatively higher at E15.5 than at E13.5 in males. The first cluster contained \( Fkbp7, Fkbp9, Bmp2k, Jak2 \) and \( Foxc1. \) The second cluster contained \( Mafb \) and \( Ifg1, \) followed by the third cluster of \( Sfrp2, Upk1b, Fap, Mkx, Tgfb2, Sox7 \) and fourth cluster, which contained \( Fbln7. \) Cluster five contained three genes of which none were remarkable. Clusters six through ten contain genes whose expression intensities were comparatively higher at E13.5, or prior to the onset of sexual differentiation. These genes include; cluster six: \( Wnt6, Wnt7a, Mxs1, Mxs2, Dlx1; \) cluster seven: \( Notch1, Hoxa9, Fkbp1b, Pitx2, Foxk2; \) cluster eight: \( Sox9, Tbx3; \) and cluster nine: \( Meis1, Meis2, Lix1, Senp6, Dlx2, Abce1, Frem2, \) and \( Fras. \)

The annotated genes were sorted into their biological function as guided by Affymetrix Mouse 430 2.0 annotation Gene Ontology terms. Genes involved in the most dimorphic biological processes included “transport protein” which constituted 10% of the genes shown to be more highly expressed at E15.5 (Figure 3-7), while only 2% of genes more highly expressed genes at E13.5 had this biological function (Figure 3-8). The
remaining 46% and 23% of the genes in the E13.5 and E15.5 male comparison respectively had no known function.

**Targets of the Androgen Receptor**

**Targets of The AR at the Onset of Sexual Differentiation**

Androgen signaling, mediated by AR and its ligands testosterone and DHT, masculinizes the genital tubercle resulting in the formation of a penis. The direct and indirect targets of the ligand-receptor complex in the genital tubercle are largely unknown. The pharmacological agent flutamide acts as an anti-androgen by directly binding to and blocking the AR (21), and was used in these studies to antagonize AR signaling. AR mRNA is not known to be present in the embryo until after E12.5 (28), and androgen production begins at E13 (27, 29), shortly after Leydig cell differentiation. Given that AR protein is not detected until E14 (26), we hypothesized that antagonism of AR prior to this stage would have minor, if any, effects on the genitalia.

To test this hypothesis, we asked which of the 5691 genes that exhibited sexual dimorphism in untreated males (E13.5, E15.5) versus untreated females (E13.5, E15.5), when contrasted within sexes and between sexes, were targets of AR. From those that showed dimorphism between males and females at both stages (5691 genes), 3026 genes also presented as significantly different within the flutamide comparisons described below.

To identify targets (direct or indirect) of AR in E13.5 genital tubercles, a series of paired comparisons were performed. A female control (corn-oil treated) versus male control comparison at E13.5 was carried out to identify sex-specific differences in the context of the corn oil vehicle. To identify which of these sex-specific differences were due to AR activity, a female control versus male flutamide treated comparison at E13.5
was performed. We therefore searched for genes that were significantly different in the first (control) comparisons but were then not differentially expressed in the second (male flutamide: female vehicle) comparison. We predicted that this experiment would determine if there is an interaction between the pharmacological agent, and anti-androgen receptor blocker flutamide, and genes present and involved in sexual differentiation at its onset.

The comparison between female and male control mice at E13.5 yielded 62 genes. Of those 62 genes, 39 were then not significant in the comparison between female corn-oil treated and male flutamide treated at E13.5. Genes present in this comparison include Grem2, Rab40b, Casp6, and Srd5a3 (Object 3-9). The data suggest that these 39 genes are either direct or indirect targets of AR.

The annotated genes were sorted into their biological functions using Affymetrix Mouse 430 2.0 annotation Gene Ontology terms (Figure 3-9). The largest number of genes fell into the biological processes of “carbohydrate metabolism” and “enzyme/amino acid and protein metabolism,” which both held 13% each. In descending order we then at 10%, “transport protein,” 9%, “cell proliferation, differentiation, and apoptosis,” 6%, “signalizing transduction” and “ion transport,” 3%, “immune related molecules”, “ECM/ cell adhesion and tissue remodeling,” and “transcription factors.” The remaining 34% of the genes in the comparison between male and female controls at E13.5, that were then were not significant in the comparison when the male was treated with flutamide at the same stage, had no known function.

**Targets of the AR After the Onset of Sexual Differentiation**

In parallel to the flutamide comparison performed at the onset of sexual differentiation, we compared female control versus male control genitalia at E15.5 to identify sex-
specific differences in the presence of corn oil. We next compared female controls versus male flutamide-treated comparison at E15.5. This was followed by a cross comparison of these two data sets to identify genes that respond to AR antagonism.

The comparison between female and male control mice at E15.5 yielded 49 genes. Of those 49 genes, 34 were then not significant in the comparison between female corn-oil treated and male flutamide treated at E15.5, suggesting that these 34 genes are androgen-responsive. Genes present in this pool include *Frem2, Igfl, Eps15, Tagln3, Smoc1*, and *Rhoa* (Object 3-10).

The annotated genes were sorted into their biological function as guided by Affymetrix Mouse 430 2.0 annotation Gene Ontology terms (Figure 3-10). The largest number of genes fell into the biological processes of “enzyme/ amino acid and protein metabolism” which constituted 17% of the genes. The remainder of the genes categorized into their biological functions in descending order were 10%, “ECM/ cell adhesion,” “signaling transduction,” and “cell proliferation, differentiation, and apoptosis,” and 7%, “transcription factors.” The remaining 46% of the genes that presented in the comparison between male and female controls at E15.5, and then were not significant in the comparison when the male was treated with flutamide at the same stage, had no known function.
Figure 3-1. Gene ontology analysis corresponding to the dimorphically expressed genes in the male E13.5 versus female E13.5 comparison. These depict the genes more highly expressed in males in this comparison.
Figure 3-2. Gene ontology analysis corresponding to the dimorphically expressed genes in the male E13.5 versus female E13.5 comparison. These depict the genes more highly expressed in females in this comparison.
Figure 3-3. Gene ontology analysis corresponding to the dimorphically expressed genes in the male E15.5 versus female E15.5 comparison. These depict the genes more highly expressed in males in this comparison.
Figure 3-4. Gene ontology analysis corresponding to the dimorphically expressed genes in the male E15.5 versus female E15.5 comparison. These depict the genes more highly expressed in males in this comparison.
Figure 3-5. Gene ontology analysis corresponding to the differentially expressed genes in the female E13.5 versus female E15.5 comparison. These depict the genes more highly expressed in females at E13.5.
Figure 3-6. Gene ontology analysis corresponding to the differentially expressed genes in the female E13.5 versus female E15.5 comparison. These depict the genes more highly expressed in females at E15.5.
Figure 3-7. Gene ontology analysis corresponding to the differentially expressed genes in the male E13.5 versus male E15.5 comparison. These depict the genes more highly expressed in males at E15.5.
Figure 3-8. Gene ontology analysis corresponding to the differentially expressed genes in the male E13.5 versus male E15.5 comparison. These depict the genes more highly expressed in males at E13.5.
Figure 3-9. Gene ontology analysis corresponding to the dimorphically expressed genes at E13.5 that are also targets of the androgen receptor.
Figure 3-10. Gene ontology analysis corresponding to the dimorphically expressed genes at E15.5 that are also targets of the androgen receptor.
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CHAPTER 4
DISCUSSION

The objective of this study was to identify genes dimorphically expressed between males and females at key stages of sexual development, and to identify possible targets of androgen receptor during the hormone dependent phase of sexual differentiation. Below I discuss the significance of a subset of genes that are known have roles in other developmental processes and diseases.

Dimorphically Expressed Genes Before Onset of Sexual Differentiation

Activin type 1 receptor - Acvr1

These receptors transduce signals for a variety of members of the transforming growth factor beta superfamily of ligands including AMH, activin, bone morphogenic proteins (BMPs) and Nodal (32). As expected due to its relation with AMH, this gene is upregulated in males at E13.5. This gene may also hint as to why there is a large discrepancy between the quantity of dimorphically expressed genes at E13.5 and E15.5. Patterning of external genitalia is under genetic control in males and females until around E15.5 in the mouse (30), after which morphological changes due sexual differentiation are first seen. This could account for the difference in number of dimorphically expressed genes between females and males at E13.5, and females and males at E15.5 (265 versus 28, respectively). This idea is supported by the presence of the Tgf beta receptor, Acvr1. Tgf beta genes have been implicated in genital tubercle patterning and outgrowth; for example, Bmp7 expression is located in the urethral epithelium, and Bmp2 and Bmp4 are expressed in the mesenchyme at this stage (16).
Dimorphically Expressed Genes After the Onset of Sexual Differentiation

**Fraser syndrome 1 - Fras1**

The *Fras1* gene encodes a membrane protein that belongs to the *FRAS1* family. This extracellular matrix protein is thought to be required for maintaining the integrity of the skin epithelium and is expressed mainly in sites of epithelial-mesenchymal interactions. The protein localizes to the basement membrane, and plays a role in epidermal-dermal interactions during morphogenetic processes. Mutations in this gene are associated with Fraser syndrome and the murine equivalent, mouse ‘bleb’ mutants. Interestingly, Fraser syndrome is characterized by developmental abnormalities including malformations in the genitalia. In the microarray analysis, this gene is expressed at a level significantly higher in males and females at E13.5 as compared to E15.5, as depicted in the temporal comparisons. It is also expressed at a level significantly higher in females at E15.5 compared to males at E15.5. *Fras1* has been shown to down regulate BMP4 in the mesonephric duct (33). At E14.5, BMP4 is expressed in the mesenchyme surrounding the urethral plate in the genital tubercle (16), although its expression at E15.5 in the genital tubercle has yet to be published. The absence of *Fras1* in males at E15.5 would suggest that BMP4 in the genital tubercle would be expressed at higher level and could indicate morphological remodeling.

**Targets of Androgen Action**

The targets of androgen action during formation of secondary sex characteristics are generally unknown. The aim of this investigation was to implicate target genes of androgen-androgen receptor complex through the use of anti-androgen flutamide. These are genes implicated as androgen targets at the onset of sexual differentiation.
Androgen Targets at the Onset of Sexual Differentiation, E13.5

In the untreated comparisons, we detected a higher number of dimorphically expressed genes (female versus male at E13.5 produced 265 genes: female versus male at E15.5 produced 28 genes) than in the parallel corn oil comparisons (female versus male at E13.5 produced 62 genes: female versus male at E15.5 produced 49 genes). Thus, corn oil alone reduces the number of differences in gene expression between males and females. The action of phytoestrogens can serve as a possible explanation for the difference in the number of dimorphically expressed genes. Phytoestrogens are plant derived xenoestrogens that are proposed to possess hormone-like properties in humans and other mammals, i.e., mimicking natural hormones, inhibiting the action of hormones, and inducing abnormal gene expressions. Exogenous estrogenic compounds that bind to the estrogen receptors (ERs) can block or alter endogenous estrogen functions via an ER-mediated response, in reproductive and developmental stages. (34) This hypothesis is reinforced by the phytoestrogen genistein, which has been shown to induce hypospadias in mice (35), as does the administration of exogenous estrogens in utero (36). Conversely, in ERKO mouse models, the adult females are partially masculinized, presenting with an elongated os clitoris (37). Along with providing evidence of phenotypic deviations due to modification of ER activation, this also disproves the traditional belief that androgens are necessary for masculinization of the male external genitalia, and in the absence of androgen the default female state will persist. Instead, a balance between androgens and estrogens are necessary for the proper morphology of adult external genitalia.

The possible action of phytoestrogens in the corn oil experiments could have increased ER signaling in males, making their downstream gene expression patterns more similar to that of a female, thereby resulting in a decreased number of significantly
different genes. This can be viewed by the untreated comparisons at E13.5 presenting with 265 significantly different genes and the corn oil comparisons at E13.5 presenting with 62 significantly different genes. Conversely, the phytoestrogens could have increased the expression of ER targets to a level that would make genes that were not significantly different in the untreated experiments, then significantly different in the corn oil experiments. This can be viewed by the untreated comparisons at E15.5 presenting with 28 significantly different genes, and the corn oil comparisons at E15.5 presenting with 49 significantly different genes.

Validation of these findings could include qPCR of an identified a gene whose expression was significantly different in the untreated comparison and not significant in the corn oil treated comparison at E13.5 and E15.5. Or, in a analogous fashion, identify the genes that were not present in the untreated comparisons and which were then present in the corn oil experiments to identify the genes are possibly antagonized by ER activation.

**Steroid 5 alpha reductase 3- Srd5a3**

The *Srd5a3* gene encodes an enzyme involved in the production of DHT from testosterone, and thus is involved in the maintenance of the androgen-androgen receptor activation pathway (38). It is also necessary for the N-linked glycosylation of proteins. Defects in SRD5A3 are the cause of congenital disorder of glycosylation type 1Q in humans, which cause a range of abnormalities mainly due to the metabolic absence of glycosylated proteins. SRD5A3 sequence predicts a steroid 5a-reductase domain, and mutation of the SRD5A2 gene in human causes male pseudohermaphroditism resulting from an enzymatic block of the conversion of testosterone to DHT (39). Still, biochemical and clinical investigations in the patients with SRD5A3 mutations displayed
no abnormal sexual abnormalities that would have suggested a primary defect of steroid metabolism (40). Further investigation will have to be performed to understand the role of Srd5a3 in sexual development.

**Gremlin 2- Grem 2**

A related Gremlin gene, Grem1 is an antagonist of Bmp2 and Bmp4, which are both localized to the mesenchyme of the genital tubercle from E11.5-14.5, and are also implicated in proliferation and outgrowth of the genital tubercle. BMP2 and BMP4 are possible downstream targets of Shh, and in its absence are down-regulated resulting in an increase in apoptosis in the genital tubercle (16). Through this gene expression analysis, Grem2 is implicated as a possible downstream target of androgen receptor, and if, like Grem1, it is an antagonist of Bmp2 and 4, it may play a role in regulating organogenesis, patterning, and tissue differentiation. This AR-mediated inhibition of Bmp2 and 4 would allow for proliferation and outgrowth of the genital tubercle in males. It has been shown that suppression of BMP signaling through mutations of the receptor Bmpr1a has resulted in reduced apoptosis, which reinforces this hypothesis. (41) Unfortunately, Grem2 knockout mouse has yet to be developed, so this hypothesis cannot be directly tested. The upstream disruption of Grem2 via anti-androgens provides an intriguing explanation for congenital birth defect localized to the external genitalia.

**Androgen Targets After the Onset of Sexual Differentiation, E15.5**

**Insulin like growth factor 1- IGF-1**

Igf1 is an endogenous activator of the AKT signaling pathway, a stimulator of cell growth and proliferation, and a potent inhibitor of programmed cell death. Igf1 has been implicated in the embryo as necessary for proper growth of Sertoli and androgen producing Leydig cells through its ability to stimulate cell growth and proliferation (42).
An AR/Igf1 positive feedback system has also been identified in prostate cells but it has never been implicated in genital development (43). As a possible target of the AR, the down regulation of Igf1 through anti-androgens during sexual development is an interesting topic that will need to be address further to elucidate its possible role in masculinizing the genital tubercle.

**SPARC related modular calcium binding - Smoc1**

Smoc1 is a matricellular protein possibly involved in angiogenesis as well as growth factor signaling, proliferation, and migration in the embryo. As a matricellular protein, it has the capability to interact with hormones, cell-surface receptors and other bioeffector molecules (44). Smoc1 is expressed in the murine fetal gonads beginning at E11.5 through E15.5, but its expression has not been characterized in the genital tubercle (45). Further work will have to be done to see if Smoc1 is implicated in the morphologic changes necessary for development and virilization of the genital tubercle.

**Fras related protein 2 – Frem2**

Frem2, like Fras1 that was expressed temporally in males and females and dimorphically at E15.5, encodes a membrane protein that belongs to the FRAS1 family. It plays a role in epidermal-dermal interactions during morphogenetic processes. Unlike Fras1, Frem2 loss of function experiments have resulted in defects in developmental events associated with coordinated migrations and cellular rearrangements of all three germ layers. This suggests that Frem2 may provide a substrate for rearrangements during embryogenesis by altering the extracellular matrix. Frem2 is expressed during development and precedes morphogenetic rearrangements in many tissues, with its loss leading to defects of these tissues (46). With Frem2 being implicated as a target of AR in the genital tubercle during sexual differentiation, in conjunction with its roll of providing
a foundation for tissue rearrangements in embryogenesis, it is an interesting candidate for masculinization of the tubercle.
CHAPTER 5
CONCLUSION

Taken together the results presented here are consistent with the idea that the formation of male and female external genitalia is not a binary switch; it is a continuum from absolute male to absolute female mediated by differential regulation of hundreds of genes. While the early establishment of chromosomal sex may define male or female, the manifestation of phenotypic sex largely involves the regulation of genes important in key cellular processes rather than broad pattern formation. In identifying the genes that are dimorphically expressed at key stages in sexual development, and differentially expressed within sexes during sexual development, than maybe we can understand the continuum fully, and what causes the deviations from absolute male and absolute female.

This gene expression analysis lays a foundation for further study and identification of the interaction between the genes differentially expressed in so that we may understand the molecular mechanisms within the androgen driven, morphological conversion from indifferent genital tubercle to phenotypic male.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Jennifer Angelica Fernandez was born in 1986 in Monterey, California. The youngest of two children, she grew up in a military household, moving across the world and finally settling in Melbourne, Florida. She earned her Bachelor of Science in Integrative Biology the University of Florida in 2008. Upon completion of her bachelor’s degree she entered a master’s degree program in Zoology, also at the University of Florida. A short year and a half later, she preemptively began a Doctorate of Medical Dentistry program at the University of Pennsylvania, only to return a year later to finish her master’s degree.

Upon completion of her master’s degree program in December 2011, she will be continuing her dental degree in Philadelphia, which will be completed in May 2014. She plans to continue her education in a specialty program.