# MOLECULAR MECHANISMS OF SEX DETERMINATION

# AND TESTIS DIFFERENTIATION

By

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MOLECULAR MECHANISMS OF SEX DETERMINATION

AND TESTIS DIFFERENTIATION

Abstract

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Decreasing sperm counts in human populations over the last century is a trend of increasing concern. Several recent studies have shown that embryonic environment and exposures can lead to adult onset and trans-generational disease including reduced sperm count and motility in rodent models. To better understand how atypical sexual development can lead to reduced fertility, it is critical to determine the mechanisms of the SRY directed program of male sexual differentiation, its disruption, and the fetal basis of adult onset disease. In this thesis, the mechanisms involved in mammalian testis development are investigated. Transcriptional regulation during sexual differentiation was investigated using a genomic microarray approach to determine how transcriptional and morphological events are orchestrated. Cellular pathways and processes affected by genes regulated in testis differentiation included Wnt and Notch signaling, cellular differentiation, proliferation, focal contact, RNA localization, and development. Transcriptional alterations of testis differentiation by the fungicide vinclozolin were also investigated using a microarray approach to identify candidate mechanisms of action leading to adult onset and trans-generational transmission of disease. The genes and processes affected suggest apoptosis and vascular formation may be affected by vinclozolin treatment and transcriptional and epigenetic mechanisms may be important for mediation of affects seen with

vinclozolin treatment. These studies have identified many new candidate genes with known functions that suggest roles which may help to fill in gaps in the understanding of testis development and its disruption leading to adult onset trans-generational disease. To connect candidate genes and processes in the sex determination cascade to one another, their regulation and function will also need to be studied. In this thesis, *Nt3* transcriptional activation was investigated as a direct target of SRY action. Results show SRY and SOX9 have the ability to activate the *Nt3* promoter in a site specific manner providing preliminary evidence that it may be one of the missing links directly downstream of *Sry* for induction of male sex determination. The sum of work in this thesis has contributed to the understanding of functional and disrupted sex determination and testis development. These studies have significantly contributed to information on gene regulation during sex determination and provide multiple new candidate genes.

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# CHAPTER 1

## INTRODUCTION:

### The Complexity and Complications of Mammalian Sex Determination

Dimorphic sexual phenotypes are required for mammalian reproduction. The two distinct sexual phenotypes develop through a process called sex determination. Atypical development associated with sex determination can lead to infertility and intersex disorders [1;2]. Several recent studies have shown that embryonic environment and exposures can lead to adult onset and trans-generational disease including reduced sperm count and motility [3;4]. Decreasing sperm counts in the general population over the last century is a trend of increasing concern [5]. It is estimated that as many as 10-15 percent of couples struggle with infertility and annually tens of thousands of these individuals will turn to assisted reproductive technologies in the United States alone [6].

Many couples affected by infertility have conditions arising from genetic disorders or atypical sexual development. Intersex disorders are estimated to affect two percent of individuals, with a conservative estimate of 1 in 2000 requiring surgery for gender assignment [7]. Proper gender assignment is not always clear and surgeries are not always successful or appropriate, causing distress for parents and affected children as they try to fit into the sexually dichotomous social systems heavily ingrained into our society [8].

To better understand the atypical sex determination and development that can lead to infertility and intersex disorders, it is critical to determine the mechanisms of normal sexual differentiation, disruption, and the fetal basis of adult onset disease. In this thesis, the mechanisms involved in normal and disrupted sex determination and testis development will be

discussed. In a simplistic view, mammalian sex determination can be broken down into three distinct parts: chromosomal, gonadal, and phenotypic.

### Chromosomal Sex Determination

Chromosomal sex is determined at conception in mammals by the paternal inheritance of an X or Y chromosome, with the maternal sex chromosome contribution always being an X in normal fertilization events. If the fertilizing haploid sperm carries a Y chromosome then the chromosomal sex of the resulting individual is male. If the sperm carries an X then the chromosomal sex is female. In mammals it is the presence of the Y chromosome, not the ratio of sex chromosomes, which determines sex. This is demonstrated in cases where the number of inherited sex chromosomes is not equal to two. Individuals with an XO or XXX karyotype develop as phenotypic females and XXY or XYY individuals develop as phenotypic males [9;10].

Primary sex reversed individuals in which chromosomal sex did not match gonadal sex were helpful in identification of the specific Y chromosome gene acting as the master sex determination switch [11]. In many phenotypically male XX individuals, translocation of a portion of the Y chromosome was identified. These regions contained the Sex determining Region of the Y chromosome (SRY) gene which confers maleness [12-14]. It was also found that in XY sex reversed female individuals, mutations in SRY had often occurred leading to the female phenotype. These observations have lead to the understanding that the basis of chromosomal sex determination in mammals is the transmission of the master regulator SRY.

### Development of the Bipotential Gonad

Gonadal sex determination is necessarily preceded by the development of the gonad, which then has two distinct possible developmental fates, the testis or ovary. This bipotential

gonad contains four basic bipotential cell types including primordial germ cells, somatic germ cell supportive cell precursors, steroidogenic cell precursors and interstitial cells. These cell types have several distinct developmental origins (reviewed in [2]).

The gonad forms from the urogenital ridge adjacent to the mesonephros. The urogenital ridge arises from mesodermal tissue and can be seen in the mouse at embryonic day nine (E9) as a layer of coelomic epithelium [15-17]. Primordial germ cells arise extra embryonically from pluripotent epiblasts in the yolk sac [18]. These primordial germ cells migrate from the extra-embryonic sites in the yolk sac, through the mesentery of the gut and the mesonephros to colonize the genital ridge between E9.5 and E10 in mouse [19-21], and E10-11 in the rat [22]. Then, the ventro-medial surface of the genital ridge thickens and forms what is now called a bipotential gonad around E10-11.5 in the mouse [15;23] and E12 in the rat [22].

Several genes are important in the development of a bipotential gonad. Genes affecting these early stages of sexual development include *Wt1*, *Nr5a1* (referred to as *Sf1*), *Lim1*, and *Emx2* [24-27]. Knockouts of these genes in mouse show the beginnings of gonadal development but gonads regress by E14.5. The knockouts can also affect kidney and/or brain development. *Wt1* is expressed in the genital ridge of the mouse at E9.5, and later in the supportive cells of the developing gonads [28]. In the absence of WT1, *Sf1* is not expressed. In *Wt1* and *Sf1* mutants, gonads do not form [26] leading to sterile phenotypic females for both XX and XY individuals. In normal development these and other genes direct the development of bipotential gonads primed for sex determination.

## Gonadal Sex Determination, SRY, and SOX9

Once a bipotential gonad has formed, there is a critical period in which the sexual fate of the gonad is decided. It is the expression of the sex determining gene SRY in pre-Sertoli cells of

a developing embryos gonad that determines gonadal sex. *Sry* expression is regulated by autosomal genes including WT1, FOG2, and GATA4, but *Sry* is only present and expressed in XY males [29;30]. It is expressed transiently in mouse only in developing Sertoli cells between E10.5 and E12.5, where it must play its functional role [15;30-32]. It has been shown that SRY is required for the differentiation of Sertoli cells [32-34]. SRY also induces migration of mesonephric cells to the genital ridge [35] and the proliferation of cells in the genital ridge [17] although these actions are likely through indirect mechanisms. Other downstream actions include Leydig cell differentiation and vascularization.

Although SRY was determined to be the critical factor for sex determination in the early 1990's [12-14], it remains to be determined how SRY directs the differentiation of the sexes. The expression pattern of SRY described above has given insight. Further insights have come from studying the structure of SRY. This is a single intron gene [36], and the human mRNA is about 830 nucleotides with a 77 nucleotide 5' sequence and 137 nucleotide 3' sequence surrounding an ORF encoding a 204 amino acid protein. The mouse has an mRNA that encodes a 395 amino acid protein with a 237 nucleotide 5' end and a very large 3481 nucleotide 3' end [15;37]. Between species there is little homology, suggesting rapid sequence evolution, and there is only one highly conserved area which contains the HMG box region [38]. This region is a 79 amino acid motif that can be found in many High Mobility Group (HMG) proteins that associate with DNA [39]. Studies have shown that the HMG box of SRY binds to DNA in a sequence specific manner [40-43] and is able to induce a bend of 60-85$^{o}$ in the DNA [44;45]. Nuclear localization signals have also been found to flank the HMG region allowing SRY to enter the nucleus [46;47]. These features of SRY have lead to the idea that it is an architectural transcription factor [48], and SRY may act as a conventional activator or repressor. Mouse SRY

transcriptional activation requires a C terminal glutamine-rich domain not present in other species [49], suggesting SRY may function differently between mammalian species. It is also interesting to note that a role for SRY in mRNA splicing has been discussed [50].

No direct targets of SRY have been conclusively identified. It has however been suggested that SOX9 may be a direct downstream target of SRY and convey male differentiation [51;52]. SOX9 is critical for male sex determination [53;54] and its over expression leads to XX sex reversal. Haploinsufficiency in humans leads to XY sex reversal. These observations suggest that SOX9 is necessary and sufficient for male sex determination and can substitute for SRY in this role [55]. It is interesting to note that in mice gonad specific SOX9 knockouts, but not heterozygous SOX9 mutants, are sex reversed [56;57]. This suggests a dosage sensitivity to SOX9 in humans, not seen in mice.

SOX9, like SRY, is an HMG box factor with about 87% amino acid similarity to SRY in that region. It has been shown to bind similar sequences and bend DNA [58]. SOX9 is also expressed in Sertoli cells, at the time of sex determination, with increased expression in testis beginning at the peak of SRY expression [51;59-61]. Furthermore, SRY is down-regulated after SOX9 expression is up-regulated suggesting that the only direct target of SRY may be SOX9, which then suppresses SRY and promotes the events of testis differentiation. Whether or not SRY and SOX9 form a regulatory loop, the similarities in structure and function require investigation of the involvement of both genes when considering direct regulatory targets for one or the other.

Unlike SRY, direct targets for SOX9 regulation have been identified including Anti Mullerian Hormone (AMH) [62;63], Vanin1 [64], and Prostaglandin D synthase [65]. Of these a role for AMH and PDGS have been characterized. AMH is critical for regression of the

Mullarian ducts in developing males [65]. It is regulated by SOX9 in cooperation with SF1, GATA4, WT1, and DAX1. PDGS is up-regulated by SOX9 and acts through inter-cellular signaling mechanisms to upregulate Sox9 expression in other interstitial testis cells to induce Sertoli cell differentiation. However, the role and regulation of AMH and PDGS only provide a link between SOX9 regulation and a small portion of the events of testis determination. Many genes and interactions downstream of SRY and SOX9 remain to be discovered.

## Testis Differentiation and Development

In male gonadal sex determination, SRY expression leads to a complex cascade of events including Sertoli, germ, and steroidogenic cell differentiation in addition to vascularization, testis cell proliferation, and testis cord formation resulting in the development of a testis (reviewed in [29]). The proper differentiation of the gonad is indeed both complex and imperative for functional gametogenesis and hormone synthesis. In the male, this process begins with SRY induced pre-Sertoli cell proliferation and differentiation. Sertoli cells form from proliferating supportive cell precursors within the coelomic epithelium between E10.5 and E11.5 in the mouse and E13 in the rat, and then generate aggregates with the primordial germ cells [16;66;67]. Although germ cells are required for spermatogenesis, they are not required for testis development [68;69]. It is the differentiating Sertoli cells that orchestrate subsequent events in testis differentiation.

One process coordinated by Sertoli cells is testis cord formation. After aggregation of germ and Sertoli cells, the Sertoli cells accumulate extra-cellular matrix proteins on their basal surfaces and become polarized [70]. At E11 in the mouse, mesenchymal cells begin to migrate from the mesonephros into the gonad in a male specific manner, and these mesonephric cells are required for further organization of the testis. By E12.5 peritubular myoid cells have begun to

surround germ cell aggregates and help form the basal lamina and seminiferous cords [17;71;72], which occurs in the rat at E13 [22;67]. This cord formation is the first morphologically significant event in sex determination.

Differentiation of steroidogenic cells is another important hallmark of gonadal differentiation. Leydig cells, responsible for testosterone production, are found in the gonad by E11.5 in mouse and originate from steroidogenic cell precursors also found in the mesonephros [73-75]. The production of hormones by Leydig cells is critical in the subsequent development of the male phenotype.

Within the developing testis cords, immature germ cells first cluster in the center of the cord and then are directed toward the basal lamina. It is once again the Sertoli cells that direct the differentiation of these prespermatogonia. Once in this spermatogonial niche, germ cells proliferate and are then maintained in a premeiotic state until puberty [76]. The proper localization of germ cells to this niche and communication with the Sertoli cells is critical for male fertility.

After the various testicular cell types come together to form the structured seminiferous cords, there is a sex-specific growth in the male gonad that includes proliferation of germ, Sertoli, peritubular, and other interstitial cells [77;78]. By E15 the mouse testis is twice the size of the ovary and cells continue to proliferate until puberty [79]. Development of the testis is complete at puberty. At this time Sertoli cells are no longer mitotic, the testis cords form lumens to become tubules, and spermatogenesis initiates.

Several factors produced in the testis have been implicated in its development and testis cord formation. Dmrt-1 is expressed at E10.5 but is present in both sexes at this time. By E12.5, expression of Dmrt-1 is restricted to Sertoli and germ cells in the developing testis. Male Dmrt-1

mutants are sex reversed or infertile and females appear normal, suggesting a critical role specific to males [80;81]. Growth factors are important players in gonadal development also. Of particular interest is FGF9, expressed only in male gonads from E11.5 to E12.5. FGF9 mutants show reduced proliferation of coelomic epithelium and reduced Sertoli cell numbers, while FGF9 knockouts often show sex reversal [82]. The neurotropic growth factor Nt-3 has also been implicated in testis development. The low-affinity nerve growth factor receptor is expressed in mesonephros of XY individuals prior to cord formation, and later in peritubular cells [83]. The TrkC high affinity receptor for NT3 has also been shown to be expressed in mesonephros prior to cord formation and later in peritubular cells, and TRKC expression is important in cord formation [83-85]. The role of NT3 will be further discussed below. Another set of signaling receptors involved in testis development are the insulin receptors. XY mice with all three insulin receptors knocked out are sex reversed [86]. Although many factors involved in testis differentiation have been discovered, much remains to be learned about this complex process, including gene regulation and signal transduction pathways.

**Neurotrophin 3 and Testis Cord Formation**

One specific factor important for male gonadal sexual differentiation is NT3. NT3 and other members of the neurotropic growth factor family have been extensively studied for their role in neuron growth and development [87-89]. However, these growth factors and their receptors are involved in non-neuronal mesenchymal-epithelial interactions and tissue morphogenesis [90-95] and are also involved in cell-cell interactions in the postnatal testis [96-99]. NT-3 is highly conserved between human, rat, and mouse [100], and acts through the high affinity receptor kinase TrkC and the low affinity receptor p75NTR. p75NTR has been shown to mediate neurotropin responses to the Trk receptors [101]. The Trk receptors stimulate several

signaling pathways including PI3K [102;103] affecting cell survival and differentiation, chemotaxis, and protein trafficking [104]. Of interest here is the role of NT-3 in the embryonic testis. It was suggested that there is a chemotactic factor that directs mesonephric cell migration into the testis [72]. The mesonephros is not required for Sertoli cell differentiation, however, cells migrating from the mesonephros are required for testis cord formation and development of a functional testis [71;105]. In the mouse and rat, p75NTR is expressed in mesonephric cells that migrate to the developing testis [83;85;106]. NT-3 is expressed in Sertoli cells at E14 in the rat just before cord formation, and TrkC is expressed at E14 in the mesonephros around its ducts. TrkC is expressed at E16 in the interstitium, and in the peritubular cells at E18 [83;107]. Blocking of the high affinity receptors with receptor specific blocking agents or receptor IgG molecules blocks cord formation, demonstrating these receptors are required for cord formation and testis differentiation [83;108]. Cupp et al [107;108] were able to show that blocking of NT-3 with antisense molecules also inhibits cord formation, whereas addition of NT-3 caused mesonephric cell migration. TrkC and TrkA knockout mice show a reduced number of cords forming and NT-3 knockouts appear normal. This suggests that compensation occurs between neurotrophin receptors and ligands [83;109]. PI3K inhibitors have also been shown to inhibit cord formation [110] supporting the involvement of Trk receptor signaling in cord formation.

## Ovary Differentiation and Development

In female gonadal sex determination, which occurs in the absence of SRY, the bipotential gonad initiates a series of events leading to ovary formation. Supporting cells begin to differentiate into granulosa cells but require the presence of meiotic germ cells for complete differentiation and follicle formation [11;69;111]. In the absence of meiotic oocytes, pre-follicular cells degenerate and supportive cells can apparently trans-differentiate into Sertoli cells

resulting in ‘streak gonads’ [112]. This requirement for the presence of germ cells for ovarian development contrasts with the male where apparently normal testes develop in the absence of germ cells. It is also interesting to note that extra gonadal germ cells, such as those that inappropriately migrate to the adrenal gland or those cultured in lung, develop as female. The extra gonadal germ cells then undergo meiosis and immediately proceed to form developing oocytes [76]. A proximity to pre-Sertoli cells is required for male germ cell sex determination, while the ovarian environment is required for meiotic arrest in oocytes [76;113].

Although ovarian differentiation is considered the default developmental pathway, it requires its own complex cascade of events. WNT4 is known to be critical for ovarian development. WNT4 regulates *Dax1* which is expressed at E10.5 in the bipotential gonad, then repressed in males at E12.5 [114]. *Dax1* is thought to be an SRY antagonist as its over-expression masks the effects of SRY and causes XY sex reversal. Mutation of WNT4 or DAX1 can cause streak gonads to form. A few other genes involved in ovarian development have been identified including FOXL2 involved in follicle formation [29], BMP2, and FST. In general, however, the process of ovary differentiation and development is less understood then testis development and much remains to be discovered about early ovarian differentiation events.

**Phenotypic Sex Determination and Development**

Phenotypic sex refers to all sexually dimorphic characteristics outside of the gonad. This includes development of the genital ducts, external genitalia, secondary sex characteristics appearing at puberty, and development of brain regions associated with gender identity which is sometimes considered a separate, fourth dimension of sex determination [115]. All of these phenotypic sex characteristics develop in response to secreted AMH and/or sex steroids produced by the gonad.

Two sets of genital ducts, the Mullerian and the Wolffian, develop from the mesonephros in male and female embryos. In male embryos the production of AMH by differentiating Sertoli cells in the gonad causes an apoptotic regression of the Mullerian ducts [63]. SOX9, in conjunction with SF1, regulates AMH which is produced from E11.5 to E12.5 in Sertoli cells of the developing testis [116]. It is the influence of testosterone produced by fetal leydig cells, however, that promotes development of the Wolffian ducts into epididymis, vas deferens, and seminal vesicles. In the female, the absence of testosterone leads to degradation of the Wolffian ducts leaving the Mullerian ducts to develop into oviducts, uterus, and vagina. In contrast to male ductile development, the differentiation of female structures from the Mullerian ducts is not dependent on steroidal hormones or any other ovarian produced signal. However, stabilization of these structures requires expression of several genes including WNT4 and DAX1 [117;118].

External genitalia form from the urogenital sinus. This sinus initially develops identically in both sexes with a urogenital slit, genital tubercle, urethral folds, and labioscrotal swellings. In males, the urogenital sinus differentiates in response to dihydrotestosterone (reviewed in [119]). Testosterone, produced in the developing testis, is converted to dihydrotestosterone by 5α-reductase expressed in the urogenital sinus. Dihydrotestosterone then binds the androgen receptors of the urogenital sinus and promotes differentiation into a penis with corpus spongiosum and penile urethra, scrotum, prostate, and bulbourethral glands. In the absence of androgens, the urogenital sinus feminizes and develops into the clitoris, labia, and separated urinary and vaginal openings. The vagina then fuses with the developing Mullerian ducts to complete proper formation.

## Disruption of Sexual Differentiation by Environmental Exposures

In addition to a host of genetic aberrations, the embryonic environment and exposures to certain chemicals are increasingly implicated in altering development and decreasing reproductive capacity [120;121]. Epidemiological studies have revealed increasing problems in male reproductive tract development and fertility linked to the embryonic developmental environment [122;123]. Causes of altered development may include higher maternal age, inhibited fetal growth, or exposure to sex steroids or endocrine disrupting compounds.

Endocrine disrupting compounds can act on hormone receptors as hormone agonists or antagonists. They have been implicated in a host of reproductive abnormalities in wildlife and human populations [124-128]. Diseases in humans which may be caused by endocrine disruptors include decreased sperm counts and gonadal dysgenesis, hypospadias, and cryptorchidism [129]. In other species sterility and intersex disorders have been observed. In certain populations of particularly sensitive aquatic species, feminization occurs pervasively [126;130-133]. Several endocrine disrupting compounds increasingly used in developed countries have been shown to inhibit male sexual development [134-136]. Since embryonic exposures, especially to endocrine disruptors, are largely controllable, it is necessary to gain a better understanding of the alterations of sexual development induced and mechanisms of action of these compounds. Such information will assist in implementing appropriate guidelines and use restrictions by organizations such as the Environmental Protection Agency.

## Vinclozolin Alteration of Sexual Development

Vinclozolin is a chemical compound used as a fungicide that acts as an anti-androgen endocrine disruptor by inhibiting androgen receptor function [136;137]. Vinclozolin is currently approved by the Environmental Protection Agency for use as a fungicide on both ornamental and

food crops. As discussed above, androgen function is required for male reproductive development and vinclozolin exposure of neonates has been shown to affect both embryonic development and adult onset disease states. Exposure of rats between E12 and postnatal day 3 (P3) revealed malformations in the reproductive tracts and renal systems of male offspring with highest sensitivity between E14 and E19 [125;138]. These alterations are due in part, if not completely, to interference of androgen signaling. However, embryonic vinclozolin exposure during gonadogenesis and sex determination, prior to androgen receptor expression, can cause increased apoptosis in the pubertal and adult rat testes and decreased sperm numbers and motility [4;139]. It is suspected that the alterations leading to these adult onset disease phenotypes are caused by altered Sertoli or germ cell development leading to altered germ cell maturation and sub-fertility however the mechanisms of the fetal basis of adult onset diseases are not known.

Also of great interest is the fact that reduced sperm count and motility due to vinclozolin treatment during gonadal sex determination is transmissible through at least three subsequent generations [4;140]. Transgenerational disease transmission was seen when pregnant F0 mothers were injected with vinclozolin from E8-E14. This means that the F1 generation was exposed during gonadal development and the germline for the F2 generation within those developing gonads also was exposed to vinclozolin treatment. However the F3 generation males which were not exposed to vinclozolin also show increased testis cell apoptosis and reduced sperm counts and motility [4]. For this trans-generational aspect of vinclozolin induced disease, an epigenetic mechanism based on DNA methylation changes has been proposed [140]. The actions of vinclozolin on the embryo at the time of treatment and the resulting mechanisms of action leading to trans-generational disease phenotypes however, currently remain unknown.

## Scope of the Thesis

This thesis aims to obtain a better understanding of the regulation and disruption of gonadal sex determination and differentiation in mammals. The obtained results are presented in three parts. In the first part (chapter two) transcriptional regulation during sex determination and differentiation was investigated. Since relatively few genes have been identified for such a complex and critical process, a microarray approach was taken to identify novel candidate genes. Specific focuses included potential direct mechanisms of action of SRY in relaying gonadal and phenotypic sex, how processes of gonadal sex determination including differentiation of various bipotential cell types are regulated downstream of SRY and SOX9, and how morphological events of sexual differentiation are orchestrated through transcriptional control. In chapter three, the alterations of testis differentiation by vinclozolin are investigated using a microarray approach to identify candidate mechanisms of action of vinclozolin on the embryonic gonad which may lead to a better understanding of the resulting adult onset and trans-generational transmission of disease. The roles of altered Sertoli or germ cell development, and the involvement of epigenetic regulation are considered. In the fourth chapter, transcriptional regulation of Neurotrophin 3 by SRY and SOX9 is investigated. Promoter mutational and activation assays were conducted by transfection of cultured cells, and attempted promoter/protein interaction assays are discussed. This work was done to determine if SRY or SOX9 could directly regulate *Nt3* to gain a better understanding of the SRY directed program of male sex determination.

The above areas of investigation contribute to a better understanding of normal sexual differentiation. This may then provide a groundwork for better understanding the causes of some infertilities, aberrations of sexual development, and adult onset trans-generational disease.

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# CHAPTER 2

# REGULATION OF THE GONADAL TRANSCRIPTOME DURING SEX DETERMINATION AND TESTIS MORPHOGENESIS: COMPARATIVE CANDIDATE GENES

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**ABSTRACT**

Gene expression profiles during sex determination and gonadal differentiation were investigated to identify new potential regulatory factors. Embryonic day 13 (E13), E14, and E16 rat testes and ovaries were used for microarray analysis, as well as E13 testis organ cultures that undergo testis morphogenesis and develop seminiferous cords *in vitro*. A list of 109 genes resulted from a selective analysis for genes present in male gonadal development and with a 1.5-fold change in expression between E13 and E16. Characterization of these 109 genes potentially important for testis development revealed that cytoskeletal-associated proteins, extracellular matrix factors, and signaling factors were highly represented. Throughout the developmental period (E13–E16), sex-enriched transcripts were more prevalent in the male with 34 of the 109 genes having testis-enriched expression during sex determination. In ovaries, the total number of transcripts with a 1.5-fold change in expression between E13 and E16 was similar to the testis, but none of those genes were both ovary enriched and regulated during the developmental period. Genes conserved in sex determination were identified by comparing changing transcripts in the rat analysis herein, to transcripts altered in previously published mouse studies of gonadal sex determination. A comparison of changing mouse and rat transcripts identified 43 genes with species conservation in sex determination and testis development. Profiles of gene expression during E13–E16 rat testis and ovary development are presented and candidate genes for involvement in sex determination and testis differentiation are identified. Analysis of cellular pathways did not reveal any specific pathways involving multiple candidate genes. However, the genes and gene network identified influence numerous cellular processes with cellular differentiation, proliferation, focal contact, RNA localization, and development being predominant.

## INTRODUCTION

Prior to gonadal sex determination, primordial germ cells migrate from extra-embryonic sites in the yolk sac through the mesentery of the gut and the mesonephros to colonize the genital ridge at embryonic day 10 (E10)–E11 in the rat (Jost *et al.* 1981, Ginsburg *et al.* 1990). A bipotential gonad is formed at E12 in the rat and has the potential to develop into either a testis or an ovary (Jost *et al.* 1981). Sex determination begins at E12.5 in the rat when the sex determining region of the Y chromosome (*Sry*) gene is expressed in the Sertoli cells of males. *Sry* is a mammalian sex determining factor leading to male development (Koopman *et al.* 1990). In the male, Sertoli cells arise from proliferating supportive precursor cells between E12.5 and E13 in the rat and coincides with the onset of *Sry* expression. Sertoli cell precursors form aggregates with the primordial germ cells (Magre *et al.* 1980, Karl *et al.* 1998). Mesenchymal peritubular myoid precursor cells migrate from the mesonephros into the gonad in a male specific manner and surround Sertoli/germ cell aggregates to promote formation of seminiferous cords around E14 in the rat (Magre *et al.* 1980, Jost *et al.* 1981, Buehr *et al.* 1993, Martineau *et al.* 1997, Levine *et al.* 2000, Schmahl *et al.* 2000, Cupp *et al.* 2003). Once peritubular myoid cells and Sertoli cells are in contact, an extracellular matrix is secreted and separates the testis cords from the interstitial tissue (Tung *et al.* 1984, Kanai *et al.* 1992). This cord formation is the first morphological event in sex determination and is imperative for proper testis development.

Vasculature development and coelomic vessel formation is also characteristic in testis development (Yao *et al.* 2006). During this embryonic period Leydig cells, which are later responsible for testosterone production, arise from the mesenchymal interstitial cells (Merchant-Larios & Moreno-Mendoza 1998, Capel 2000, Nishino *et al.* 2001). After seminiferous cord

formation, there is sex-specific growth in the male gonad that includes proliferation of Sertoli, germ, peritubular, and interstitial cells (Mittwoch *et al.* 1969, Chubb 1992, Levine *et al.* 2000).

A number of genes have previously been shown to be critical for sex determination and testis development (Morrish & Sinclair 2002, Yao *et al.* 2002, Jameson *et al.* 2003). *Sry* was determined to be an essential factor for sex determination in the early 1990s (Berta *et al.* 1990, Koopman *et al.* 1990, Sinclair *et al.* 1990), however, it is presently unknown how *Sry* directs sexual differentiation. *Sox9*, a high mobility group (HMG) domain transcription factor closely related to *Sry*, causes sex reversal when over-expressed in the female or when inactivated in the male (Jost *et al.* 1981, Ginsburg *et al.* 1990, Wagner *et al.* 1994, Vidal *et al.* 2001). Doublesex and mab3 related in testis 1 (*Dmrt1*) is turned off in the developing ovary and is expressed in the developing testis of a number of species. Male *Dmrt1* mutants are sex reversed or infertile, while females appear normal (Raymond *et al.* 1999, 2000). The dosage-sensitive sex reversal – adrenal hypoplasia congenita gene on the X chromosome gene 1 (*Dax1*) is an orphan nuclear receptor thought to be an SRY antagonist since *Dax1* over-expression masks the effects of *Sry* in sex reversal (Swain *et al.* 1998). *Dax1* is expressed prior to sex determination in the bipotential gonad, then is repressed in the male during sexual differentiation, while persisting in the ovary (Swain *et al.* 1996). Anti-Müllerian hormone (AMH) is a secreted factor produced by Sertoli cells responsible for the regression of the Müllerian ducts in the mesonephros leaving the Wolffian ducts to develop a subsequent male reproductive tract (Munsterberg & Lovell-Badge 1991, De Santa Barbara *et al.* 1998). SOX9, in conjunction with steroidogenic factor 1, is thought to regulate the *Amh*/Müllerian inhibiting substance expression (De Santa Barbara *et al.* 1998, Jamin *et al.* 2002). Fibroblast growth factor 9 (*Fgf9*) is expressed in male gonads early in testis development and *Fgf9* null mutants show some sex reversal (Colvin *et al.* 2001). In addition, the

neurotropic growth factor NT3 has been shown to be important for testis cord formation (Levine *et al.* 2000, Cupp *et al.* 2003). NT3 is a Sertoli cell product that acts as a chemotactic agent (Cupp *et al.* 2003) to promote cell migration of peritubular cell precursors from the mesonephros by binding its receptor TRKC (Levine *et al.* 2000, Cupp *et al.* 2002, Cupp *et al.* 2003). The insulin family of receptors is also involved in testis development. Male XY mice with all three insulin receptors knocked out are sex reversed (Nef *et al.* 2003). Although several growth factors and transcription factors have been shown to be involved in sex determination and testis cord formation, the present study uses a genomic approach to identify novel factors and signal transduction events. Further identification of factors involved in gonadal development will help elucidate the genomic control of sex determination and differentiation.

Sexual differentiation is required to produce the dimorphic sexes, essential for mammalian reproduction. A delay in sexual differentiation and testis cord formation can lead to sex reversal, infertility, or gonadal dysgenesis. A better understanding of embryonic testis development will help determine how these abnormalities arise. Numerous factors are anticipated to be involved in the key events of embryonic testis development including cellular proliferation, cell migration, cell associations, extracellular matrix remodeling, and vascularization that leads to testis morphogenesis. The present study was designed to identify new candidate genes involved in sex determination and testis development using a genomic approach involving a microarray analysis of gene expression during embryonic gonadal differentiation. The gene expression in E13, E14, and E16 male and female gonads were compared in order to identify sex differences during this developmental period. In addition, rat E13 testis organ cultures, which develop seminiferous cords *in vitro*, were used and compared with *in vivo* testis development (Martineau *et al.* 1997, Cupp *et al.* 2000, 2003, Levine *et al.* 2000, Uzumcu *et al.* 2002).

Comparative microarray analysis was also used to narrow the candidate list of potential regulatory factors for testis development. The rat microarray data obtained herein were compared with data produced for gonadal differentiation in mouse (Nef *et al.* 2005, Small *et al.* 2005, Beverdam & Koopman 2006). Gene lists resulting from these comparative microarray analyses will assist in identifying potential candidate genes in gonadal sex determination.

## MATERIALS AND METHODS

### Animals

Sprague–Dawley rats were kept in a temperature controlled environment and given food and water *ad libitum.* Estrous cycles of female rats were monitored by cellular morphology from vaginal smears (Uzumcu *et al.* 2002). Rats in early estrus were bred overnight and matings confirmed by sperm positive smears, denoted day 0 of pregnancy. Animals were euthanized at E13, E14, and E16 of pregnancy, and gonads were collected for RNA isolation and histology. Sex was determined by PCR using primers specific for *Sry* on genomic DNA isolated from embryo tails as previously described (Levine *et al.* 2000). All procedures were approved by the Washington State University Animal Care and Use Committee.

### Organ cultures

Rat gonads from E13 embryos were dissected with mesonephros intact and cultured 3 days as previously described (Cupp *et al.* 2000). Briefly, gonads were placed in drops of medium on Millicell CM filters (Millipore, Bedford, MA, USA) floating on 0.4 ml of CMRL 1066 medium (Gibco BRL) supplemented with penicillin–streptomycin, insulin (10 µg/ml), L-glutamine (350 µM), transferrin (10 µg/ml), and BSA (0.01%). Media were changed on the second day of culture. Gonads were maintained in culture for 3 days at which time testis cords formed and testes were used for histological analysis or separated from mesonephros and used for RNA collection. Embryonic cultures undergo similar testis morphogenesis after 3 days of culture as that seen *in vivo* (Levine *et al.* 2000, Cupp *et al.* 2003). Analysis of E13 testis viability in culture demonstrated no abnormal histology, with similar morphology as observed *in vivo* (Levine *et al.* 2000, Cupp *et al.* 2003).

### Histology

Tissue specimens were fixed in Bouin's solution for 1 h and embedded in paraffin using standard procedures. Serial sections of 5 µm were stained with hematoxylin and eosin (H&E) using standard procedures by the Histology Core Laboratory of the Center for Reproductive Biology, Washington State University. Sections were visualized by light microscopy.

### RNA preparation

Gonads without mesonephros were collected from female and male Sprague–Dawley rat E13, E14, and E16 embryos. Stage of development was confirmed by counting tail somites of each embryo. Gonad samples were stored in TRIZOL at –20 °C (Invitrogen) until extraction following the manufacturer's protocol. Two separate gonadal sample sets were collected for each E13, E14, and E16 testis and ovary, and E13 cultured testis for replicate analysis. Generally 20–30 gonads were combined for an individual sample. Each separate RNA sample was used for a single microarray chip, such that two separate experiments involving two separate sets of animals and RNA isolations were performed.

### Microarray analysis

High quality RNA samples were assessed with gel electrophoresis and required a minimum $OD_{260/280}$ ratio of 1.8. At least 5 µg RNA per sample was delivered to the Center for Reproductive Biology, Genomics Core Laboratory, Washington State University for processing as previously described (McLean *et al.* 2002, Small *et al.* 2005). RNA was transcribed into cDNA, and cDNA transcribed into biotin-labeled RNA. Biotin-labeled RNA was then hybridized to the rat RAE230A arrays containing ~16 000 transcripts (Affymetrix, Santa Clara, CA, USA) and labeled with phycoerythrin-coupled avidin. Hybridized chips were visualized on an Affymetrix Scanner 3000 (Affymetrix). Once raw data were obtained, they were processed using GeneChip Operating Software (GCOS) version 1.1 (Affymetrix) and analyzed by Genespring

version 7.2 (Silicon Genetics, Redwood City, CA, USA), and Pathway Assist software (Stratagene, La Jolla, CA, USA).

### Bioinformatics and statistical analysis

Initial analysis of microarray data was completed as previously described (Small *et al.* 2005). Microarray hybridization data were examined for physical anomalies on the chip and background noise above a value of 3. Default GCOS statistical values were used for the analysis. All probe sets were scaled to a mean of 125, where signal correlates to the amount of transcript in the sample. An absolute analysis was performed with GCOS to assess the relative abundance of the transcripts on the RAE230A chip based on signal and detection calls (present, absent, or marginal). This information was imported into GeneSpring 7.2 (Silicon Genetics) and normalized using the recommended default normalization methods. This includes setting signal values below 0.01, total chip normalization to the 50th percentile, and normalization of each gene to the median, which allows visualization of data based on relative abundance for a given sample, rather than by comparison with a specific control value (Small *et al.* 2005). The reproducibility between replicate chips was determined and an $R^2 > 0.95$ was judged sufficient to allow two chips to be used per data point, with a $P < 0.05$ confidence. The criteria to consider chip number has been previously described (Chen *et al.* 2004).

Gene expression during sexual differentiation was determined using data restriction and analytical tools within the GeneSpring software. Transcripts with raw signal values above 75 were selected. Previously a raw signal of 50 has been determined to be near background for an absent/present call for expression of most genes. Therefore, a signal of 75 was selected to minimize the inclusion of false positive calls in the analysis. In contrast, a signal of > 100 does not include low expressing genes and excludes positive signals. Therefore, a signal of 75 was

selected as the optimal cut-off. Transcripts with an average fold change of 1.5 or greater in signal intensity between the developmental stages were also selected. Transcripts expressed differentially in a statistically significant manner were determined using a one-way ANOVA parametric test with variances not assumed equal and $P < 0.05$. Statistics were applied to all time points for both testis and ovary samples to determine when statistically relevant changes occurred. Two replicates for each sample were prepared and this allowed a 2 x 2 factorial comparison in the experiment. Unsupervised cluster analysis within the set of transcripts expressed above a signal of 75 allowed for organization of samples by relatedness based on similarity of the expression profiles between different genes and samples (Eisen *et al.* 1998). Gene expression data from mouse embryonic gonads at the time of sex determination produced by Small *et al.*(2005) were obtained from the gene expression omnibus available through NCBI. This data came from E11.5, E12.5, E14.5, and E16.5 mouse embryonic gonadal RNA hybridized to MGU74v2 arrays (Small *et al.* 2005). The raw data were analyzed by GCOS and GeneSpring 7.2 in the same manner as the rat chips above. The finalized mouse list comprised transcripts with a signal above 75, and statistically significant change in expression of 1.5-fold or more. A comparison of mouse genes present with those in the analogous rat list was made.

The rat genome 9999 program included in the GeneSpring software was used to search available promoters in finalized gene lists up to 2500 bases (Chaudhary *et al.* 2005) for the putative SRY binding element (A/T)AACAA(A/T) which would be expected to appear at a frequency of 4096 bps at random. Pathway Assist software (Stratagene) was used to further analyze lists of genes produced in GeneSpring as described previously (Asirvatham *et al.* 2006). The software excludes redundant and non-annotated genes. The final list is then used to produce shortest pathways for identification of cell processes affected and connections between genes of

interest. Each connection was then verified using the PubMed/Medline hyperlink given for each node.

## RESULTS

The Affymetrix RAE230A chip represents ~16 000 transcripts of the rat genome and allows for a large portion of the rat transcriptome to be evaluated. In the present study, male and female developmental periods E13, E14, and E16 were evaluated to examine gonadal development during sex determination and gonadal differentiation. E13 testes cultured for 3 days form testis cords and were then used to elucidate transcripts consistently involved in testis development and cord formation in vivo and in vitro. Histological analysis of the testis from E13, E14, E16, and cultured E13 testis verified the progress of testis cord formation at each stage of development (Fig. 1). No cords were observed at E13 in the male or in the female samples. No significant morphology was observed in the ovary except for the development of oocyte nests at E16 (Fig. 1C). Testis cords with aggregated germ cells and Sertoli cells were seen in both E14 and E16 testis and in cultured E13 testis (Fig. 1E–G).

The transcripts expressed in the E13, E14, and E16 testis and ovary samples and the relationships of the transcriptomes were investigated. A comparison of the duplicate chips for each developmental time point had an R2 > 0.96 demonstrating that the animal, sample, and microarray chip variability was negligible suggesting two chips are adequate. Expression profiles obtained for each sample were analyzed by determining the number of genes present above a raw signal value of 75 in at least one time point of the male or female developmental periods (i.e. E13, E14, or E16). A dendrogram and hierarchical clustering analysis of E13, E14, and E16 samples using genes with a signal above 75 separately in male and female gonadal development are shown for male and female sample sets (Fig. 2). The cluster analysis using GeneSpring software (i.e. link relationships shown on the left side of each dendrogram set) revealed that expression was similar between the E13 and E14 testis (Fig. 2A). The E13 and E14 ovary were

also similar (Fig. 2B). The E13 testis and E13 ovary were also found to be similar (data not shown). E16 ovary clustered distally to E13 and E14 ovary transcriptomes. E16 testis also clustered distally to E13 and E14 testis transcriptomes. This suggests that the later E16 time points are more divergent. A second set of dendrograms were produced for male and female transcriptomes using genes present above 75 with a minimum of a 1.5-fold significant change in the developmental period studied. This analysis was done separately for male (Fig. 2C) and female (Fig. 2D) gonadal development. The E13 cultured testis was included in analysis of the male samples. Although the E13 culture testis and E16 testis transcriptomes appear similar (Fig. 2C), the E13 cultured testis clustered distally to the freshly isolated E13, E14, and E16 testis samples (Fig. 2C). A comparative analysis of transcripts expressed in the E13 cultured testis that have formed testis cords to those expressed in vivo at E16 in the testis was performed. Regulated genes with similar expression changes in vivo and in vitro are more likely to be important candidates in testis development and cord formation, while those not similar may not be essential for these processes. Subtracting those transcripts not consistently regulated in vivo and in vitro allowed the candidate regulatory gene list for involvement in testis cord formation and development to be reduced. The pattern of expression changes was identified by selecting genes that changed over the entire developmental period (E13, E14, and E16). Genes increasing in expression are represented by a color change from blue to red, and genes decreasing in expression are represented by a color change from red to blue. Examples of genes with both increasing and decreasing expression are prevalent in the differentiating testis (Fig. 2C) and ovary (Fig. 2D).

The number of genes expressed above a raw signal of 75 was determined individually for male and female E13, E14, and E16 samples. Genes expressed at each time point were organized

in a Venn diagram (Fig. 3). From the 16 000 genes on the chip, 7740 transcripts were expressed in the male gonad in at least one time point. There were 6560 genes with a signal above 75 in all the three (E13, E14, and E16) testis samples. The remaining 1180 genes were expressed in only one or two of the time points analyzed (Fig. 3A). In the female gonad, 7489 transcripts had a raw signal over 75 in at least one time point. Expression similar to the male was seen with 6472 genes above a signal of 75 in all the three E13, E14, and E16 ovary samples. The remaining 1017 ovary transcripts had a signal above 75 in only one or two time points (Fig. 3B). The similar numbers of expressed genes in the male and female developmental periods suggest that the male and female transcriptional controls of gonad development are equally active.

Genes known to be involved in sex determination and gonadal development; (*Sry*, *Dax1*, *Wnt4*, *Amh, Vanin, Fgf,* and *Wt1*) were used to determine whether expression in the microarray was comparable with that demonstrated in previous studies (Table 1). Expression trends throughout the developmental period matched previously published expression trends for these genes. The *Sry, Vanin, Fgf9,* and *Amh* genes all were expressed in the testis with negligible expression found in the ovary during this developmental period. *Dax1* was initially present in both sexes and then downregulated in the testis as previously described (Swain *et al.* 1996). As expected, the *Wnt4* gene was predominantly expressed in the ovary.The *Wt1* gene is required for gonadal development, hence is expressed in both sexes. Unfortunately, *Sox9* is not in the list as it is not on the RAE230A rat chip. This short list of sex determination genes was selected to validate the microarray and experimental approach.

To identify novel genes that were differentially regulated between E13, E14, and E16 testis or ovary samples, genes with a statistically significant 1.5-fold increase or decrease between time points were determined separately for male (Fig. 4A) and female samples (Fig.

4B). Male and female developmental periods had approximately the same number of statistically significant changes in gene expression between E13, E14, and E16. There were 160 genes with at least a 1.5-fold change in expression in the testis and 175 changing in the ovary between E13, E14, and E16 samples. In the male, the majority of changes occurred between E13 and E14 with 46 genes increasing and 36 genes decreasing in expression (Fig. 4A). Between E14 and E16, the male had 40 genes increase and 16 genes decrease. In the female (Fig. 4B), the majority of gene expression changes occurred between E14 and E16 where there were 43 genes with increased expression and 37 with decreased expression. Between E13 and E14 in the ovary, 36 genes had decreased expression and only 18 increased. The similar number of regulated genes during E13–E16 development in male and female suggests that both have active transcriptional regulation during this development period. Interestingly, the majority of changes in the female occurred later than in the male suggesting that there is a delay in transcriptional activation in gonadal differentiation of the female as compared with the male.

A comparative and subtractive analysis between genes in the in vivo E13, E14, and E16 testis developmental period and genes expressed in the E13-cultured testis was performed to narrow the list of potential candidate genes for involvement in testis cord formation and morphogenesis (Fig. 4C). This allowed elimination of genes not essential for seminiferous cord development, as required genes would need to be expressed both in vivo and in vitro for gonadal and cord development. The testis culture comparison reduced the number of genes regulated in the E13–E14 period to 29 increasing and 18 decreasing, and in the E13–E16 period to 46 increasing and 35 decreasing. Comparative and subtractive analyses lead to a reduction in the list of overall candidate genes from 160 (Fig. 4A) to 109 (Fig. 4C and Table 2). The 51 genes subtracted in this analysis are presented in Supplementary Table 1, which can be viewed online

at www.reproduction-online.org/supplemental/. The 109 gene list represents genes regulated during embryonic E13–E16 testis development and contains potential candidate genes associated with male gonadal sex determination and testis morphogenesis. As discussed below, further subtraction of the female expressed genes provides a male enhanced and regulated list (Fig. 4D).

Functional categorization of the 109 gene list (Table 2) revealed that 23 of the genes are cytoskeletal and extracellular matrix factors and 21 are signaling factors (Fig. 5). Other represented categories include 16 metabolism genes, 9 growth factors, 7 receptors, and 6 transcription/translation factors (Fig. 5). The categorizations were made based on the major cellular function of the gene. The 109 gene list was imported into Pathway Assist (Stratagene) to evaluate cell processes and signaling pathways affected by the 109 genes (Fig. 6). The number of arrows pointing to or from each cellular function box for this gene network indicates the connectivity (Asirvatham et al. 2006) and was used to determine major cell processes affected by the gene set. The resulting gene network indicated that proliferation, differentiation, and maturation (i.e. development) are all affected. Focal contact, RNA localization, and pathogenesis are also connected to the gene network (Fig. 6). A further analysis of 130 different known cellular and signaling pathways using GeneSpring KEGG pathways did not identify any specific pathways where multiple genes (> 2) from the 109 list were involved (data not shown). Therefore, no specific pathways were identified to be influenced by multiple genes in the 109 list. In contrast, major cellular processes were influenced by the 109 list of genes, Fig. 6.

The list of 109 rat genes was compared with mouse genes regulated during E11.5, E12.5, and E14.5 of mouse gonadal development from a previously generated microarray database (Small et al. 2005; available at www.ncbi.nlm.nih.gov/geo). The 15 genes found in both the 109 rat list and identified in the mouse are noted with an ‘S’ in Table 2. The lack of some

homologous transcripts on the mouse and rat chips limits the number of genes in both analyses. From the 109 rat gene list, 68 genes were present on the mouse microarray chip. A calculation of the random chance genes would overlap between the rat and mouse chips indicated that 0.75 transcripts may potentially randomly overlap. Therefore, the 15 genes identified significantly exceed any random overlap expected. Genes appearing in both the rat and mouse analyses can be considered good candidates for involvement in sex determination and testis development.

The 109 genes differentially regulated in the male throughout the developmental period were screened for potential SRY binding elements in promoters. The frequency of the SRY element to randomly appear is every 4096 bp. From the 109 gene list, 21 genes had searchable promoters. From these 21 genes, 13 promoters were found to contain putative SRY binding elements and are marked with an asterisk in Table 2. Although the number of genes identified with potential SRY elements is in part due to random appearance of the SRY element, the lack of an element likely precludes direct binding and regulation by an HMG box protein such as SRY. Therefore, the presence of an SRY element simply indicates the potential for regulation, but does not indicate functional relevance.

Genes with a raw signal above 75 in any female samples (E13, E14, or E16) were removed from the list of the 109 genes to reveal regulated transcripts with enriched expression in the testis versus the ovary. This resulted in 34 genes that were differentially regulated between E13, E14, and E16 in the testis and enriched in the male rat gonad (Fig. 4D). These 34 genes are indicated in bold in Table 2. A similar reduction of the 175 genes expressed above a signal of 75 and with a 1.5-fold change in the ovary resulted in no gene candidates that were both differentially regulated between E13, E14, and E16 in the ovary and female enriched.

To determine whether any expressed genes were female enriched, genes expressed in male and female gonads above a given microarray signal at any time point were determined. This was done for raw microarray signal cut-off values of 75, 100, 150, 200, 250, and 500 (Fig. 7A). Genes expressed above a signal of 75 in the opposite sex were then subtracted to find the number of gender-enriched genes at each raw signal cut-off value (Fig. 7A). The numbers of genes that are female enriched at any cut-off value during this developmental period are fewer than those that are male enriched. Genes enriched above a signal value of 75 represent the portion of genes form the Venn diagrams in Fig. 3 that are gender-enriched. Genes enriched in the male and female above 100 are listed in Supplementary Table 2, which can be viewed online at www.reproduction-online.org/supplemental/. A cut-off of 100 was used for Table S2 due to the large number of genes present above 75, which would make a prohibitively lengthy list.

Genes that are differently regulated between the sexes at a single time point were identified (Fig. 7B) to allow comparison with previously reported mouse studies (Nef et al. 2005, Beverdam & Koopman 2006). Genes with a raw microarray signal of at least 75 and a statistically significant 1.5-fold increase between testis and ovary were identified at each time point individually (Fig. 7B). Genes identified in this analysis will be referred to as gender-enhanced to distinguish them in the discussion herein from the enriched genes represented in Fig. 7A. For each time point, more genes were over-expressed in the female than in the male. There were 62 transcripts found with an expression 1.5-fold higher in the female than in the male at E13, and 30 transcripts with 1.5-fold higher expression in the male at E13. At E14, 36 transcripts had enhanced expression in the female and 25 in the male. At E16, 103 transcripts had enhanced (> 1.5-fold increase) expression in the female and 81 had enhanced expression in the male. A combined total of 316 genes were differentially regulated between the sexes at a given time point

(E13, E14, or E16; Supplementary Table 3, which can be viewed online at www.reproduction-online.org/supplemental/). The list of 109 genes regulated in the male between E13, E14, and E16 (Table 2) was then compared with the 316 genes differentially regulated between the sexes at a single time point (Supplementary Table 3). The 33 genes in both lists are underlined in Table 2 and indicate genes regulated between the sexes and regulated over the developmental period of E13, E14, and E16 in the testis.

Comparison of differentially regulated genes for individual developmental time points between the sexes identified 316 total genes (Fig. 7B and Supplementary Table 3). A direct comparison of genes differentially regulated between the sexes in the rat at E13, E14, and E16 was made to lists of genes differentially regulated between sexes in the mouse at E10.5, E11.5, and E12.5. A list of genes generated in a similar manner for the mouse at individual developmental time points (Nef et al. 2005) was used for comparison, revealing 19 genes differentially regulated between the sexes in both the mouse data and rat data marked with an ‘N’ (Table 3). A similar comparison was made between the rat and the mouse data generated by Beverdam & Koopman (2006). Genes found to be differentially expressed in the rat between the sexes, as shown in Fig. 7B, or changing throughout the developmental period for testis or ovary, as shown in Fig. 4C and B respectively, were compared with published lists of genes regulated in mouse gonadal sex determination (Beverdam & Koopman 2006). This revealed 17 conserved genes between the Beverdam & Koopman mouse data and the rat data from the present study marked with a ‘B’ (Table 3). Previous comparison of the rat 109 list to mouse data by Small et al.(2005) revealed a total of 15 genes conserved and marked with an ‘S’ in Table 2. Three of these genes from the Small et al.(2005) mouse comparison also appear in the comparisons with the Nef et al.(2005) or Beverdam & Koopman (2006) mouse data and are also marked with an

‘S’ in Table 3. Genes identified as being regulated in the mouse and rat during this developmental period of sex determination and gonadal development appear to be conserved in sex determination and are potential candidates for further consideration. A complete list of candidate genes was compiled and is shown in Table 4. Genes included in the list had to appear in at least two of the comparative analysis lists, one of which had to be the novel rat list from the present study. The rat and mouse lists in which each gene appeared are indicated in Table 4. The candidate regulatory gene list contains 36 candidate genes including 25 potentially conserved between rat and mouse, and 23 genes in three or more lists (Table 4). In addition, 11 novel genes were identified in the present study and are also listed. The present study presents this list of candidate genes in gonadal sex determination for both testis and ovary development that are conserved between rodent species.

## DISCUSSION

The present study used a microarray analysis to identify new potential candidate genes for rat sex determination and differentiation. Whole gonads were used such that all the different somatic cells and germ cells are present. Clearly, changes in the individual cell type transcriptomes will contribute to the total gonadal transcriptome and, as such, correlations of the present data to individual cell types must be made with caution. The present analysis used two different samples and two different microarray chips to obtain the individual data points. Criteria to assess chip number has been previously described (Chen et al. 2004). Previous studies have demonstrated two chips for each data point, assuming an $R2 > 0.95$ and confident $P < 0.05$, provide a critical and statistically relevant analysis (McLean et al. 2002, Kezele et al. 2005, Small et al. 2005). The reproducibility of the different microarrray chips used in this study was assessed as variance between samples and had an $R2 > 0.96$ for each dataset.

Genes known to be involved in sex determination had patterns of expression that match what is known from previous literature (Table 1). Some of these genes were eliminated from the candidate male development lists due to the stringency of the analyses. For example, FGF9 and WT1 do not exhibit the 1.5-fold statistically significant change in expression parameter used in the study. The elimination of some potentially important genes allowed for a stringent selection of candidate genes. Analysis of genes expressed with a raw signal of at least 75 revealed that approximately half the genes on the rat RAE230A chip were considered present in the embryonic testis and ovary. Approximately 2% had a 1.5-fold statistically significant change in the male and female E13, E14, and E16 developmental periods. The list of 160 genes with a 1.5-fold change in the male was reduced to 109 genes by comparison with the gene expression levels from the 3-day cultured embryonic testis. This narrowed the list of genes to be considered as candidates

critical for testis development and cord formation by ~32%. The list of subtracted genes is presented in Supplementary Table 1. Since the cultured embryonic E13 testis undergoes cord formation and testis differentiation similar to in vivo, those genes regulated both in vitro and in vivo are assumed to be important. Although potential subtraction of important genes is a limitation, selection of regulated genes present from the in vivo and in vitro studies is likely to identify candidate genes for cord formation. The subtraction of genes expressed in the ovary above a raw signal of 75 from the 109 gene list revealed 34 male-enriched genes. In contrast, the subtraction of genes expressed above a signal of 75 in the male from the 175 ovary gene list yields no female-enriched genes. Therefore, the stringent subtraction analysis used revealed no ovary-enriched genes that were regulated during the E13–E16 developmental period.

Increased transcriptional changes occur in the male between E13 and E14, while major changes in the female were delayed and occur between E14 and E16. When considering genes enriched in each sex for a given signal cut off, Fig. 7A, more genes appear to be enriched in the testis. The increased number of male-enriched genes suggests that there is an active female developmental process, but a reduced number of unique transcripts at this point in female development. These observations support the concept that it is the male process of development which requires expression of unique transcripts for testis determination. In contrast, when looking at the number of transcripts differentially regulated between the sexes at a single time point, as was done in a previous mouse analysis (Nef et al. 2005) and shown in Fig. 7B, there appear to be more female-enhanced genes than male. At only a single time point, a sex-enhanced gene may be present due to up- or downregulation in that sex. Therefore, it is not until gene expression is considered in the context of the developmental time course that it can be determined in which sex it is regulated and enhanced. The 33 genes identified in the 109 list of

genes and in the 316 list of genes gender enhanced are particularly interesting candidates for further consideration in male sex differentiation and development. The 33 gene list includes 10 out of the 21 signaling factors, 4 out of the 9 growth factors, and 3 out of the 7 receptors in Table 2. These genes are interesting because they are potential regulatory gene candidates and warrant further investigation. The 33 gene list does not include many cytoskeletal and extracellular matrix factors despite the fact that this is the largest category in the 109 list. Signaling is the second largest represented functional gene category in the 109 gene list and the largest category represented by genes also testis-enriched or -enhanced. Genes appearing in this category such as Tgfb3, Sfrp4, and Jag1 suggest involvement of specific signaling pathways in testis development. There appears a high level of connectivity of these genes with other genes of interest in a gene network built from the 109 list (Fig. 6). These pathways have the potential to influence gonadal differentiation. A thorough pathway analysis of over 130 different cellular and signaling pathways revealed no specific pathways where multiple (> 2) genes within the 109 list are present. Clearly, a large number of critical cellular processes are influenced (Fig. 6); however, subsets of grouped genes involved in specific pathways were not identified. The assumption that defined specific pathways involving multiple-regulated genes are needed for male sex determination is questioned and instead observations suggest that a larger number of cellular processes influenced by a smaller number of different genes are likely. The concept that a smaller group of genes that influence larger gene networks is critical for development and disease etiology has been suggested (Schadt et al. 2005).

Recently, three studies have investigated genome wide transcriptional regulation of sex determination in the mouse using multiple time points (Nef et al. 2005, Small et al. 2005, Beverdam & Koopman 2006). Several others have also been done for selected genes and

developmental periods (Koopman & Koopman 2002, Smith et al. 2003). The study by Small et al.(2005) focused on transcriptional changes throughout the gonadal developmental period, while the study by Nef et al.(2005) focused on changes between the sexes at E10.5, 11.5, 12.5, and 13.5 separately. The Beverdam study (Beverdam & Koopman 2006) examined changes between the sexes and between E10.5 and E11.5 in mouse. These studies were able to identify genes that were regulated early in gonadal differentiation in a sex-enhanced manner. The gonadal development and transcriptional processes in mouse and rat are similar. Investigation of the gonadal transcription during sex determination in the rat and comparison with the mouse allowed identification of genes conserved in mammalian gonadal sex determination.

The list of candidate genes involved in sex determination and gonadal development was compiled from genes identified in the present rat study (Table 4). The majority of the genes in this list also appeared in one of the mouse studies used for comparison. There was a subset of 11 genes that appear in several of the analyses performed on the rat data and did not appear in the mouse analyses. These genes may be novel in rat gonadal development, but the absence of some on the mouse microarray chips must be considered. The 36 genes in this candidate list (Table 4) had 15 genes conserved in mouse and rat testis development. To confirm the validity of the approach used in the present study, several genes known to be essential for testis development were also identified. VANIN1, A5D3, and IGFBP7 were all identified as significant candidate genes using this genomic approach. VANIN1 is a membrane-linked protein that has been shown to be expressed in a sex-specific manner in the developing testis (Wilson et al. 2005). A5D3 is highly expressed in the testis and contains potential leucine zipper and phosphorylation sites (Blomberg et al. 2002). A5D3 has also been found in a study of vitamin A deficient synchronized testis where it was called VAD4 (Luk et al. 2003). Insulin-like growth factor

binding protein 7 (IGFBP7) belongs to a family of proteins that regulates IGF function. Interestingly, it has been shown that IGF receptors are required for testis development (Zhou & Bondy 1993, Nef et al. 2003).

A developmental signaling pathway suggested to have a role in sex determination is the WNT signaling pathway (Kim et al. 2006 ). WNTs are secreted proteins implicated in cell growth, migration, and differentiation. Secreted frizzled related protein 4 (SFRP4) is a secreted lipoprotein receptor complex similar to the frizzled receptors in the WNT signaling cascade, but without the transmembrane activation domain (Hewitt et al. 2006). SFRP4 is thought to antagonize cell survival and inhibit WNT signaling by binding WNT without activating its signaling cascade. SFRP4 has been implicated in placental growth and ovulation (Drake et al. 2003, Hewitt et al. 2006). Wnt5a also appears on the candidate gene list. WNT5A has been shown to activate signaling cascades in a manner dependant on the receptor to which it binds (Mikels & Nusse 2006). A potential role for WNT5A and SFRP4 in gonadal development is yet to be determined and the present observations support a role of the WNT signaling pathway in sex determination.

Another developmental signaling pathway potentially involved in sex determination involves the notch pathway. JAGGED1 is a notch signaling ligand involved in cell fate decisions (de La Coste & Freitas, 2006), and has been found in mouse testis where it may function in cell fate decisions during spermatogenesis (Dirami et al. 2001). DELTEX4 homolog belongs to a family of proteins originally identified in Drosophila able to interact with the ankrin repeats in notch to mediate notch signaling (Ordentlich et al. 1998, Kishi et al. 2001). Identification of these transcripts in the present study suggests that the notch pathway may be involved in male sex determination.

A number of other signal transduction-related genes were present in the candidate list, but did not group to specific pathways. Suppressor of cytokine signaling 2 is an inhibitor of JAK/STAT signaling (Leung et al. 2003, Leroith & Nissley 2005). Inositol 1,4,5-triphosphate receptor 1 has an intrinsic calcium ion channel opened in response to InsP3 (Mignery et al. 1990). CXCR4 is a G-protein-coupled chemokine receptor with a c-x-c motif (Habasque et al. 2002, Khan et al. 2005, Smith et al. 2005). Endothelin receptor type A is also a G-protein-coupled receptor expressed in Sertoli and peritubular myoid cells (Ergun et al. 1999). Osteoglycin is a keratan sulfate proteoglycan (Iozzo & Murdoch 1996, Osawa et al. 2006). Adenylate cyclase 7 catalyzes conversion of ATP to cAMP (Suzuki et al. 1998). These genes affect a number of different signal transduction pathways and are likely important for sex determination.

There are 11 genes proposed as testis development candidates that were not found in the mouse studies. Although these 11 genes did not cross rodent species analysis, they display a high probability of significant impact on sex determination in the rat, and hence require further scrutiny. This includes the c-fos-induced growth factor, a secreted factor involved in cell growth and morphogenesis (Orlandini et al. 1996). NPPC is the precursor protein for CNP, which activates a Gi signal cascade (Hobbs et al. 2004, Anand-Srivastava 2005). Unc-5 homolog B is a netrin receptor that plays a role in morphogenesis of the vascular system (Lu et al. 2004) and oligodendrocyte precursor motility (Jarjour et al. 2003). PAWR is a pro-apoptotic protein containing a leucine zipper and death domain, and is important for cell sensitization to apoptotic stimuli (Boosen et al. 2005). Transforming growth factor (TGF)ß3 is a growth factor that is a growth inhibitor and present in the testis and involved in spermatogenesis (Jarjour et al. 2003, Lui et al. 2003). Nuclear protein 1 (NUPR1 or p8) is an HMG-I/Y like protein that functions as a

transcriptional regulator (Hoffmeister et al. 2002). Neuregulin 1 is a critical signaling factor in cell–cell interactions (Falls 2003). The p21 activated kinase (PAK3) is a serine–threonine kinase involved in apoptosis (McPhie et al. 2003, Boda et al. 2004). A phosphoprotein enriched in astrocytes and a dual specificity phosphatase are also included in the testis development candidate list. Although these 11 candidate rat genes did not appear on the mouse lists, individually they are viable candidates for roles in sex determination due to the correlations observed in the rat data provided.

There are ten candidate genes listed for ovarian development in Table 4. Follistatin and WNT4, known to be involved in ovarian development (Schneyer et al. 2004, Yao et al. 2004, Yao 2005), appear on the ovary development candidate list. These observations help validate the experimental approach and this ovary list. IGFBP2 is involved in growth inhibition in fetal development, and is abundant in Leydig cells (Wang et al. 1994, Schneyer et al. 2004, Terrien et al. 2005, Yao 2005). AMH receptor 2 (AMHR2) is known to bind AMH to promote Müllerian duct regression in the developing male, and to negatively regulate postnatal Leydig cell differentiation (Jamin et al. 2002, Mendis-Handagama et al. 2006). A role in the fetal ovary has not been identified. FGF receptor 2 is a receptor tyrosine kinase. A role for FGFR2 in male sex determination has been identified (Schmahl et al. 2004), however, a role in female development has not. ENPP2 (also known as autotaxin) promotes cell motility, angiogenesis, and myelination (Moolenaar 2002). Kallikrein 1 is a secretory serine protease (Clements et al. 1994). Solute carrier family 37 member 2 is homologous to the GlpT antiport sn-glycerol 3-phosphate transporter family in bacteria (Bartoloni et al. 2000). Msx1 is a homeobox gene (Blin-Wakkach et al. 2001, Ramos & Robert 2005). The genes identified in Table 4 are candidates for

involvement in female sex determination and early gonadal development and should be considered for further investigation.

This study characterizes transcriptional regulation of sex determination in the rat on a genomic scale and compares regulated genes in the mouse and rat. It has provided a resource for identifying candidate genes in mammalian sex determination. Profiles of expression of over 8000 genes present between E13 and E16 in the rat testis and ovary and transcripts expressed in cultured E13 testis are available (www.skinner.wsu.edu). These expression profiles provide information on genes regulated during the time of gonadal sex determination and testis development in the rat and can be used in comparative studies with the mouse and other organisms to gain insight into potentially conserved regulatory mechanisms. A functional analysis of the identified candidate genes is now required to help elucidate their potential significance in gonadal sex determination and differentiation process. Observations from the present study suggest that a smaller group of genes regulated during sex determination may have a role in influencing a large number of different cellular processes (i.e. gene networks), rather than larger groups of genes specific to selected pathways. This genomic analysis of the gonadal transcriptome during sex determination has provided a global assessment of genes and pathways potentially involved in sex determination and gonad development.

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**Table 2-1.** List of genes known to be involved in sex determination. The highest and lowest expression (Exp.) values detected in the microarray analysis for each of these genes are given. Signals denoted non-detectable (ND) were considered absent for the microarray analysis and generally have an expression < 50 unless indicated.

| Name | High exp. | Signal | Low exp. | Signal | GenBank | Description |
|---|---|---|---|---|---|---|
| *Sry* | E13 testis | 174 | E14 ovary | ND | AF275682 | *Rattus norvegicus* sex-determining region Y protein |
| *Vanin* | E16 testis | 169 | E13 ovary | 31 | BI289085 | Vanin 1 |
| *Fgf9* | E14 testis | 80 | E13 ovary | ND | D14839 | Fibroblast growth factor 9 |
| *Dax1* | E13 testis | 175 | E16 testis | 66.4 | NM_053317 | Nuclear receptor subfamily 0, group B, member 1 |
| *Amh* | E16 testis | 953 | E16 ovary | ND | AI059285 | UI-R-C1-lb-f-01-0-UI.s1 UI-R-C1 *Rattus norvegicus* |
| *Wt1* | E14 ovary | 234 | E16 ovary | 125 | NM_031534 | Wilms tumor 1 |
| *Wnt4* | E16 ovary | 249 | E16 testis | 63 | NM_053402 | Wingless-type MMTV integration site family, member 4 |

**Table 2-2.** List of 109 genes expressed in the rat testis from E13 to E16 with a signal of at least 75, a 1.5-fold or greater significant change, and with similar expression in E13 testis cultured for 3 days. Genes in bold are not in the ovary above a signal of 75 at E13, E14, or E16. Underlined genes were also differentially expressed between the sexes in at least one time point. An '*' denotes genes found to contain a potential SRY binding element in there promoter. An 'S' denotes genes identified in analysis of mouse data from Small *et al.*(2005).

| Gene symbol | High exp. | Signal | | GenBank | Description |
|---|---|---|---|---|---|
| Cytoskeletal and extracellular matrix | | | | | |
| *Tgfbi* | E13 | 1359 | | BG379319 | Transforming growth factor ß induced |
| *Podxl* | E13 | 468 | | AF109393 | Podocalyxin-like |
| *Tpm1* | E13 | 223 | | AF370889 | Tropomyosin-α |
| *Epb4.1l3* | E13 | 142 | | NM_053927 | Erythrocyte protein band 4.1-like 3 |
| *Crtl1* | E13 | 125 | | NM_019189 | Cartilage link protein 1 |
| *Mmp16* | E13 | 102 | * | NM_080776 | Matrix metalloproteinase 16 |
| *Sponf* | E14 | 112 | * | M88469 | F-spondin |
| *Col1a1* | E16 | 1646 | | BBI285575 | Collagen, type 1, α1 |
| | E16 | 771 | | BI296340 | Similar to cadherin 11 |
| | E16 | 530 | | BM389149 | Immunoglobulin superfamily/RNase inhibitor |
| | E16 | 505 | S | AI177055 | Similar to myosin, light polypeptide kinase |
| *Bgn* | E16 | 477 | | NM_017087 | Biglycan |
| | E16 | 302 | S | AI176126 | Similar to procollagen, type VI, α3 |
| | E16 | 292 | | BM384071 | Tubulin, ß2 |
| | E16 | 252 | | BG672591 | Plastin 3 (T-isoform) |
| | E16 | 237 | | BM391858 | Similar to dynein, axonemal, heavy polypeptide 11 |
| | E16 | 203 | | BF406693 | Similar to Laminin α-4 chain precursor |
| | E16 | 183 | | AI008689 | Similar to transforming acidic coiled-coil containing protein |
| | E16 | 170 | | BE110691 | Similar to EH-domain containing 2 |
| ***Tagln*** | **E16** | **148** | **S** | **NM_031549** | **Transgelin** |
| | E16 | 123 | S | BI296640 | Similar to Epsin 2 |
| | E16 | 108 | | BI287851 | Similar to procollagen, type VI, α2 |

| | | | | | |
|---|---|---|---|---|---|
| ***Ril*** | **E16** | **103** | | **NM_017062** | **Reversion-induced *LIM* gene** |
| Signaling | | | | | |
| *Bambi* | E13 | 512 | | AF387513 | BMP and activin membrane-bound inhibitor |
| *Gucy1b3* | E13 | 237 | * | NM_012769 | Guanylate cyclase 1, ß3 |
| ***Gucy1b3*** | **E13** | **232** | * | **BF399387** | **Guanylate cyclase 1, soluble, ß 3** |
| *Ren1* | E13 | 183 | * | J02941 | Renin 1 |
| | E14 | 88 | | BG371889 | Similar to phosphodiesterase 6G, cGMP-specific |
| | E16 | 502 | | AI639128 | Similar to wingless-type MMTV integration site 5A |
| ***Sfrp4*** | **E16** | **241** | * | **AF140346** | **Secreted frizzled-related protein 4** |
| | E16 | 237 | | BF287964 | Similar to annexin A11 |
| ***Socs2*** | **E16** | **224** | | **BM384088** | **Similar to suppressor of cytokine signaling 2** |
| | E16 | 151 | S | AI408442 | Similar to deltex 4 homolog |
| | **E16** | **142** | | **BI296275** | **Similar to monocyte to macrophage differentiation-associated 2** |
| ***Pawr*** | **E16** | **136** | * | **U05989** | **PRKC, apoptosis, WT1, regulator** |
| ***Wnt5a*** | **E16** | **134** | * | **NM_022631** | **Wingless-type MMTV integration site 5A** |
| | E16 | 129 | | AI406490 | Similar to tyrosine kinase, non-receptor, 2 |
| | E16 | 126 | | BF283621 | Similar to Ras GTPase-activating-like protein |
| ***Sh3kbp1*** | **E16** | **118** | | **AF230520** | **SH3-domain kinase-binding protein 1** |
| | **E16** | **108** | | **AI178741** | **Similar to PRA1 family 2 (mouse)** |
| | **E16** | **101** | S | **AI071649** | **Similar to adenylate cyclase 7** |
| | **E16** | **91** | | **BE112895** | **Similar to phosphoprotein enriched in astrocytes 15** |
| ***Dusp6*** | **E16** | **85** | | **AA957292** | **Dual specificity phosphatase 8** |
| | **E16** | **85** | | **BI277482** | **Similar to MAP kinase interacting kinase** |
| Metabolism | | | | | |
| | E13 | 286 | | BI284270 | Similar to glucan (1,4-α-), branching enzyme 1 |
| | E13 | 228 | | AA799700 | Similar to selenophosphate synthetase 2 |
| | E13 | 221 | | BI289467 | Similar to expressed in non-metastatic cells 4, protein |

| | | | | | |
|---|---|---|---|---|---|
| | E13 | 170 | | BF420664 | Similar to ubiquitin-specific protease 29 |
| *Pppr2b2* | E13 | 141 | S | NM_022209 | Protein phosphatase 2 (formerly 2A) |
| *Slc2a3* | E13 | 93 | | AA901341 | Solute carrier family 2, member 2 |
| *Nedd4a* | E14 | 873 | | BG379338 | Similar to ribonucleotide reductase M2 |
| | E16 | 468 | | AI411530 | Similar to aminoacylase 1 |
| | E16 | 414 | | AI227941 | Similar to tumor-related protein |
| | E16 | 232 | | BG673187 | Similar to four and a half LIM domains 1 |
| | **E16** | **169** | **S** | **BI289085** | **Similar to vanin 1** |
| ***Enpep*** | **E16** | **157** | | **AF214568** | **Aminopeptidase A** |
| ***Gatm*** | **E16** | **134** | **S** | **NM_031031** | **Glycine amidinotransferase** |
| | E16 | 128 | | BF406832 | Similar to leprecan-like 2 |
| *Tm6p1* | E16 | 119 | | NM_139107 | Fasting-inducible integral membrane protein |
| | E16 | 100 | | AI412948 | Similar to ataxin 7-like 3 |
| Growth factor | | | | | |
| *Bmp4* | E13 | 137 | * | NM_012827 | Bone morphogenetic protein 4 |
| | E13 | 115 | S | BG671943 | Similar to ephrin A5 |
| | E16 | 647 | | BG375362 | Similar to latent transforming growth factor ß-binding protein 4 |
| | **E16** | **239** | | **AI170324** | **Similar to C-fos-induced growth factor (Figf)** |
| **Nppc** | **E16** | **184** | * | **NM_053750** | **Natriuretic peptide precursor C** |
| **Jag1** | **E16** | **118** | * | **NM_019147** | **Jagged 1** |
| | **E16** | **117** | **S** | **BG664221** | **Similar to osteoglycin** |
| **Tgfb3** | **E16** | **102** | * | **NM_013174** | **Transforming growth factor, ß 3** |
| **Nrg1** | **E13** | **78** | | **U02319** | **Neuregulin 1** |
| Receptor | | | | | |
| *Grinl1a* | E13 | 300 | | BG664035 | Glutamate receptor, ionotropic |
| *Gpr48* | E13 | 285 | S | BI300274 | G-protein-coupled receptor 48 |
| | E13 | 196 | | AI072459 | Similar to Eph receptor A4 |
| *Itpr1* | E13 | 185 | *S* | J05510 | Similar to inositol 1,4,5-triphosphate receptor 1 |
| *Cxcr4* | E13 | 128 | | AA945737 | Chemokine receptor (LCR1) |
| *Lrp4* | E13 | 85 | | AI070976 | Low-density lipoprotein receptor-related protein 4 |

| | | | | | |
|---|---|---|---|---|---|
| | E14 | 123 | | BI275605 | Similar to unc-5 homolog B |
| Transcription and translation | | | | | |
| *Egr1* | E13 | 171 | | NM_012551 | Early growth response 1 |
| *Hoxa5* | E13 | 163 | | BE107303 | Similar to Homeo box A5 |
| | E14 | 147 | | BE107296 | Ribosomal protein S6 kinase polypeptide 6 |
| | E16 | 319 | | AW253720 | Nuclear factor I/B |
| | E16 | 135 | | AI409308 | Max interacting protein 1-negative reg. of cell proliferation |
| ***Nupr1*** | **E16** | **91** | ***** | **NM_053611** | **Nuclear protein 1** |
| Ion transport | | | | | |
| *Scn4b* | E13 | 313 | | AI137995 | Sodium channel, voltage-gated, type IV, ß |
| *Lgals9* | E13 | 107 | | U72741 | Lectin |
| *Nme7* | E16 | 345 | | AI232036 | NME7 |
| Other/unknown | | | | | |
| | E13 | 851 | | AI599621 | EST251324 cDNA clone REMEH36 |
| | E13 | 566 | | AA956417 | *Rattus norvegicus* transcribed sequences |
| | E13 | 246 | | AI175861 | *Rattus norvegicus* transcribed sequences |
| | E13 | 222 | S | AA924756 | Similar to ES neuronal differentiation 2 |
| | E13 | 167 | | BI294768 | Hypothetic protein–DNA-binding domain |
| | E13 | 143 | | AW529759 | *Rattus norvegicus* transcribed sequences |
| | **E13** | **104** | | **AA799470** | ***Rattus norvegicus* transcribed sequences** |
| | **E13** | **88** | | **AI236229** | **Similar to RNA binding motif protein 24** |
| | **E13** | **77** | **S** | **AI178384** | **Similar to kelch-like 6** |
| | E14 | 172 | | AA860014 | Similar to hemoglobin: SUBUNIT = ζ |
| | **E14** | **92** | | **AI410969** | **Hypothetical protein MGC27854** |
| | **E14** | **76** | | **AI101385** | ***Rattus norvegicus* transcribed sequences** |
| *A5D3* | E16 | 1514 | | AY007690 | A5D3 protein |
| | E16 | 203 | | AI102758 | Transcribed sequence-similarity to ref:NP_080909.1 |
| | **E16** | **183** | | **BE103235** | ***Rattus norvegicus* transcribed sequences** |
| | **E16** | **178** | | **AI009714** | **Similar to serum deprivation response** |
| | E16 | 157 | | AA891255 | Hypothetic protein |
| | E16 | 147 | | BM388789 | *Rattus norvegicus* transcribed sequence |

| | | | | |
|---|---|---|---|---|
| | E16 | 147 | BI281129 | Unknown |
| | **E16** | **138** | **BI274243** | **Transcribed sequence-similarity** |
| | E16 | 112 | AI410305 | *Rattus norvegicus* transcribed sequences |
| | E16 | 111 | AI716904 | Predicted hypothetical protein |
| | **E16** | **79** | **BE099060** | **Hypothetical protein LOC311430** |
| ***Pak3*** | **E16** | **75** | **NM_019210** | **P21 (CDKN1A)-activated kinase 3** |

**Table 2-3.** List of genes conserved in the rat and mouse. Genes present in the Nef *et al.* 2005 (N) or Beverdam & Koopman (2006) (B) or Small *et al.*(2005) (S) studies are indicated. The time in the mouse and rat that the gene is expressed (Exp) differently between the sexes is indicated for mouse and rat. Genes in bold also appear in the 109 list.

| Study pools | Mouse exp. | Rat exp. | Name | GenBank | Description |
|---|---|---|---|---|---|
| Testis | | | | | |
| **N, B** | **E11.5** | **E16** | ***A5D3*** | **AY007690** | **A5D3 protein** |
| **N, B** | **E11.5** | **E16** | | **BI296275** | **Monocyte-macrophage differentiation** |
| **N** | **E12.5** | **E16** | *Tcf21* | BE113336 | Transcription factor 21 |
| **N** | **E12.5** | **E16** | | AI227742 | Bcl-2-related ovarian killer protein |
| **N** | **E12.5** | **E16** | | AI179988 | Ectodermal-neural cortex 1 |
| **N, B** | **E12.5/11.5** | **E14, E16** | *Gatm* | NM_031031 | Glycine amidinotransferase |
| **N** | E12.5 | E16, E13 | *Ednra* | NM_012550 | Endothelin receptor type A |
| **N, S** | **E12.5** | **E16** | ***Jag1*** | **NM_019147** | **Jagged 1** |
| **N, S** | **E12.5** | **E14** | ***Gatm*** | **NM_031031** | **Glycine amidinotransferase** |
| **N** | E12.5 | E13 | | AI233246 | Insulin-like growth factor binding protein 7 |
| **B** | E10.5 | E13, E16 | | AI410924 | *Rattus norvegicus* transcribed sequences |
| **B** | E11.5 | E14 | | **B1296340** | ***Rattus norvegicus* transcribed sequences** |
| **B** | E11.5 | E13–E16 | | **BG664221** | **Transcribed sequence similar to osteoinductive factor** |
| **B** | E11.5 | E13–E16 | | AI103641 | *Rattus norvegicus* transcribed sequences |
| **B** | E11.5 | E13–E16 | | **BM384088** | **Transcribed sequence similar to** ß |
| Ovary | | | | | |
| **NSB** | E11.5 | E13 | *Fst* | NM_012561 | Follistatin |
| **N** | E12.5 | E16 | *Amhr2* | NM_030998 | Anti-Müllerian hormone type 2 receptor |
| **N** | E12.5 | E16 | *Axin2* | BF398114 | Axin2 |
| **N** | E12.5 | E16 | | AI172116 | Zinc finger protein 672 |
| **N** | E12.5 | E14 | *Igfbp2* | NM_013122 | Insulin-like growth factor binding protein 2 |
| **N** | E12.5 | E14 | *Enpp2* | NM_057104 | Ectonucleotide pyrophosphatase/phosphodiesterase 2 |

| | | | | | |
|---|---|---|---|---|---|
| **N** | E12.5 | E14 | | BI291872 | Solute carrier family 37 member 2 |
| **N** | E12.5 | E13 | | AI412658 | Fibroblast growth factor receptor 2 |
| **N** | E12.5 | E13 | *Wnt4* | NM_053402 | Wingless-type MMTV integration site family, member 4 |
| **B** | E11.5 | E13, E14 | *Klk1* | NM_012593 | Kallikrein 1 |
| **B** | **E11.5** | **E14, E16** | ***Cxcr4*** | **AA945737** | **Chemokine receptor (LCR1)** |
| **B** | E11.5 | E16 | | AI102517 | *Rattus norvegicus* transcribed sequences |
| **B** | E11.5 | E16 | *Msx1* | NM_031059 | Homeo box, msh-like 1 |
| **B** | E11.5 | E16 | | BG374285 | *Rattus norvegicus* transcribed sequences |
| **B** | E11.5 | E13–E16 | *Ifi27I* | NM_130743 | Interferon, α-inducible protein 27-like |
| **B** | E11.5 | E13–E16 | | AI172218 | Simlar to RIKEN Cdna 2810002N01 |
| **B** | E11.5 | E13–E15 | *Bzrp* | AI008680 | Benzodiazepin receptor |

**Table 2-4.** Final compiled candidate list of genes for involvement in sex determination and gonadal development. Genes were selected based on the number of rat analysis (rat lists) and mouse lists they appeared in and potential function. 109 and 316 refer to gene lists in Table 2 and Supplementary Table 2 respectively.

| Name | High exp. | Signal | Rat lists | Mouse lists | GenBank | Description |
|---|---|---|---|---|---|---|
| Testis development candidates | | | | | | |
| A5D3 | E16 | 1514 | 109,316 | NB | AY007690 | A5D3 protein |
| | E16 | 840 | 316 | N | AI233246 | Insulin-like growth factor binding protein 7 |
| *Sfrp4* | E16 | 241 | 109,316,M,* | B | AF140346 | Secreted frizzled-related protein 4 |
| *Socs2* | E16 | 224 | 109, M | N | BM384088 | Similar to suppressor of cytokine signaling 2 |
| | E13 | 222 | 109,316 | S | AA924756 | Similar to ES neuronal differentiation 2 |
| *Itpr1* | E13 | 185 | 109,316 | S | J05510 | Inositol 1,4,5-triphosphate receptor 1 |
| *Cxcr4* | E16 | 178 | 109,316 | B | AA945737 | Similar to chemokine receptor (LCR1) |
| | E16 | 169 | 109,M | S | BI289085 | Similar to vanin 1 |
| | E16 | 151 | 109,316 | S | AI408442 | Similar to deltex 4 homolog |
| | E16 | 142 | 109,316,M | NB | BI296275 | Similar to monocyte to macrophage differentiation- |
| *Ednra* | E16 | 126 | 316 | NS | NM_012550 | Endothelin receptor type A |
| *Jag1* | E16 | 118 | 109,316,M,* | N | NM_019147 | Jagged 1 |
| | E16 | 117 | 109,M | SB | BG664221 | Similar to osteoglycin |
| | E16 | 106 | 316 | B | AI410924 | *Rattus norvegicus* transcribed sequences |
| | E16 | 101 | 109,316,M | NS | AI071649 | Similar to adenylate cyclase 7 |
| | E16 | 239 | 109,316,M | | AI170324 | Similar to C-fos induced growth factor (Figf) |
| *Nppc* | E16 | 184 | 109,316,M,* | | NM_053750 | Natriuretic peptide precursor C |
| *Pawr* | E16 | 136 | 109,316,M,* | | U05989 | PRKC, apoptosis, WT1, regulator |
| *Wnt5a* | E16 | 134 | 109,316,M,* | | NM_022631 | Wingless-type MMTV integration site 5A |

| | | | | | | |
|---|---|---|---|---|---|---|
| | E14 | 123 | 109,316 | | BI275605 | Similar to unc-5 homolog B |
| *Tgfb3* | E16 | 120 | 109,M,* | | NM_013174 | Transforming growth factor, ß 3 |
| | E16 | 91 | 109,316,M | | BE112895 | Similar to phosphoprotein enriched in astrocytes 15 |
| *Nupr1* | E16 | 91 | 109,M,* | | NM_053611 | Nuclear protein 1 |
| *Dusp6* | E16 | 85 | 109,316,M | | AA957292 | Dual specificity phosphatase 8 |
| *Nrg1* | E13 | 78 | 109,316,M | | U02319 | Similar to neuregulin 1 |
| *Pak3* | E16 | 75 | 109,M | | NM_019210 | p21 (CDKN1A)-activated kinase 3 |
| Ovary development candidates | | | | | | |
| *Igfbp2* | E16 | 1478 | 316 | N | NM_013122 | Insulin-like growth factor binding protein 2 |
| *Amhr2* | E14 | 964 | 316 | N | NM_030998 | Anti-Müllerian hormone type 2 receptor |
| *Fst* | E16 | 406 | 316 | NSB | NM_012561 | Follistatin |
| | E14 | 332 | 316 | N | AI412658 | Similar to fibroblast growth factor receptor 2 |
| *Wnt4* | E16 | 249 | 316 | N | NM_053402 | Wingless-type MMTV integration site family, member 4 |
| *Enpp2* | E16 | 233 | 316 | N | NM_057104 | Ectonucleotide pyrophosphatase/phosphodiesterase 2 |
| *Klk1* | E13 | 219 | 316 | B | NM_012593 | Kallikrein 1 |
| | E14 | 135 | 316 | N | BI291872 | Similar to solute carrier family 37 member 2 |
| *Msx1* | E16 | 130 | 316 | B | NM_031059 | Homeo box, msh-like 1 |
| | E16 | 128 | 316 | B | BG374285 | *Rattus norvegicus* transcribed sequences |

M indicates male-enhanced genes from Table 2. An asterisk indicates genes with an SRY element in the promoter. S, Small *et al.*(2005) data; N, Nef *et al.*(2005) data; B, Beverdam & Koopman 2006 data.

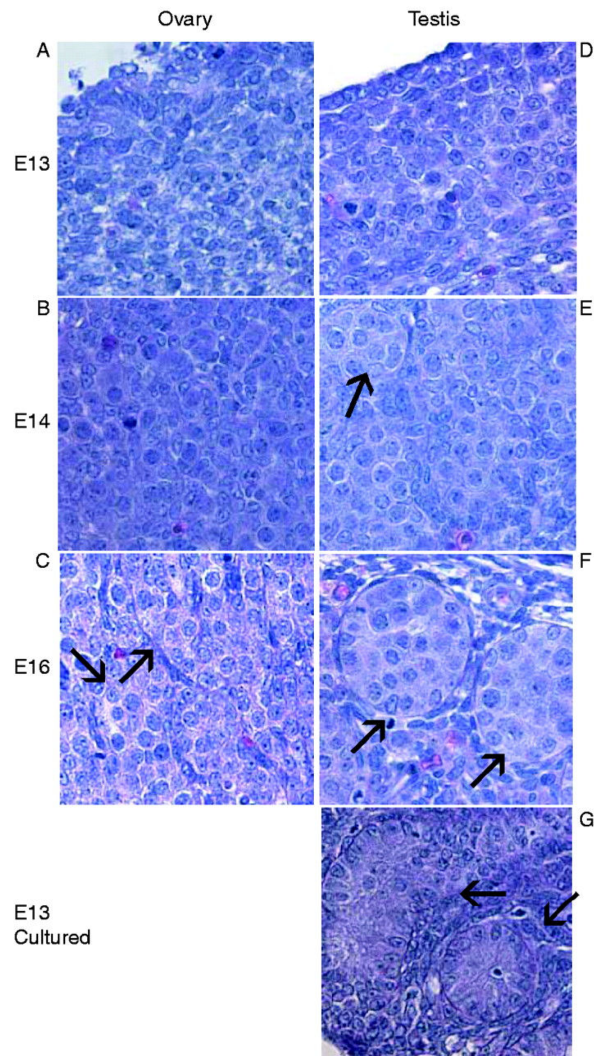


**Figure 2-1**. Histology of embryonic rat testis and ovary. Tissue sections from E13 (A), E14 (B), and E16 (C) ovary were analyzed. Tissue sections from E13 (D), E14 (E), and E16 (F) testis, as well as from E13 testis cultured for 3 days (G) were fixed and stained for morphological analysis. Serial sections were stained. Black arrows indicate testis cords in testes or oocyte nests in ovaries.

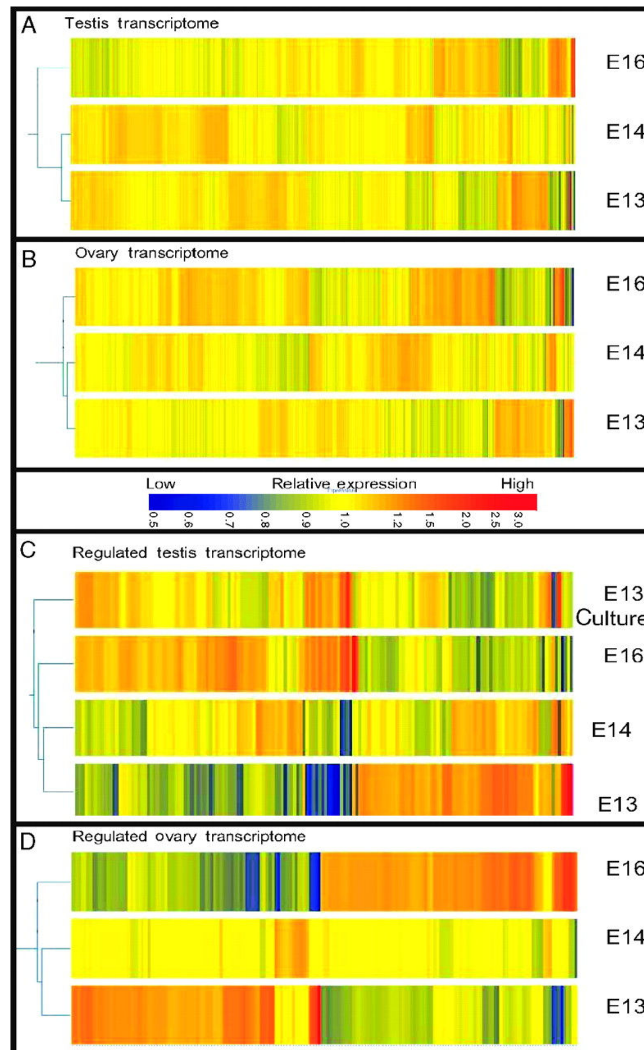


**Figure 2-2.** Dendrogram analysis of microarray data reveals the relative relatedness of gonadal transcriptomes. Dendrograms were produced in GeneSpring 7.2 using an unsupervised cluster analysis. Genes are clustered by pattern of expression. Sample sets or time points are clustered by relatedness of gene expression patterns as indicated by the left margin connective illustrations (i.e. links). (A) Dendrogram of male E13, E14, and E16 testis gene expression above a signal value of 75. (B) Dendrogram of female ovary gene expression above a signal of 75 at E13, E14, and E16. (C) Male gonadal genes for E13, E14, E16, and E13 cultured testis expressed above a signal of 75 and with a 1.5-fold significant change during the developmental period. (D) Female gonadal genes for E13, E14, and E16 expressed above a signal of 75 and with a 1.5-fold significant change.

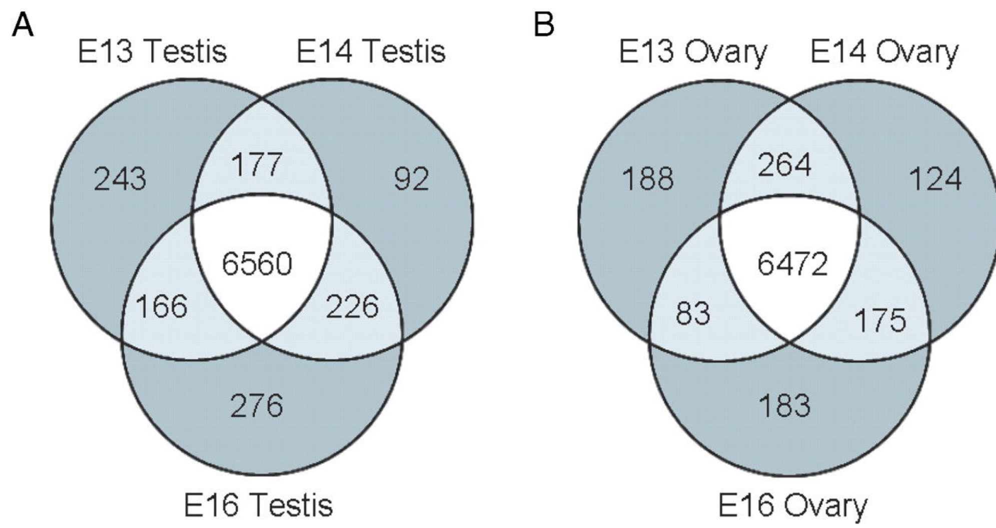


**Figure 2-3.** Expressed gene numbers in male and female gonadal development during sexual differentiation. Venn diagrams comparing numbers of genes expressed above a raw signal of 75 were produced in Genespring 7.2. Genes expressed over 75 in E13, E14, and E16 in the rat testis (A) and ovary (B) are compared.

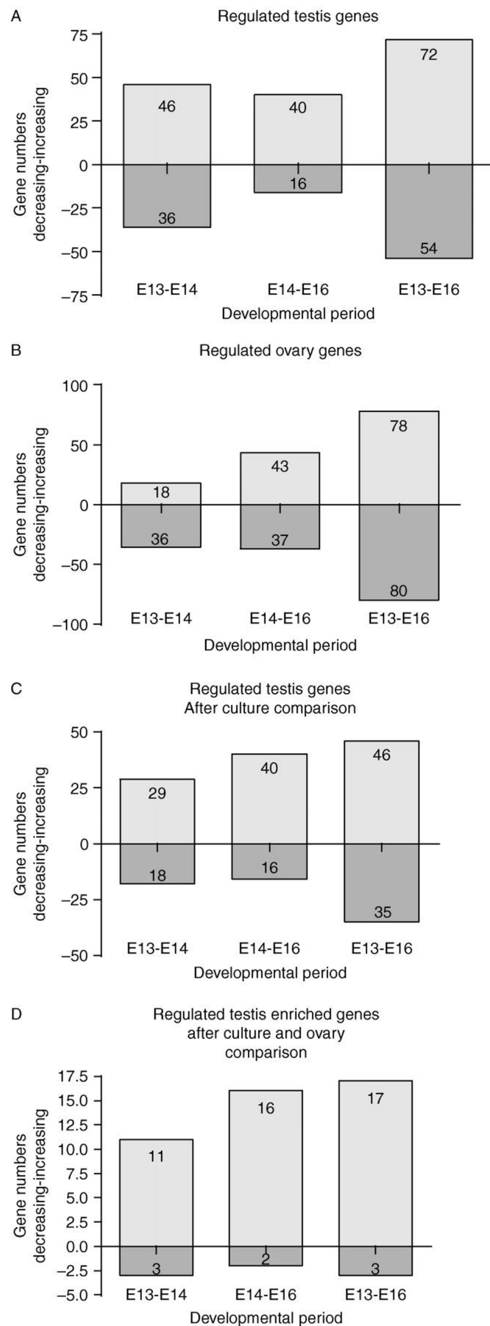


**Figure 2-4.** Numbers of regulated genes from testis and ovary developmental periods. (A) Numbers of genes expressed during testis development that have an expression signal of at least 75 and a statistically significant increase or decrease of 1.5-fold between each time interval are represented in a bar graph. (B) Numbers of ovary development genes with a signal of at least 75 and a statistically significant 1.5-fold increase or decrease. (C) The number of candidate genes for involvement in testis development and cord formation was reduced by comparison with E13 testis culture. Genes that are expressed above a raw signal of 75, have a 1.5-fold increase or decrease in the male time course and have similar patterns of expression changes from E13 to cultured testis as from E13 to E14 or E13 to E16. (D) Candidate testis development genes after subtraction of ovary expressed genes and comparison of testis organ cultures.

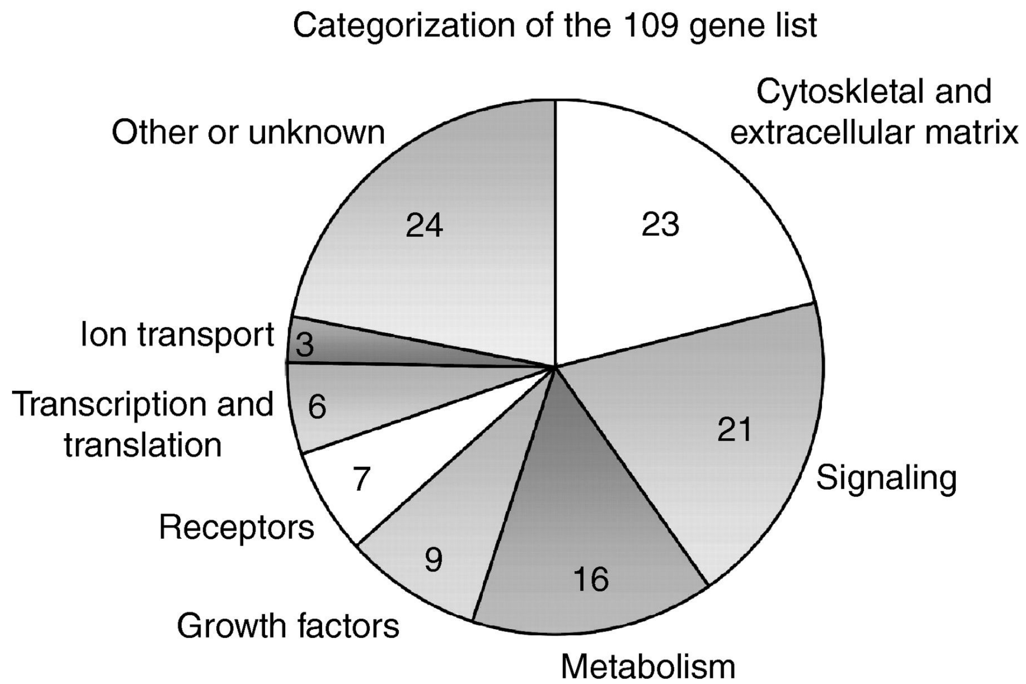


**Figure 2-5.** Functional categorization of the 109 gene list. The number of genes in each functional category of the 109 gene list of male-enhanced transcripts regulated in testis development.

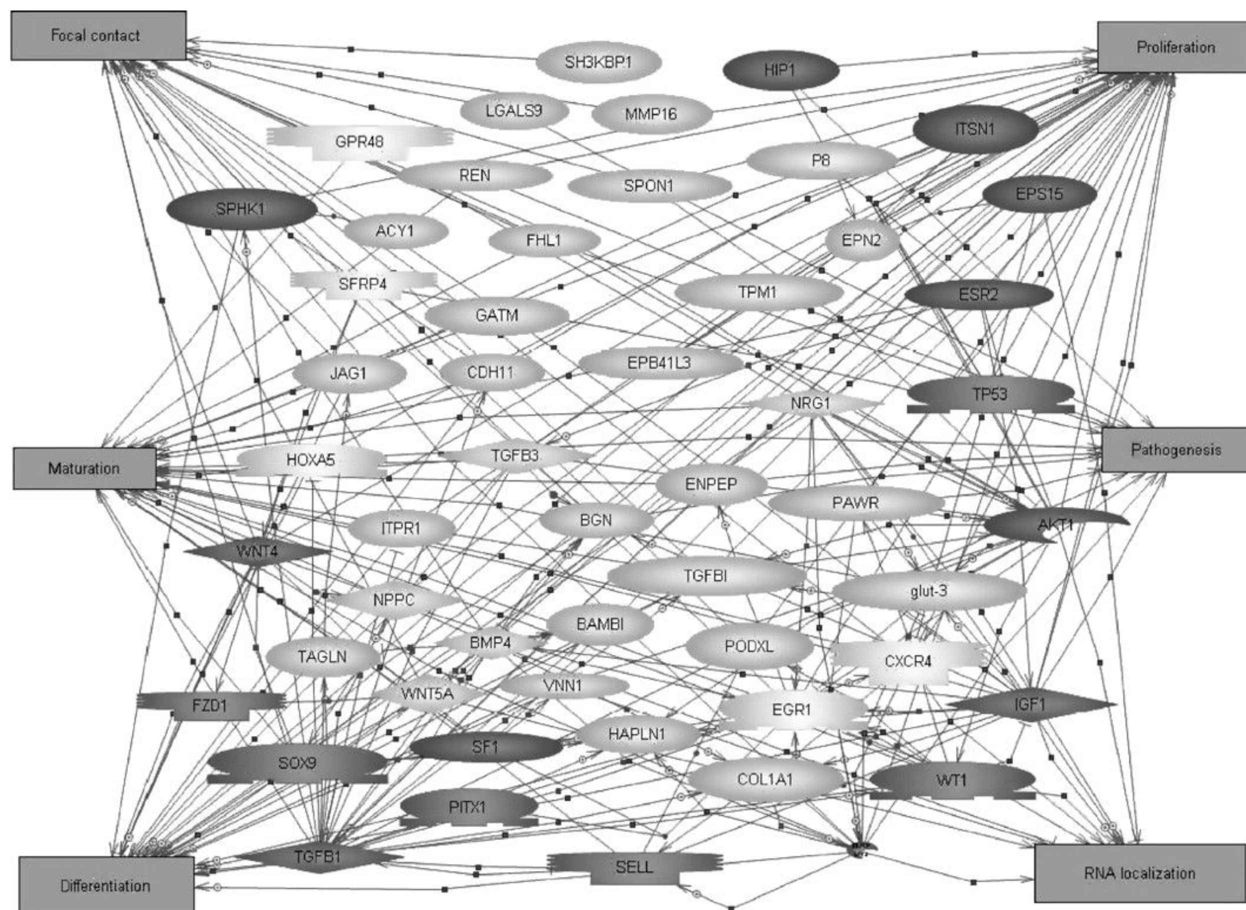


**Figure 2-6.** Functional gene network analysis of testis development genes. The 109 gene list was analyzed by Pathway Assist. Cell processes involved in testis development were determined based on the number of arrows connected to each box (connectivity). Rectangles are the cellular processes, light shaded shapes are a subset of the 109 list, and dark circles represent interconnecting proteins not on the 109 list.

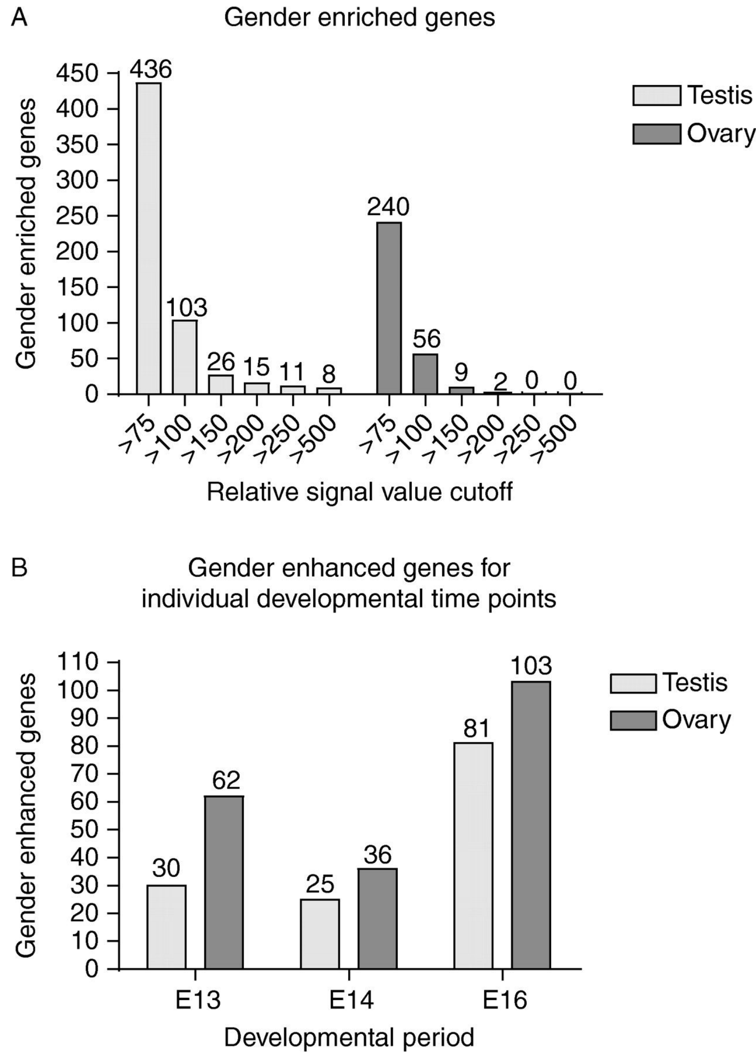


**Figure 2-7.** The number of gender-enriched (A) enhanced (B) transcripts between E13 and E16 in the testis and ovary. (A) All genes gender-enriched in the male and female time courses with given signal cut off values. Genes expressed above a signal of 75 in any time point of one sex were removed from lists of genes above a specified signal cut-off in at least one time point of the other sex to obtain the number of gender-enriched genes for a given signal cut off. (B) Numbers of gender-enhanced genes at a single given time point with at least a 1.5-fold change between the sexes were determined.

# CHAPTER 3

# ALTERATION OF THE DEVELOPING TESTIS TRANSCRIPTOME FOLLOWING EMBRYONIC VINCLOZOLIN EXPOSURE[1]

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**ABSTRACT**

The current study investigates the effects of in utero vinclozolin exposure on the developing rat testis transcriptome to investigate the direct actions of vinclozolin on the embryonic testis which are known to indirectly lead to trans-generational adult onset disease states. Testis differentiation is a complex process critical for reproductive function. Vinclozolin, a fungicide, is one of many environmental endocrine disrupting chemicals increasingly implicated in dysfunctional male reproductive development. It has been suggested that embryonic exposure to such compounds may be particularly disruptive to proper development and function and lead to adult onset and trans-generational disease states. When embryos are exposed to vinclozolin during gonadal sex determination, testis cell apoptosis in pubertal and adult offspring is increased and sperm motility is decreased. A better understanding of the mechanisms of action leading to these disease states is required to better asses the environmental impacts of vinclozolin, and to provide insight into how embryonic exposures cause adult onset disease which is transmissible between generations. To identify candidate mechanisms of vinclozolin action directly on the differentiating testis, a genomic microarray comparison of control and treated testis around the time of sex determination was analyzed for changes in gene expression. A total of 567 candidate genes were identified and the major cellular functions and pathways associated with these transcripts were examined. Interestingly genes regulated during normal sex determination do not appear to be altered by vinclozolin treatment suggesting disruption of genes not required for sex determination may play a role in adult onset trangenerational disease induction. Categorization by major known function of all 576 genes altered by *in utero* vinclozolin exposure reveals that transcription, signaling, and cytoskeletal and extra cellular matrix associated transcripts are highly represented. Specifically known functions

of genes identified in this analysis suggest the involvement of *Wnt* and calcium signaling, vascular development and epigenetic mechanisms as potential mediators of vinclozolin action.

# INTRODUCTION

The ability of embryonic vinclozolin treatment at the time of sex determination to cause increased testis cell apoptosis and decreased sperm motility in the adults transgenerationally has raised several important questions [1;2]. Discovery of the mechanism of action leading to these phenotypes would lead to a better understanding of vinclozolin actions on the developing embryo outside of androgen disruption, and also give insight into regulation of adult onset diseases. This study investigates vinclozolin induced transcriptional alteration in testis during sex determination and early testis development.

Sex determination and testis development are complex processes required for the perpetuation of mammalian species. Germ cells originate in the yolk sac and begin to migrate to the genital ridge at embryonic day ten (E10)- E11 in the rat [3;4]. Gonadogenesis occurs at E12 and gonadal sex determination is initiated at E12.5. Subsequently, in chromosomal males carrying the Sex determining Region of the Y chromosome (SRY), Sertoli cells differentiate and aggregate with primordial germ cells [4;5]. Testis cords begin to form at E14 and are surrounded by Peritubular myoid cells. Extracellular matrix forming the basal lamina of the testis cords is secreted, and vasculature and Leydig cells form in the subsequent few days of development [6-9]. There is also a male specific proliferative event in the developing gonad [10;11]. At puberty, the testis cords become seminiferous tubules and spermatogenesis is initiated. Proper development of the testis is critical for full reproductive capacity. Mutation of genes involved in the key events of embryonic testis development including cellular proliferation, germ cell colonization, sex determination, cell migration, cell associations, and vascularization can cause sub-fertility, intersex disorders, or sex reversal (reviewed in [12-14]). The embryonic

environment and exposures to chemicals such as endocrine disrupting compounds can also alter development and decrease reproductive capacity [15].

Endocrine disrupting compounds can act as hormone agonists or antagonists and have been implicated in a host of reproductive abnormalities in wildlife and human populations [16-20]. Several endocrine disrupting compounds have been shown to inhibit male sexual development [21-23]. Vinclozolin is one such compound that is used as a fungicide for turfgrass, ornamental plants, grapes and other fruits and vegetables. Vinclozolin and two of its major metabolites, enanilide and butenoic acid, act as anti-androgens by inhibiting androgen receptor (AR) function [23;24]. AR function is required for male reproductive development and expression of AR first appears in testis and reproductive tract tissues of the rat between E14 and E15 [25-27]. Vinclozolin exposure of neonates has been shown to affect both embryonic development and adult onset disease states. Maternal oral vinclozolin treatment of rats between E12 and postnatal day 3 revealed malformations in the reproductive tracts and renal systems of male offspring with highest sensitivity between E14 and E19 [17;28]. Embryonic vinclozolin exposure during gonadogenesis and sex determination, from E8-E14 through maternal intraperitoneal injection, caused increased apoptosis in pubertal and adult rat testis and decreased sperm numbers and motility in males for three generations [2;29]. These adult onset transgenerational disease phenotypes are presumably caused by a cascade of events beginning at the time of vinclozolin exposure of the first generation. However, AR is not yet expressed in the developing embryos at the time of treatment so alternative mechanisms of action for vinclozolin must be considered.

Vinclozolin exposure from E8-E14 in rat overlaps with several complex events critical to testis function including gonadogenesis, sex determination, testis differentiation, and epigenetic

reprogramming of the germ line. Transcriptional regulation during rat sex determination and testis differentiation between E13 and E16 has been investigated [30]. That study produced a list of 109 candidate transcripts involved in normal testis morphogenesis processes such as cellular differentiation, proliferation, focal contact, RNA localization, and development. Specific signaling pathways were not found to be highly affected in that study but indentified genes suggest regulation of several cellular processes. Identified genes included known players in testis development such as *Sry*, *Vanin*, *Fgf9*, and *Amh*, and many novel gene candidates including *Tgfb3*, *Sfrp4*, *Wnt5a*, *Deltex4*, and *Jag1*. These genes which may play a critical role in sexual differentiation may also be targets for the action of vinclozolin. Epigenetic mechanisms should also be considered as they have previously been implicated as causative in some adult onset diseases [31-34], and in the action of vinclozolin on the testis transcriptome at E16 leading to adult onset and transgenerational disease [35]. However, potential mechanisms of vinclozolin action on the developing testis during the time of exposure from E8-E14 are yet to be elucidated. The current study investigates gene transcription overlapping the E8-E14 vinclozolin treatment and testis differentiation at E13, E14, and E16. Specifically, changes in gene expression that may point to those actions of vinclozolin leading to altered development were investigated.

## MATERIALS AND METHODS

### Animals

Sprague–Dawley rats were kept in a temperature controlled environment and given food and water ad *libidum*. Estrous cycles of female rats were monitored by cellular morphology from vaginal smears [36]. Rats in early estrus were bred overnight and mating confirmed by sperm positive smears, denoted as day 0 of pregnancy. Pregnant rats were injected with 100mg/kd/day of vinclozolin, or DMSO vehicle only, daily on days E8-E14 of pregnancy to expose pups *in utero* during this time. Animals were euthanized at E13, E14, and E16 of pregnancy, and embryonic gonads were collected for RNA isolation and histology. Sex was determined by PCR using primers specific for *Sry* on genomic DNA isolated from embryo tails as previously described [37]. All procedures were approved by the Washington State University Animal Care and Use Committee.

### Organ Cultures

Embryonic cultures undergo similar testis morphogenesis after 3 days of culture as that seen *in vivo* [37;38]. Rat gonads from E13 embryos were dissected with mesonephros intact and cultured 3 days as previously described [39]. Briefly, gonads were placed in drops of medium on Millicell CM filters (Millipore, Bedford, MA, USA) floating on 0.4 ml of CMRL 1066 medium (Gibco BRL) supplemented with penicillin–streptomycin, insulin (10 µg/ml), L-glutamine (350 µM), transferrin (10 µg/ml), and BSA (0.01%). Gonadal pairs were split with one gonad treated with 50uM vinclozolin in DMSO and the contra-lateral gonad with DMSO only as a vehicle control. Alternatively, one gonad was treated with 5uM flutamide with Ethanol and the contra-lateral in ethanol only. Media and treatments were changed daily. Gonads were maintained in

culture for 3 days at which time testis cords formed and testes were used for histological analysis or separated from mesonephros and used in RNA collections.

### Histology

Tissue specimens were fixed in Bouin's solution for 1 h and embedded in paraffin using standard procedures. Serial sections of 4 µm were stained with hematoxylin and eosin (H&E) using standard procedures by the Histology Core Laboratory of the Center for Reproductive Biology, Washington State University. Sections were visualized by light microscopy.

### RNA Preparation

Sprague–Dawley rat embryonic gonads without mesonephros from E13, E14, and E16 control and treated testes were collected (a total of 6 conditions). Stage of development was confirmed by counting tail somites of each embryo. E13 gonads cultured for three days with 50uM vinclozolin, 5uM flutamide, or vhehical controls of DMSO or ethanol respectively were also collected (total of 4 additional conditions). Two separate gonadal pools of 10-30 gonads were collected for each treatment group for replicate analysis and stored in TRIZOL at –80 °C (Invitrogen) until RNA extraction following the manufacturer's protocol. Each separate RNA pool was used for a single microarray chip, for a total of 12 chips for *in vivo* analysis and 8 for *in vitro* analysis. High quality RNA samples were assessed with gel electrophoresis and required a minimum $OD_{260/280}$ ratio of 1.8.

### Microarray and Statistical Analysis

At least 3 µg RNA per sample was delivered to the Center for Reproductive Biology, Genomics Core Laboratory, Washington State University for processing. Briefly, RNA was transcribed into cDNA, and cDNA transcribed into biotin-labeled RNA. Biotin-labeled RNA was then hybridized to the rat RAE230 2.0 arrays containing 31,099 transcripts (Affymetrix,

Santa Clara, CA, USA) and labeled with phycoerythrin-coupled avidin. Hybridized chips were visualized on an Affymetrix Scanner 3000 (Affymetrix). CEL files containing raw data were then processed and analyzed using R software and Bioconductor packages [40;40;41]. Theses CEL files have also been deposited with the NCBI gene expression and hybridization array data repository (GEO, http://www.ncbi.nlm.nih.gov/geo).

Microarray hybridization data were examined for physical anomalies on the chip by pseudochip and reisidual error visualizations. Analysis continued only when no anomalies were identified. For all further analyses, microarrays were split into three experiments; E13, E14, and E16 control and treated (12 chips), in vitro vinclozolin treated and control (4 chips), and in vitro flutamide treated and control (4 chips). Quality Assurance of microarray data was completed using Affy QC Report [42]. Hybridization and housekeeping controls, RNA degradation, sample clustering, NUSE plots, LPE plots, and RLE plots all showed high quality data for each analysis(not shown) and no chips were removed.

Array signal values were calculated using RMA, GCRMA, PLIER, and MAS 5. MAS 5 pre-processing was chosen for further analysis for its ability to best separate background from signal and standardize samples in this experiment. Gene expression per chip for *in vivo* conditions was scaled to a signal value of 210, calculated as the median of average 2% trimmed mean chip values. The calculated target value for conditions in the *in vitro* vinclozolin treated testis experiment was 218, and 107 for conditions of *in vitro* flutamide treated testis experiment. Signal values correlate to the amount of transcript in the sample.

To determine the effects of vinclozolin treatment on gene expression during sexual differentiation, a linear model was applied to each gene. First, unexpressed or unchanged genes were removed by filtering in an unbiased manner. Present-marginal-absent (PMA) calls were

determined using a P value cut off for absent of greater then 0.06, and less then 0.04 for presence. Unexpressed genes were then defined as less than a signal cut off value at which 99.5% of genes absent across all samples and were excluded. This value of $log_2$ 6.68 for in vivo treated conditions (6.972 for in vitro vinclozolin treated conditions, and 6.638 for in vitro flutamide conditions) was used to filter the data on expression value and removed 15,930 genes presumed absent leaving 15,169 genes for further analysis. A filter on range was also applied to remove genes that did not change. Genes with a range of less then 1 across all chips in the experiment were excluded leaving 8,259 genes for further analysis. Differential expression analysis was performed on the filtered gene list using a linear model on $log_2$ signal values which considered time, treatment, and the interaction between time and treatment to identify significantly changing genes. Comparisons of interest were extracted through contrasts (mediated t-statistic) where a raw p value of .05 was considered significant.

Post filtering and visualization of data was achieved using GeneChip Operating Software (GCOS) version 1.4 (Affymetrix) and Genespring version GX 7.3.1 (Silicon Genetics, Redwood City, CA, USA). Sample biased post filters were applied to obtain more conservative gene lists. Specifically, a gene was kept for consideration if it was expressed above 104 in at least one treatment group in a comparison. Also, for each pair of control and treated groups (i.e, E13, E14, E16, and Cultured E13) genes changing by a factor of less then 1.5 were removed.

## RESULTS

In the present study, in utero vinclozolin treated (daily 100mg/kg/day maternal interparetineal injections from E8-E14) and control testis from E13, E14, and E16 rats were compared to evaluate transcriptional changes during gonadal morphogenesis. Histological analysis of E13, E14, and E16 revealed no significant differences in morphology between control and treated animals (Fig. 1A-D). At E13 the morphological changes associated with gonadal sex determination have not begun and structures associated with this process are not yet visible. Control and treated E13 testis both lack visible structures and are indistinguishable (data not shown). At E14 pre-Sertoli cell/germ cell aggregates are visible and the beginnings of testis cords are seen forming in both control and treated animals (Fig. 1A and B). By E16, testis cords have fully formed with Sertoli and germ cells surrounded by peritubular myoid cells in testis of both control and treated animals (Fig1C and D). The lack of changes seen in histological testis sections between control and vinclozolin treated animals suggests that vinclozolin does not drastically alter morphological processes of embryonic sexual differentiation.

Adult trans-generational phenotypes do occur with embryonic vinclozolin treatment [1;2]. To determine what transcriptional changes occur in embryonic testis, which may initiate a cascade of events leading to adult onset disease, even in the absence of embryonic histopathologies, control and treated testes were compared using a genomic microarray analysis. Specifically, rat E13, E14, and E16 control and treated RNA samples were hybridized to the RAE230 2.0 chips for comparison. The Affymetrix RAE230 2.0 chip contains probe sets for 31,099 transcripts and allows for the majority of the rat transcriptome to be evaluated. Quality assurance analysis (data not shown) of raw data revealed high quality data and all chips were used in further analysis. A dendogram with a condition tree showing relatedness of treatment

groups was generated in GeneSpring from transcripts expressed in at least one condition of the *in vivo* treated or control samples (Fig. 2A). The condition tree reveals that control and treated samples for each individual time period cluster closest together indicating that their transcription profiles are more similar. The E13 and E14 conditions also cluster closer to each other than to E16 conditions. Together this shows that transcriptional changes with treatment for each time period are more subtle then transcriptional changes between the time periods E13, E14 and E16 during testis differentiation. Furthermore, the relatedness of E13, E14, and E16 samples agrees with the findings of a previous study of transcriptional changes during gonadal development [30] in which E16 also clustered distally to E13 and E14 samples. This supports the validity of the approach in the current study and demonstrates the large number of transcriptional changes involved in testis morphogenesis.

Genes significantly changed in the differentiating testis due to vinclozolin treatment were identified by extracting genes significantly changed between vinclozolin treated and control samples with a 1.5 or higher fold change for E13, E14, and E16. This revealed a total of 576 altered genes (Fig. 3A) representing approximately two percent of transcripts on the RAE230 2.0 chip. Interestingly nearly all genes identified are altered at only one time point with only 26 transcripts altered in any two time points and none altered in all three (Fig. 3A, genes appearing in multiple lists are indicated in the x-list column of supplemental tables by indicating other regulated time points E13, E14, or E16). Forty-five genes had altered expression at E13. These genes are listed in Supplemental Table 1 categorized by major gene function. At E14 a total of 241 genes had altered expression (Supplemental Table 2). At E16 there were 316 altered genes (Supplemental Table 3). A dendogram clustering altered transcripts for E13, E14, and E16 separately shows that an equal number of genes are increasing and decreasing with treatment at

E14 (Fig. 2C), while at E13 and E16 there are more genes that increase after vinclozolin treatment then decrease (Fig. 2B and D). A total of 422 transcripts increased after vinclozolin treatment with 34 increased at E13, 121 at E14, and 279 at E16 (Fig. 3B). Only 163 total transcripts decreased after vinclozolin treatment with 11 decreased at E13, 120 at E14, and 37 at E16 (Fig. 3C). Together this suggests a general increase in transcriptional abundance following vinclozolin treatment.

Categorization by major known function of all 576 genes altered by in utero vinclozolin exposure reveals that transcription, signaling, and cytoskeletal and extra cellular matrix associated transcripts are highly represented (Fig. 4). Development, translation and protein modification, protein binding, epigenetic, proteolysis, growth factor, immune response, cell cyle, and electron transport were also represented by multiple transcripts. Interestingly, transcripts that play a role in protein translation and modification are over-represented at E13 suggesting greater sensitivity of this cellular process to vinclozolin treatment prior to gonadal differentiation.

To identify cellular signaling pathways affected by vinclozolin, Kegg Pathway Analysis [43] was performed on the 576 genes found to be altered by *in vivo* vinclozolin exposure. Nineteen pathways were affected by two or more of the 576 genes altered by *in vivo* vinclozolin treatment. These pathways are listed in Table 1 with the number of genes and the associated impact factors (as calculated in Kegg Pathway Analysis) indicated for each time point. Impact factor calculation by Kegg considers the total number of genes in the pathway, the number of genes from the submitted list appearing in the pathway, and the role those genes play in the pathway. The majority of pathways identified are affected by vinclozolin altered genes from the later time points of E14 and E16 suggesting that vinclozolin may initiate a cascade of events

affecting a larger number of signaling pathways with time. Calcium signaling is affected at E13, E14, and E16 by vinclozolin treatment. Wnt signaling is affected at E13 and E14. MAPK, Gap junction, and cell adhesion pathways are also among those affected by vinclozolin altered genes at E14 and E16. The majority of these pathways are affected by the same small subset of genes with a total of 35 of the 576 transcripts appearing in a represented pathway. This suggests that a few genes may be responsible for a large number of signaling mediated effects including altered transcript levels of other identified genes. Therefore specific known pathways may not be as critical in mediation of vinclozolin altered expression as various cellular processes or gene-gene interactions in general.

In the current study organ cultures were used to compare the actions of vinclozolin *in vivo* and *in vitro*. E13 gonads cultured *in vitro* for three days develop testis cords and have been used as a model for study of testis development [29;44;45]. Previously a dose of 500uM vinclozolin was shown to reduce testis cord formation but a dose of 200uM or 50uM vinclozolin did not induce morphological changes in cultured testis [29]. In this study a dose of 200uM also reduced testis cord formation (data not shown) so a 50uM vinclozolin dose was used for the current *in vitro* studies (Fig1 E and F). The lack of change in testis histology in treated cultures compared to controls was desirable so that transcriptional changes could be compared to *in vivo* treatments that also lacked morphological change compared to controls. This comparison was made to determine if vinclozolin altered transcription was similar between the treated organ cultures and in vivo samples and determine if treated cultures could be used as a model for in vivo treatments. To help determine if vinclozolin was acting by an anti-androgenic mechanism the anti-androgen flutamide was also used to treat cultured testes at a dose of 5uM that did not cause morphological change (data not shown). Testis treated with 50uM vinclozolin or 5uM

flutamide were compared to respective vehicle controls to identify significantly changed transcripts with at least a 1.5 fold change between the treated and control conditions. Organ cultures treated with vinclozolin versus vehicle control revealed 19 altered transcripts (Supplemental table 4). Three of these transcripts were also among the 576 altered by *in vivo* vinclozolin treatment (marked with a "V" the "cross listed" column of supplemental tables) including *Sap18* and two ESTs. Organ cultures treated with flutamide versus vehicle control revealed 43 altered transcripts (Supplemental table 5). Four of these transcripts were also among the 576 altered by *in vivo* vinclozolin treatment (marked with an "F" in "Cross listed" column of supplemental tables) and included *Pdgfb*, *Ncor1*, *Mthfd21*, and an EST. The transcripts altered by *in vitro* vinclozolin or flutamide treatments did not share any common transcripts. This seems to suggest that vinclozolin and flutamide are not acting in the same manor and therefore that Vinclozolin is not acting through an anti-androgenic mechanism similar to flutamide, as previously suggested [46].

Based on the findings of the current study, a list of 38 candidate genes for future investigation of vinclozolin action on differentiating testis was compiled (Table 2). These genes were chosen for their higher fold changes, interesting cellular functions, or appearance in multiple lists compared in this study. The list of candidates includes genes that affect epigenetics, extra cellular matrix, signaling, and development. The present study presents this list of candidate genes for consideration in future studies of the mechanism of direct actions of vinclozolin during testis development that are known to later lead to adult onset disease.

# DISCUSION

The current study investigates vinclozolin induced alterations in gene expression in differentiating testes. Previous studies have shown that intra-peritoneal injection of 100mg/kg/day vinclozolin from E8-E14 of pregnancy resulted in increased apoptosis in testicular cells at P20 and P60, and decreased sperm number and motility in male offspring [29]. These phenotypes were also shown to persist for at least three subsequent generations [2]. These trans-generational adult disease phenotypes are presumed to result from changes induced during the time of embryonic exposure which are coincident with testis development. The current study was designed to investigate the mechanisms of vinclozolin action in the rat embryo, specifically on the differentiating testis.

It was hypothesized that alterations in the transcriptome of differentiating testis following vinclozolin treatment would be observed and that these alterations would be helpful in determining the mechanism of vinclozolin actions directly on the embryonic testis, which are known to indirectly lead to adult onset trans-generational disease. Transcriptional alterations affecting testis differentiation that lead to adult onset disease are expected to be subtle since no morphological changes were observed and previously no differences in fertility, testis weight, anogenital distance, body weight, testicular decent, or testosterone levels were seen with vinclozolin treatment during testis differentiation were observed [29]. In this study histological analysis of testes from E13 (not shown), E14, and E16 embryos (Fig. 1A-D), also showed no apparent morphological changes between control and treated testis during differentiation. Both control and treated testes appeared to develop normally as previously described [30]. This suggests few or no vinclozolin induced alterations of the gross morphological processes involved in sex determination. Results of this study also indicate that the transcriptomes of control and

treated testes are more similar than E13, E14, or E16 testes. This was expected due to the lack of morphological changes with treatment.

To identify vinclozolin induced alterations, transcripts with statistically significant changes in expression between control and treated samples were extracted for E13, E14, and E16. The lack of overlap in the 576 altered transcripts between developmental time points may be due to the different environments throughout testicular differentiation and development that are available for vinclozolin to act on. Alternatively, a cascade of events initiated by vinclozolin treatment leading to new alterations in transcription over time may explain the lack of overlapping alterations. The ratios of increasing to decreasing transcripts suggest a general increase in transcriptional abundance following vinclozolin treatment (Fig. 2). This general trend would be consistent with vinclozolin causing a cascade of events mediated by transcriptional regulators or post transcriptional modifiers leading to increased transcript abundance.

Cellular processes known to be affected by vinclozolin altered genes are also consistent with a potential role of vinclozolin in transcriptional alteration. Interestingly, genes known to affect cellular processes such as oxidative stress that are associated with toxicology were not identified following vinclozolin treatment in this study. This suggests that vinclozolin may not cause adult onset disease through classic toxicological mechanisms. The cellular functions most likely affected by vinclozolin altered genes appear to be transcription followed by signaling, extra cellular matrix, and metabolism. At the earliest time point studied, E13, RNA processing and translation is the highest represented function, suggesting transcripts associated with translational regulation are sensitive to alteration by vinclozolin during the initiation of sex determination. In a previous microarray study of normal sex determination and gonadal

development extra cellular matrix, signaling, and metabolism were highly represented, but not transcription and translation [30]. Combined observations suggest vinclozolin action is mediated at least in part through altering transcriptional regulation and possibly altered translation.

To determine if genes regulated during gonadal sex determination and testis development were altered by vinclozolin treatment, the 579 transcripts found to be altered by *in vivo* vinclozolin treatment in the current study were compared to candidate genes previously implicated in sex determination and testis development [30]. In this previous study 109 genes that were regulated between E13, E14, and E16 testis were identified as candidates for regulation of testis differentiation. Surprisingly, of these 109 genes only three (*Spon1*, *Spock2*, and *Ogn*) appear to be altered by vinclozolin during testis differentiation (indicated with a "C" in the "Cross listed" column of supplemental tables). This suggests limited involvement of those genes regulated during typical gonadal sex determination and testis differentiation in the mechanism of vinclozolin action on embryonic gonads which ultimately leads to increased testis cell apoptosis and decreased sperm count and motility.

In addition to the *in vivo* vinclozolin treatments used in the current study, the affect of *in vitro* treatments on E13 cultured gonads was investigated using microarray analysis. This was done to compare *in vivo* and *in vitro* vinclozolin altered transcripts and determine if the treated organ cultures could be used as a model system to study the effects of vinclozolin on testis development. In addition, the anti-androgen flutamide was used to treat organ cultures and microarray analysis results were compared to those of vinclozolin treated organ cultures to determine if flutamide and vinclozolin had similar effects. Results indicated that different transcripts were altered by *in vitro* vinclozolin treatment then by flutamide treatment. This seems to suggest that vinclozolin and flutamide are not acting in the same manner, however

caution must be taken in drawing conclusions about what does not overlap between microarray lists and comparisons must be interpreted with caution. The small resulting lists were likely the result of many factors including the low doses chosen for analysis. Doses were chosen that did not affect histology between control and treated samples to parallel lack of morphological effects seen in *in vivo* developing testis with or without vinclozolin treatment such that transcription alteration due to morphological changes did not make it more difficult to identify candidates for adult onset disease. These doses *in vitro* may be to low and not significantly alter testis development on any level. Another possible reason for the small numbers of altered transcript may be that testes were not exposed from E8-E14, but rather starting at E13 for three days in culture. Thus the treatment did not overlap with the formation of the indifferent gonad or germ cell reprogramming, and may not induce the same alterations as seen *in vivo*. A third explanation for the small number of detected transcriptional alterations is that transcriptional changes were relatively small and/or the background and variability of the system was sufficiently high to prohibit detection of changes with statistical significance using a replicate microarray approach. If background is high or changes in expression are small it becomes difficult to identify biological variability that is statistically significant. Sources of variability may have been introduced in the culturing of gonads and lead to a reduced number of detectable transcriptional alterations. For all of these reasons the small number of genes having been identified as altered by *in vitro* treatment for vinclozolin or flutamide suggests that treated cultures are not an overly useful model as applied in this study. However the results do indicate that flutamide and vinclozolin treatments alter different transcripts and may be acting through different mechanisms. Since flutamide is known to act as an antiandrogen, the differences may

mean that vinclozolin is acting through alternative mechanisms which is in agreement with a previous study of vinclozolin and flutamide actions on gonadal differentiation [46].

An epigenetic mechanism of action for vinclozolin has been proposed to explain the adult onset and transgenerational phenotypes associated with exposure [2;35]. In the current study, identification of altered genes in differentiating testis which may affect epigenetic gene regulation was of interest as an epigenetic mechanism may explain how an adult onset disease can result from an embryonic treatment as previously suggested [35]. The list of 576 vinclozolin altered genes from this study was compared to a previous study of E16 control and vinclozolin treated testis for F1, F2, and F3 generation animals which identified 1597 altered transcripts between control and treated F1 testes. A total 124 transcripts appeared in both studies (indicated by an A in the "cross listed" column of supplemental tables), many of which are associated with epigenetic functions consistent with the previous study. A total of twelve transcripts with epigenetic related functions were altered by vinclozolin in the current study (Fig. 4). A few of these are discussed in more detail below. Together these findings further support the idea that epigenetics play a role in vinclozolin actions on the embryonic testis that may lead to the appearance of phenotypes later in life and in subsequent generations.

From the *in vivo* analyses in this study, several genes with interesting and potentially significant functions were chosen to highlight as candidates altered by vinclozolin in the differentiating testis which may lead to adult onset trans-generational disease. This list contains 38 vinclozolin altered genes chosen for interesting expression patterns, larger changes in expression, or intriguing functional or pathway associations. Many of the candidates identified as altered by vinclozolin in the embryonic testis have known functions identified in previous studies. Although some candidates may have differing functional roles in testis development

then in previously studied tissues or systems, knowledge of the previously identified functions for these candidate genes provides useful insights. Therefore previously identified gene fuctions of identified candidates were considered. Interestingly, several genes altered at E13 by vinclozolin treatment are known to regulate transcript stability and processing, as was hypothesized from the number of up-regulated transcripts identified in this study. These included three non-coding RNAs, *Prp4b* (known to play a role in RNA processing) and *Thoc2* which has been shown to associate with ribonucleoprotein complexes and be important for mRNA quality control [47;48]. Another transcript, *Camk2d*, is known to phosphorylate histone deacetylases affecting histone localization on DNA and therefore transcriptional availability [49;50]. *Camk2d* histone phosphorylation is a calcium dependant process and is known to affect vascular smooth muscle cell proliferation and migration [51;52]. *Gtl2* is known to be a maternally imprinted region involved in micro RNA stabilization [53;54]. *Gtl2* is methylated in the male germ line during germ cell differentiation correlating with the time of treatment in this study [55]. The DNA methylation patterns that arise have been shown to persist to spermatogonia and spermatocytes [56]. The RNA stabilization role and epigenetic imprinting of *Gtl2* that persists to adult hood in the germ line makes the *Gtl2* gene and gene region particularly interesting for future studies of vinclozolin action on differentiating testis potentially leading to the previously observed induction of adult onset and transgenerational disease.

Four transcripts altered at E14 are known to be sensitive to hyper-methylation suppression. These included *Ppplr3c*, a serine/therine protein phosphatase implicated in many diverse cellular processes [57], the tumor suppressor Hic2 [58;59], and *Sox7* known to bind to beta-catenin and inhibit transcriptional activity [60;61]. The fourth gene, *Apc2*, can also affect beta-catenin function as well as cadherins and is known to be important for cellular proliferation

and differentiation decisions [62;63]. At E16, known epigenetic regulators were altered by vinclozolin. *Chd6* is an ATP dependant chromatin remodeling enzyme with DNA dependant ATPase activity [64]. *Prmt1* has been identified in embryonic germ cells [65]. It is a coactivator of AR and binds and methylates hnRNPQ for internalization of insulin receptors [66;67]. A role for these genes in mediation of vinclozolin induced disease or altered testis development is currently unknown and may help in understanding how potential epigenetic regulators or epigenetically regulated genes can affect testis development ultimately leading to adult onset disease.

In addition to *Prmt1*, other vinclozolin altered genes are known to be important for insulin signaling which is required for proper testis development. *Nedd4* is a ubiquitin ligase important for protein trafficking and signal transduction [68]. *Nedd4* is a known mediator of androgen dependant negative regulation of AR. In another signaling loop, *Nedd4* increases *Igf1* and insulin signaling and is downstream of *Igf1r* [69-71]. Igf1r signaling in mitochondria causes internalization of *Nedd4* which leads to apoptotic protection [72]. Because a loss of *Nedd4* translocation leads to a loss of apoptotic protection, *Nedd4* is another highly interesting candidate for mediation of vinclozolin action in the embryo that may lead to increased apoptosis in pubertal and adult rats.

Increased testis cell apoptosis is one adult onset disease phenotype induced by vinclozolin. Although vinclozolin treatment between E8-E14 was previously shown to increase apoptosis in the pubertal and adult rat testis and decrease sperm motility and numbers, the action of vinclozolin on the embryonic testis which leads to these adult phenotypes has not been investigated. Perhaps a reduction in apoptotic protection in the embryonic testis persists until puberty at which time this loss of protection becomes apparent. Other transcripts affecting

apoptosis were also altered by vinclozolin. *Hrmt1l1* is a protein argentine methyltransferase which can interact with ribonucleoprotein complexes and promote apoptosis through *NF-KappaB* transcription [73;74]. *Tia1* is an alternative splicing factor that can also promote apoptosis by affecting Fas receptor splice variants [75;76]. The role of these transcripts during testis differentiation and there potential role in mediating vinclozolin induced increases in apoptosis later in life would also be interesting to investigate further.

In addition to the epigenetic associated transcripts *Apc2*, *Gtl2* and *Camk2d* mentioned above, several other transcripts are known to be calcium dependent or affect calcium signaling. The prolactin receptor has been shown to regulate circulating levels of calcium [77]. It is expressed in Leydig and germ cells [78]. Prolactin pathways are present in the embryonic rat and are required for testis production [79]. Cadherin 1 is a calcium dependent transmembrane protein that mediates cell-cell adhesion and is found in type A spermatogonia and spermatocyte stem cells [80]. *Efcab2* also binds calcium but has unknown functions. Chromogranin B binds calcium and mediates calcium release by inositol 1,4,5-triphosphate receptor [81]. Given the number of transcripts implicating the potential importance of calcium in mediation of vinclozolin action, calcium signaling would be worthy of further investigation. If Calcium distribution can be significantly altered by vinclozolin, Calcium dependant functions may be altered.

Chromogranin B not only is important for calcium release in general, but also in regulating Angiotensin 2, so it is interesting to note that the Angiotensin 2 Receptor is also altered by vinclozolin. Classically known for its role in blood pressure regulation, *Agtr2* is expressed in early postnatal testis and correlated with germ cell maturation [82;83]. *Camk2d*, which is mentioned above in relation to epigenetic and calcium functions, affects migration and proliferation of vacular smooth muscle [51;52]. *Vcam1* has been implicated in atherosclerosis

[84]. *Gucyl1A3* is an angiogenic factor that is regulated by AR [85;86]. Given the number of vascular system related transcripts, vinclozolin interference with the critical vascularization events associated with testis differentiation and development should be investigated further.

Other miscellaneous transcripts affected by vinclozolin include *Pdgfr*, Osteoglycin, *Cxc4*, *Spondin 1*, and *Prss35*. *Pdgfr* is required for mesonephric cell migration and proper testis cord formation [36;87;88]. Osteoglycin has been previously implicated in testis development as well [30;89]. *Cxc4* is expressed in germ cells and important for germ cell migration and maintaining the germ cell niche [90-92]. Spondin 1 is known to induce beta-catenin signaling and also inhibits testis differentiation and promotes ovarian development [93]. *Prss35* is a serine protease up-regulated by gonadotropins for ovarian function [94]. Given that all of these transcripts have been implicated in reproductive processes, they too make interesting candidates for mediating vinclozolin action leading to altered reproductive phenotypes.

It is interesting to note that vinclozolin treatment affects expression of multiple transcripts implicating epigenetics, vascular system, apoptosis, transcription and signaling by calcium, insulin, Wnt, and AR. Of further interest is the fact that several transcripts have the potential to affect many of these processes. Investigation of vinclozolin action mediated through each of these processes individually as well as the connections between them will likely lead to a better understanding of the mechanisms of vinclozolin action on developing testes. The present study has provided a list totaling 576 candidates for further investigation, of which 38 of the more interesting altered transcripts have been highlighted. In addition, the processes and pathways most affected by vinclozolin treatment have been identified. This study has provided a better understanding of the effects of vinclozolin treatment on the developing testis and numerous ideas for the direction of future investigations.

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Table 1. Pathways Affected by Vinclozolin Altered Genes

| | E13 | | E14 | | E16 | |
|---|---|---|---|---|---|---|
| Pathway Name | Impact Factor | #Genes In path | Impact Factor | #Genes in path | Impact Factor | #Genes in path |
| Calcium signaling pathway | 4.38 | 1 | 4.79 | 3 | 6.04 | 4 |
| Leukocyte transendothelial migration | 2.74 | 1 | 4.67 | 3 | 5.57 | 3 |
| Neuroactive ligand-receptor interaction | 2.61 | 1 | 0.73 | 2 | 2.33 | 3 |
| Regulation of actin cytoskeleton | 3.03 | 1 | 4.61 | 4 | 3.66 | 1 |
| Focal adhesion | 2.91 | 1 | 5.43 | 3 | 9.86 | 1 |
| Melanogenesis | 3.11 | 1 | 4.24 | 2 | 2.99 | 1 |
| Wnt signaling pathway | 2.84 | 1 | 4.30 | 2 | | |
| MAPK signaling pathway | | | 1.33 | 2 | 3.67 | 4 |
| Gap junction | | | 10.13 | 4 | 8.32 | 3 |
| Cell adhesion molecules (CAMs) | | | 20.15 | 2 | 264.81 | 3 |
| Tight junction | | | 7.97 | 1 | 6.78 | 3 |
| Cytokine-cytokine receptor interaction | | | 0.75 | 1 | 3.54 | 3 |
| Jak-STAT signaling pathway | | | 3.85 | 2 | 5.78 | 2 |
| Adherens junction | | | 3.06 | 1 | 14.06 | 2 |
| Melanoma | | | 7.66 | 1 | 7.03 | 2 |
| Axon guidance | | | 2.55 | 2 | 2.71 | 1 |
| Colorectal cancer | | | 2.64 | 2 | 2.47 | 1 |
| Glycan structures - biosynthesis 1 | | | 2.49 | 2 | 1.91 | 1 |
| Type I diabetes mellitus | | | 12.69 | 2 | 1.47 | 1 |

Impact Factor indicates relative significance of pathways. Impact Factor is generated by KEGG pathway analysis software. Higher values indicate pathways of greater relative significance.

TABLE 2. Candidate genes for vinclozolin action on differentiating testis

| Category | Gene | V/C | Cross Listed* | Affy ID |
|---|---|---|---|---|
| Transcription | THO complex 2 (Thoc2, predicted) | 3 | E13 | 1385493_at |
| Signaling | Protein phosphatase 1, regulatory subunit 3C (Ppp1r3c) | 1.91 | A, E14 | 1373108_at |
| | Guanylate cyclase 1, soluble, alpha 3 (Gucy1a3) | 1.91 | A, E14 | 1387079_at |
| | SH3 domain binding protein CR16 | 1.91 | A, E14 | 1370648_a_at |
| | Calcium/calmodulin-dependent protein kinase II, delta (Camk2d) | 1.7 | E13 | 1371263_a_at |
| | Rho guanine nucleotide exchange factor17 (Arhgef17, pred) | 1.6 | E13 - | 1374907_at |
| | Sushi domain containing 3 (Susd3, predicted) | 1.91 | E14 | 1377351_at |
| Receptor & Binding | Angiotensin II receptor, type 2 (Agtr2) | 2.83 | E13-,E14- | 1398288_at |
| | Platelet derived growth factor receptor beta (Pdgfrb) | 3.87 | F, E14, E16 | 1379211_at |
| Protein mod. & binding | Protease, serine, 35 (Prss35) | 3.08 | - A, E14 | 1379747_at |
| | PRP4 pre-mRNA processing factor 4 homolog B (Prpf4b, yeast) | 1.9 | E13 | 1397203_at |
| | EF-hand calcium binding domain 2 (Efcab2, predicted) | 1.64 | E16 | 1375646_at |
| Immune Response | Thymus cell antigen 1, theta (Thy1) | 1.91 | E14 | 1369652_at |
| | RT1 class II, locus Bb (RT1-Bb) | 1.91 | E14 | 1371033_at |
| Growth Factors, Cyto+Chemokines | Insulin-like 3 (Insl3) | 1.91 | E16 | 1388241_at |
| | Prolactin receptor (Prlr) | 1.91 | E16 | 1370384_a_at |
| | Spondin 1 (Spon1) | 1.91 | E16 | 1370312_at |
| | Chemokine (C-X-C motif) receptor 4 (Cxcr4) | 1.91 | E16 | 1370097_a_at |
| Epigenetics | Non-coding RNA expressed in the brain, repeat sequence | 3.44 | - A, E14 | 1398716_at |
| | Non-coding RNA expressed in the brain, repeat sequence | 2.92 | - A, E14 | 1397700_x_at |
| | SNF2 histone linker PHD RING helicase (Shprh, predicted) | 1.95 | - A, E14 | 1379951_at |
| | Chromogranin B (Chgb) | 4.69 | - E14 | 1368034_at |
| | Non-coding RNA expressed in the brain, repeat sequence | 2.37 | - E14 | 1392166_at |
| | Enhancer of polycomb homolog 2 (Epc2, Drosophila, predicted) | 1.6 | - E14 | 1383467_at |
| | Similar to GTL2, imprinted maternally expressed untranslated | 3 | E13 | 1368887_at |
| | Hypermethylated in cancer 2 (Hic2, predicted) | 1.63 | E14 | 1390030_at |
| | Cytotoxic granule-associated RNA binding protein 1 (Tia1) | 1.91 | E16 | 1397692_at |
| | Chromodomain helicase DNA binding protein 6 (Chd6) | 1.91 | E16 | 1398225_at |
| | protein N-arginine methyltransferase 1 (Prmt1) | 1.91 | E16 | 1376025_at |
| | HMT1 hnRNP methyltransferase-like 1 (Hrmt1l1, S. cerevisiae) | 1.91 | E16 | 1376025_at |
| Development | Neural expressed developmentally down-regulated 4 (Nedd4) | 1.65 | - E14, E16 | 1383899_at |
| | Adenomatosis polyposis coli 2 (Apc2, predicted) | 1.91 | E14 | 1397579_x_at |
| | SRY-box containing gene 7 (Sox7, predicted) | 1.66 | E14 | 1384415_at |
| | Synaptic vesicle glycoprotein 2b (Sv2b) | 1.91 | E16 | 1369628_at |
| Cytoskeleton-ECM | Vascular cell adhesion molecule 1 (Vcam1) | 1.79 | - E14 | 1368474_at |
| | Osteoglycin (Ogn, predicted) | 3.17 | E13,C,A, - E14 | 1383263_at |
| | Similar to Myosin-binding protein H (MyBP-H) (H-protein) | 1.91 | E16 | 1378970_at |
| | Cadherin 1 (Cdh1) | 1.91 | E16 | 1386947_at |

For any gene identified in multiple lists, the other lists in which it appears are indicated in the Cross Listed column. E13= altered by vinclozolin at E13, E14= altered by vinclozolin at E14, E16= altered by vinclozolin at E16, V= altered by vinclozolin in organ cultures, F= altered by flutamide in organ cultures, A= appeared in a list of F1 E16 vinclozolin altered transcripts published by Anway et al 2008, C=appeared in a list of testis differentiation candidates published by Clement et al 2007

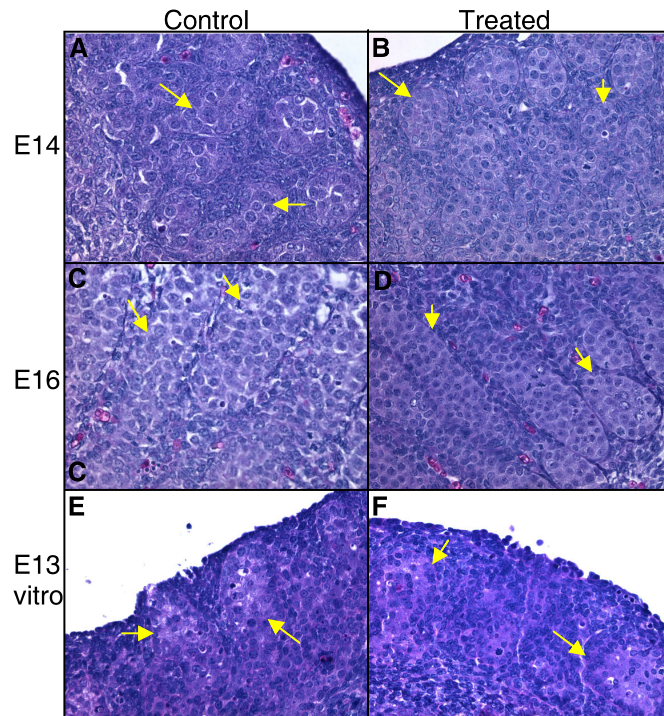


**Fig 3-1.** Histological sections of vinclozolin treated and control testis (H&E stained). A. E14 control. B. E14 treated. C. E16 control. D. E16 treated. E. E13 cultured three days control. F. E13 cultured vinclozolin treated. Arrows indicate forming cords which contain large round darker stained germ cells surrounded by lighter stained Sertoli cells.

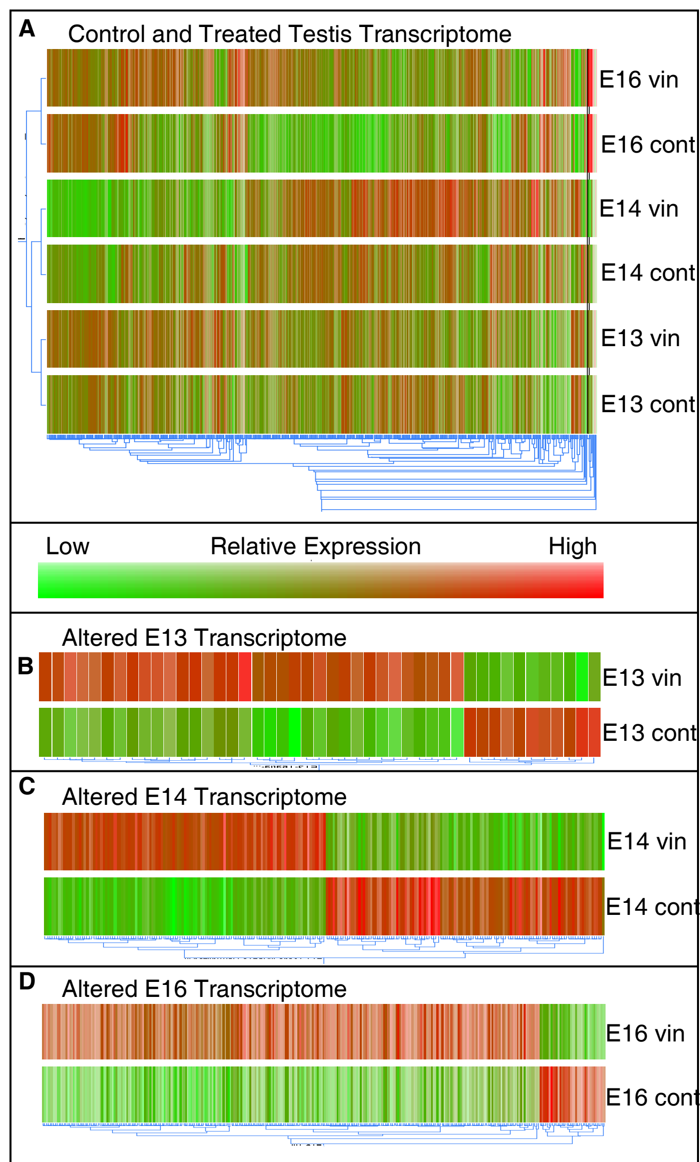


**Fig 3-2.** Dendogram of in vivo vinclozolin treated and control testis. A. All genes expressed in any of the six conditions (E13, E14, and E16 control and treated) for in vivo exposed analysis. Condition tree to the left shows relatedness of the 6 conditions. B. Genes altered by vinclozolin treatment at E13 (45). C. Genes altered by vinclozolin treatment at E14 (241). D. Genes altered by vinclozolin treatment at E16 (316). Gene trees below dendograms indicate relative relatedness of transcript expression patterns between conditions.

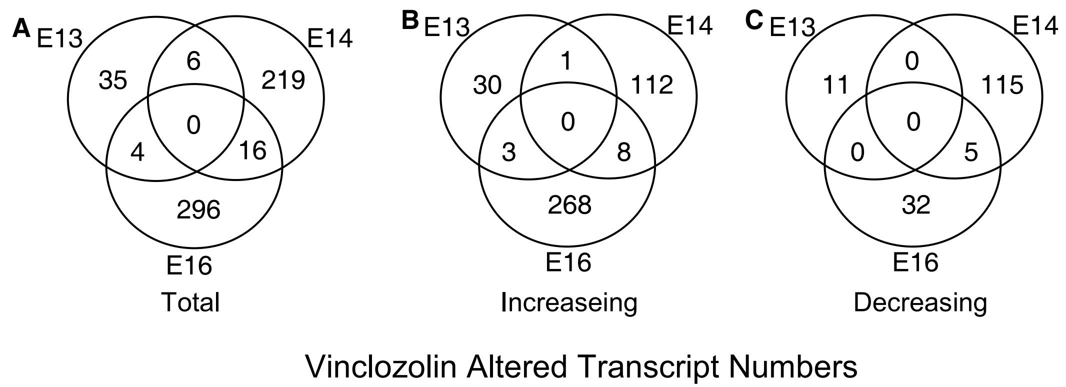


**Fig 3-3.** Venn diagrams of significantly altered transcripts with at least a 1.5 fold change in E13, E14, and/or E16 after in vivo vinclozolin exposure. A. All 576 vinclozolin altered transcripts from in vitro treated E13, E14, and E16. B. 422 increasing transcripts. C. 163 decreasing transcripts.

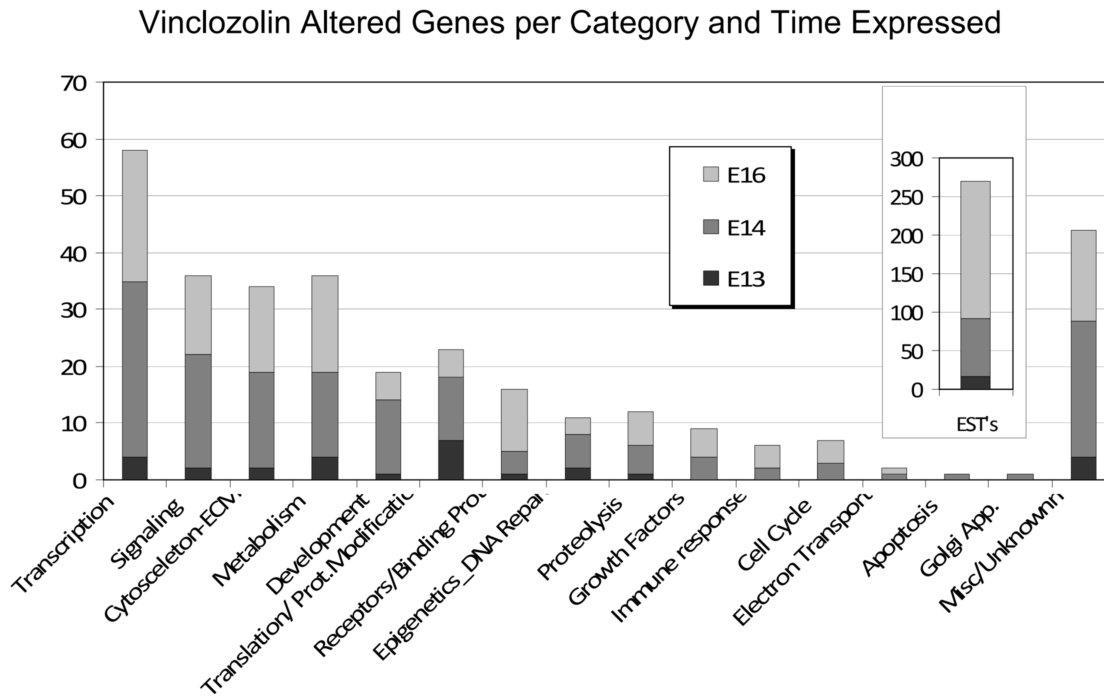


**Fig 3-4.** Categorized vinclozolin altered genes for E13, E14, and E16. Categorized by major known function. For each time period a gene is only represented once, for each category a gene is represented for each time point in which it appears (minimal overlap).

CHAPTER 4

# INFLUENCES OF SRY AND SOX9 ON THE NEUROTROPHIN 3 PROMOTER

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**ABSTRACT**

A critical role for Nt3 in gonadal sexual differentiation has been demonstrated. Nt3 is expressed in Sertoli cells and acts as a chemo-attractant for cell migration from the mesonephros into the developing testis, a process critical to the early morphological events of testis cord formation. The expression of *Nt3* suggests a potential for direct regulation of *Nt3* by the key regulators of sex determination, SRY and SOX9. Although the sex determining factor SRY has been studied for more than 18 years, a direct regulatory role by which it directs the differentiation of the testis has yet to be identified. Information on the transcriptional targets of another key regulator of testis differentiation, SOX9, is also limited to a few genes. For the few genes known to be regulated by SOX9 a response element composed of paired SOX binding sites seems to be required for promoter activation. In the current study the molecular mechanisms of male-specific *Nt3* expression during testis differentiation were investigated using immunohistochemistry, promoter mutational analysis and co-transfection of *Nt3* promoter/reporters with SRY and SOX9 in a cell culture system. Immunohistochemical results show that Nt3 is initially expressed in Sertoli cells only at E14 which is consistent with potential regulation by SRY. Results of promoter mutational and co-transfection analyses suggest that SRY and SOX9 can activate the *Nt3* promoter *in vitro* in a site specific manner. Furthermore, paired SOX binding sites are not required for *Nt3* promoter activation by SRY or SOX9. These results suggest that *Nt3* may be a direct downstream target of SRY or SOX9 regulation and investigation of the potential for SRY and SOX9 to bind directly to SOX sites in the *Nt3* promoter are currently underway.

# INTRODUCTION

Differentiation and development of dimorphic sexes is critical to mammalian reproduction. Genetic or environmental disruption of sex determination during embryogenesis can lead to morphological or epigenetic defects leading to infertility, malformation of secondary sexual structures, or adult onset trans-generational disease. To better understand how sex determination can be disrupted, the mechanisms underlying the normal process of sex determination must first be understood. The key sex determining gene *Sry* was discovered over 18 years ago. Despite intensive investigation since the identification of *Sry* a direct mechanism of action remains to be discovered. In the current study we investigate *Nt3* as a direct target of SRY transcriptional activation to identify a direct SRY action in testis differentiation.

Testis development begins with the production of a bipotential gonad followed by a critical period in which sexual fate is decided. *Sry*, the male sex-determining gene, is integral to this process [1;2]. It is first expressed at E10.5 in male mouse gonads [3;4]. *Sry* is required for the differentiation of Sertoli cells and for induction of mesonephric cell migration to the genital ridge to form seminiferous tubules [5;6]. Infertility studies often focus on the germ cells that, while required for spermatogenesis, are not required for testis development [7;8]. Sertoli cells, however, are required for both proper development and functioning of the testis [9]. As the only cells to express *Sry*, Sertoli cells are thought to be the orchestrators of testis development and altered *Sry* expression can lead to sex reversal and intersex disorders [2;3;10;11]. SRY was recently shown to act in conjunction with SF1 to upregulate *Sox9* expression for testis differentiation [12]. However, other direct actions of *Sry* critical to testis development remain elusive. No other information regarding direct regulation of other downstream genes involved in testis morphogenesis by *Sry* is available.

The expression pattern of *Sry* has given insight into its mechanism of action. *Sry* is expressed in mouse by developing Sertoli cells between E10.5 and E12.5 where it plays its functional role [3]. Further insight into the action of *Sry* has come from studying its structure. It is a single exon gene with one highly conserved area, the HMG box [3;13-15]. This motif binds to DNA in a sequence-specific manner and induces a bend in the DNA [16;17]. Nuclear localization signals have also been found to flank this HMG region [18;19]. These features of *Sry* have led to the suggestion that it is an architectural transcription factor [20], however, it may be functioning as a conventional activator or repressor. Identifying a direct functional role for SRY will help to better understand regulation of functional testis development.

SOX9, like SRY, is an HMG box factor with about 87% amino acid similarity to SRY in that region. It has been shown to bind similar sequences and bend DNA [21]. SOX9 is also expressed in Sertoli cells, at the time of sex determination, with increased expression in testis beginning at the peak of SRY expression [11;12;22;23]. This suggests that *Sox9* may be a direct target of SRY [11;24], although no direct targets of SRY have been conclusively identified. SOX9 is critical for male sex determination [25;26], as over-expression leads to XX sex reversal. Haploinsufficiency in humans and gonad specific knockouts in the mouse leads to XY sex reversal [27-29]. The similarity in expression, structure, and function between SRY and SOX9 requires both genes be considered when investigating direct regulatory targets for one or the other.

Due to its critical role in testis development and timing of expression, Neurotrophin 3 (*Nt3*) is a potential target of SRY or SOX9 binding for transcriptional activation. NT3 is required for mesonephric cell migration into the testis for cord formation [30-32]. NT3 is highly conserved between species [33] and acts through the high affinity receptor, TRKC, and the low

affinity receptor, p75LNGFR . TRKC is expressed at E14 in the mesonephros around the ducts [30;32]. In the mouse and rat, p75LNGFR is expressed in mesonephric cells that migrate to the developing testis to promote testis cord formation [30;34-36]. *Nt3* is expressed in Sertoli cells immediately following *Sry* beginning at E14 in the rat [30;32]. This is just before testis cord formation and NT3 acts as a chemo-attractant for mesonephric cells which must migrate into the testis for cord formation to occur. Its spatial and temporal expression patterns make it a candidate for SRY regulation. Consistent with that idea, a promoter search revealed potential SRY binding SOX elements in the *Nt3* promoter. In the current study, the ability for SRY and SOX9 to act at these putative SOX binding sites was investigated to determine if *Nt3* is a direct downstream target of SRY or SOX9. Results indicate that *Nt3* may in fact be regulated directly by SRY or SOX9 in a site specific manner.

## MATERIALS AND METHODS

### Histology

Sprague–Dawley rats were kept in a temperature controlled environment and given food and water *ad labidum*. Estrous cycles of female rats were monitored by cellular morphology from vaginal smears [37]. Rats in early estrus were bred overnight and matings confirmed by sperm positive smears, denoted day 0 of pregnancy. Pregnant rats were euthanized at E13, E14, and E16 of pregnancy, and embryonic gonads were collected for histological analysis. Sex was determined by PCR using primers specific for *Sry* on genomic DNA isolated from embryo tails as previously described [30]. All procedures were approved by the Washington State University Animal Care and Use Committee. Tissue specimens were fixed in Bouin's solution for 1 h and embedded in paraffin using standard procedures. Serial sections of 4 µm were stained with hematoxylin and eosin (H&E) using standard procedures. Sections were visualized by light microscopy.

### Immunohistochemistry

Embryonic testis sections were deparaffinized, rehydrated through a graded ethanol series, boiled 5 minutes in 10 mM sodium citrate buffer to expose the antigens, washed with 0.1% Triton-X solution, and then blocked with 10% goat serum (normal goat serum; Vector Laboratories Inc., Burlingame, CA, USA) for 30 min prior to incubation with 0.5 µg/ml primary anti-NT3 antibody for 15 h (Santa Cruz Biotechnology, Santa Cruz, CA). The sections were then washed with PBS and incubated with 1:3000 diluted Alexa Fluor 488 labeled secondary antibody for 45 min (goat anti-rabbit IgG; Invitrogen, Eugene, OR). Slides were mounted with Vecta-Shield plus DAPI (Vector Laboratories Inc.), sealed with coverslips, and analyzed using fluorescence confocal microscopy. Negative control experiments were performed using a non-

immune primary antibody at 1 μg/ml (rabbit IgG; Sigma, St. Louis, Mo). MIS localization was performed using 5 μg/ml primary anti-MIS antibody for 15 h (R&D Systems, Minneapolis, MN) and 1:500 diluted Alexa Fluor 488 labeled secondary antibody (donkey anti-goat IgG; Invitrogen, Eugene, OR) using the protocol above.

### Plasmid Construction

*Mouse and rat Nt3 promoter/reporter vectors:* A 636bp fragment of the mouse and rat *Nt3* promoters were amplified from respective genomic DNA (Forward: CGGGGTACC-AGGGTCTGAGGTTTGGAGAC, Reverse: GTACTAGCTAGCACTACCCAGCGGTGG-GAA) and cloned into the pGL3-Basic luciferase reporter vector (Promega, Madison, WI) using the Nhe1 and Kpn1 sites of the multiple cloning region. To generate mutant promoter/reporter constructs, the SOX binding consensus sites in the mouse and rat *Nt3* promoters were mutated using complementary oligos with a total of four point mutations to the consensus sequence $^{A}/_{T}$AACAA$^{A}/_{T}$. Oligos were PAGE purified and mutagenesis preformed using QuikChange Site-Directed Mutagenesis (Stratagene, La Jolla, CA) according to the manufactures directions. All constructed promoter vectors were sequence verified.

*SRY and SOX9 expression vectors:* A full length rat *Sry* expression plasmid with a MYC tag was produced by amplifying the single exon from rat genomic DNA (Forward: AGAGCTTTGGGAGCAGTGACAGTT, Reverse: TCTTTGCTGAGGTGCTCCTGCTAT) and cloned into pCMV-Myc expression vectors (Clontech, Mountain View, CA) using the BglII and NotI restriction sites. HA tagged Mouse SRY and SOX9 expression vectors in a pCDNA vector were kindly gifted by Dr. Peter Koopman [38].

### Cell Preparation and Culture

*Sertoli cell culture:* Sertoli cells were isolated from 20-day-old rat testes by sequential enzymatic digestion [39-41]. All animal procedures and protocols were approved by the Washington State University Animal Care and Use Committee. Decapsulated testes were minced with razor blades. Fragments were then digested with trypsin (1.5 mg/ml, Life Technologies, Gaithersburg, MD) to remove the interstitial cells followed by collagenase (1 mg/ml type I, Sigma) for removal of germ cells and then hyaluronidase (1 mg/ml, Sigma) for removal of peritubular cells. Sertoli cells were plated under serum free conditions in 24-well Falcon Plates (Falcon Plastics, Oxnard, CA) at 1 x $10^6$ cells/well. Cells were maintained in 5% $CO_2$ atmosphere in Ham's F-12 medium (Life Technologies) with 0.01% BSA at 32$^o$C.

*E13 testis cell culture:* E13 embryos were collected from timed-pregnant females as described above. Gonads from E13 animals were dissected and each pair of gonads from individual animals were placed into one well of a 24 well plate with 300 µl Ham F-12 medium until embryos could be sexed as described above. The male gonads were then pooled and digested with collagenase (1 mg/ml type I) and hyaluronidase (1 mg/ml) to disassociate the cells. All the cells from the digested testes were then cultured on 100 mm plates in Ham's F-12 with 10% bovine calf serum (Sigma). Cells initially multiplied well in culture and were split as they reached confluence 1:2 twice, at which point cell division slowed considerably. Cells were maintained in culture, changing medium every three days, until growth plaques were observed at approximately one month. These growth plaques were then collected for further propagation and frozen stocks were prepared for subsequent cell splittings such that cells were maintained below 12 subbings.

**Transfection Procedures**

*Sertoli cell transfection:* Sertoli cells cultured for 48 hours were transfected by the calcium phosphate method coupled with hyper osmotic shock (10% glycerol) as previously described [39]. Briefly, 2 µg promoter reporter plasmid with or without 1 µg expression plasmid in 150 µl transfection buffer (250 mM $CaCl_2$ mixed 1:1 (v:v) with 2x HEBES (28mM NaCl, 50 mM HEPES, and 1.47 mM $Na_2HPO_4$ at pH 7.05)) was added to each well of a 24 well plate containing $10^6$ Sertoli cells/well in 1 ml Ham's F-12 medium and incubated at 32$^o$C for 3.5 hours. Following incubation, the cells were subjected to hyper-osmotic shock. The medium was aspirated and 1 ml of 10% glycerol in Hanks' Balanced Salt Solution (HBSS, Invitrogen) was added for 3 minutes. Wells were washed twice in HBSS before fresh Ham's F-12 with 0.01% BSA and 1% serum was added. Following a 72 hour incubation cells were harvested for luciferase assays. Media was aspirated and 100 µl of 1x cell lysis solution (Promega) was added per well. Plates were frozen and thawed before cell lysate was collected. For detection of luciferase reporter activity 20 µl of Sertoli cell lysate was mixed with 50 µl of luciferase substrate (Promega) and luciferase activity detected on a Wallac Victor II 1420 instrument.

*E13 cultured testis cell transfection:* Cells between sub 8 and 12 were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were serum starved for 24 hours in Ham's F-12 medium without antibiotics. Two µg promoter reporter plasmid with or without 0.5 µg expression plasmid was mixed with 2 µl Lipofectamine 2000 in 100 µl Opti-MEM medium (Invitrogen) for each well of a 24 well plate. This 100 µl mix was added to each well containing ~90% confluent cultured E13 testis cells in 1 ml Ham's F-12 medium without antibiotics and incubated at 32$^o$C for 24 hours. After 24 hours medium was aspitrated from cells and replaced with 1ml Ham's F-12 with 0.01% BSA and 1% serum was added per well. Cells were incubated 72 hours and collected for luciferase assays as described above.

**Electrophoretic Mobility Shift Assays**

Coupled *in vitro* transcription-translation reactions were performed with the TNT Quick Coupled Transcription/Translation System (Promega). One microgram of mouse *Sry* or *Sox9* expression plasmid DNA was used per reaction according to the manufactures directions. Protein expression was verified by western analysis using an antibody against the HA tag (Clontech). Some protein was further purified using Profound HA-tag IP Application Set (Thermo Scientific, Rockford, IL) according to the manufactures directions. As probes, complementary oligonucleotides corresponding to each *Nt3* promoter putative SOX binding site and mutants were end labeled with Biotin 3' End DNA Labeling Kit (Pierce, Rockford, IL). Binding reactions were performed in a final volume of 30 µl in binding buffer (100 mM KCl, 1 mM $MgCl_2$, 10 µM $ZnSO_4$, 10 mM Tris, pH 7.5, 4% glycerol, 0.1% Triton X-100, 1 mg/mL BSA, 1 µg of poly(dIdC)/poly(dAdT), 0.5 mM DTT). Reaction mixtures containing 0.1-1 µl protein product, with or with out 4 pmol unlabeled competitive oligo, were preincubated for 10 min at room temperature followed by the addition of 20 fmol end-labeled probe. After 30 minute incubation at room temperature, DNA-protein complexes were resolved by electrophoresis on 4% polyacrylamide gels (containing 2.5% glycerol) at 100 V for 2 h in 0.5× TBE buffer. Complexes were transferred to Hybond N+ membranes (Amersham Biosciences) at 380 amps for 45 min followed by UV cross-linking on a trans-illuminator for 20 minutes. Complexes were detected using biotin detection reagents (Pierce) and exposed to X-ray film.

# RESULTS

To identify the expression pattern of NT3 in differentiating testis immunohistochemical localization was performed. NT3 was localized on E14 and E16 embryonic rat testis sections using an NT3-specific antibody. To assist in indentifying the specific cell types in which NT3 is expressed, comparison with the Sertoli cell-specific marker gene AMH was also performed. NT3 was expressed by Sertoli cells at both E14 and E16 (Fig. 1). At E14 NT3 stained Sertoli cells are observed with characteristic contorted shapes and are contrasted by germ cells with a lack of NT3 staining containing large round DAPI stained nuclei (Fig. 1C). This pattern of expression is in contrast to E16 where germ cells and Leydig cells also show apparent NT3 expression (Fig. 1E and F), whereas AMH showed the expected expression in Sertoli cells only at E16 when it is initially expressed (Fig. 1G).

The Sertoli cell only expression pattern of NT3 at the onset of testis differentiation suggested that SRY and/or SOX9 might directly regulate *Nt3* transcription. To test this hypothesis, the genomic regions upstream of the mouse, human, and rat *Nt3* transcription start sites were compared using a ClustalW alignment (available on the EMBL-EBI web server). Over 500 bp upstream of the transcription start site are highly conserved between human, mouse, and rat (data not shown). MotifFinder (available through GenomeNet) was used to check for consensus SOX binding sites to which the conserved HMG box DNA binding region of SOX proteins including SOX9 and SRY can bind. A single site was found in rat 214 base pairs upstream of the putative transcriptional start site (Fig. 2A) and two of these SOX binding sites, separated by 9 base pairs, were found in mouse 222 and 206 base pairs upstream of the transcriptional start site (Fig. 2B).

To determine whether these SOX binding sites are important for *Nt3* promoter activity a mutational analysis was performed. Introduction of four point mutations into the rat 214 site resulted in a statistically significant decrease in promoter activity compared to that of the wild type promoter transfected in P20 cultured Sertoli cells (Fig. 3A). In mouse, mutation of either the 222 or 206 site, or both sites, also resulted in a significant decrease (p-value $\geq 0.05$) in promoter activity (Fig. 3B). Similar trends in *Nt3* promoter activity were seen when this data was repeated in E13 cultured testis cells (data not shown). These observations suggest that the SOX sites are important for full *Nt3* promoter activity in mouse and rat.

To test if SRY or SOX9 expression has an effect on *Nt3* promoter activity, co-transfection of *Nt3* promoter reporter plasmids with SRY or SOX9 expression plasmids or an empty expression plasmid as a negative control was performed. When the rat *Nt3* promoter was co-transfected with a rat SRY expression vector, the promoter was stimulated significantly over *Nt3* in the absence of SRY (Fig. 4A). A similar effect was seen when mouse *Nt3* promoter was co-transfected with mSRY (Fig. 4C). A mouse SOX9 expression vector was also co-transfected with mouse and rat *Nt3* promoters. The results show that SOX9 is also able to stimulate the *Nt3* promoter of rat (Fig. 4B) and mouse *in vitro*. These results show that SRY and SOX9 have the ability to stimulate *Nt3* promoter activity.

To determine if SRY and SOX9 are acting specifically at the SOX binding sites co-transfections of protein expression vectors with mutated *Nt3* promoters were also analyzed. Results showed an inability of SOX9 or SRY to stimulate *Nt3* promoter when SOX binding sites are mutated, suggesting SRY and SOX9 are acting specifically at the SOX binding response elements for promoter activation in these assays. Interestingly, mutating only the 206 site in mouse still allows activation by SRY and SOX9, while mutating only the 222 site does not (Fig.

4D and data not shown). Together these data suggest that that SOX sites are required for *Nt3* promoter stimulation by SRY and SOX9 and that the 222 site may be more important for mouse *Nt3* activation by SOX proteins.

Since SRY and SOX9 both act in a site-specific manner in the transfection assays, it was hypothesized that they may bind directly to the SOX sites. To test if binding is direct and site specific, Electrophoretic Mobility Shift Assays (EMSA) were attempted. No conclusive data has been obtained, thus far. Using *In vitro* transcribed and translated protein products only appears to produce non specific shifts, which can not be competed off with unlabeled competitor oligo as demonstrated by the Sox9 protein/SOX site oligo EMSA in Fig. 5 (C,D, and E). This is in contrast to a positive control assays using EBNA protein binding to site-specific oligos in which adding an excess of unlabeled competitor removes the band associated with specific protein oligo interaction (Fig. 5 G). When using purified protein products, no shift is visible (Fig. 5 A and B). A modified EMSA protocol or an alternative approach will be needed to detect the ability of SRY and SOX9 to bind directly to the SOX binding sites identified in the *Nt3* promoter. Specifically comparison of SOX9 and SRY binding to oligos containing mutant or wildtype SOX binding sites corresponding to those in the *Nt3* promoter will need to be performed to determine if SRY or SOX9 has the potential to bind site specifically to the SOX sites in the *Nt3* promoter. The EMSA assays will not necessarily reflect regulation of *Nt3* in vivo however. To asses if SRY or SOX9 associate with the *Nt3* promoter *in vivo* a ChIP assay will be needed. The current study of the Nt3 promoter together with proposed EMSA and ChIP assays will lead to a better understanding of the regulation of Nt3 during sex determination.

## DISCUSSION

Previously *Nt3* was shown to act as a chemo-attractant for cell migration from the mesonephros to the developing testis, a process critical to early morphological events of testis cord formation. The expression of *Nt3* as determined in previous studies suggested a potentially direct regulation of *Nt3* by the key regulators of sex determination, SRY and SOX9 [32;34;42]. In this study the molecular mechanism of male-specific activation of *Nt3* expression during gonadal differentiation was investigated. Specifically, *Nt3* promoter regulation by SRY and SOX9 was investigated to determine if *Nt3* was a direct target of SRY action in the testis. This investigation is particularly important as *Sox9* is the only direct target of SRY action identified in the 18 years since SRY was proposed as the male sex determining gene [12].

Localization of NT3 in the developing testis of rat revealed initial expression at E14 in Sertoli cells only. The initial expression of NT3 in Sertoli cells only at the onset of testis morphogenesis at E14 is consistent with induction of *Nt3* expression by SRY or SOX9 which are initially expressed in Sertoli cells at E13 and E13.5 respectively. Interestingly, by E16 NT3 is also expressed in germ cells and Leydig cells. It has been shown that expression of SRY in pre-Sertoli cells can act in a cell autonomous manner to increase *Sox9* expression and through an inter-cellular signaling mechanism to increase *Sox9* in neighboring cell to promote Sertoli cell differentiation [43;44]. Similar mechanisms may be involved in the initiation of *Nt3* expression first in Sertoli cells, then in other testicular cell types. Specifically, *Nt3* may also initially be stimulated cell autonomously by SRY followed by inter-cellular signaling to up-regulate its expression in other cell types. Further investigation of this hypothesis would lead to a better understanding of *Nt3* regulation and testis differentiation.

Analysis of the *Nt3* promoter revealed consensus SOX family protein binding sites in the promoters for human, mouse, and rat. Interestingly, there are two SOX sites in the proximal 600 base pairs of the *Nt3* promoters in human and mouse and only a single site in the rat. For SOX9 target genes identified previously, paired SOX sites oriented in opposing directions with 3-4 bps between them appear to be important for recognition and activation [38;45;46]. In the testis however, it has been speculated that paired SOX sites may not be required for transcriptional activity. This suggestion arose after the identification of a patient with a *Sox9* mutation disrupting the ability of *Sox9* to dimerize. Several tissues where SOX9 dimerization is required were adversely affected, however the patient was not sex reversed despite the fact that *Sox9* is required for male sex determination [47]. The SOX sites identified in the mouse and human *Nt3* promoters are oriented in the same rather than opposing directions and in the rat there is only one site. This means that the potential mechanism of action by SOX9 or SRY at these sites may differ from that of other SOX9 targets and may be different between mouse and rat. Furthermore, the DNA-dependent dimerization domains found in SOX9 are not present in SRY [47-49]. Therefore, SRY action does not likely depend on dimerization or reverse-oriented paired SOX binding sites. This further supports a hypothesis that SRY may directly regulate *Nt3*.

To determine if the consensus SOX binding sites in the *Nt3* promoter are important for gene expression, mutational analysis of these sites using mouse and rat *Nt3* promoter reporter plasmids was performed. Results show that the SOX site in rat and both SOX sites in mouse are important for full promoter activity in their respective species. Co-expression of these reporter constructs with SRY or SOX9 expression plasmids showed that both SRY and SOX9 were also able to stimulate the promoters in a site-specific manner. This is in contrast to a previous study

of Prostaglandin D Synthase regulation where only SOX9 was found to stimulate SOX sites in the *Pgds* promoter and only as a dimer [38]. In the mouse *Nt3* promoter there are two SOX sites 9 base pairs apart in the same orientation. Both sites are important for full promoter activity but the two sites do not play equal roles. The mouse *Nt3* promoter can still be stimulated by SRY and SOX9 in the absence of a consensus SOX 206 site, but not when the 222 site is mutated. This suggests the 222 site is more critical for activation of the mouse promoter then the 206 site. Investigation of a protein complex with SRY or SOX9 may be useful in the future to determine the difference in the importance of the mouse SOX sites and the difference in number of sites between mouse and rat.

To determine if SRY and SOX9 have the potential to bind the *Nt3* promoter directly, EMSAs were performed. However, preliminary data is inconclusive. *In vitro* transcription/ translation produced SRY and SOX9 proteins in reticulocyte lysates cause non-specific shifts. The nonspecific shifts may mask specific DNA-protein interaction and are undesirable. The non specific shifting may be due to other proteins in the reticulocyte lysates so SRY and SOX9 proteins were further purified through immunoprecipitation using the HA tag. Use of purified SRY and SOX9 proteins in EMSAs did not cause a shift, but in the absence of a functional assay for these proteins it is difficult to determine if the proteins survived purification in a functional form. It will now be necessary to proceed with modified EMSAs or an alternative approach to determine if SRY and SOX9 bind directly to the SOX binding response elements in the *Nt3* promoter. Several methods could be used to compare wild type and mutated SOX sites in *Nt3* promoter oligos/plasmids for detection of site specific binding by SRY or SOX9. These include use of a radio labeled oligo to replace the biotin labeled oligos and address possible sensitivity issues. A hybrid EMSA/ChIP assay using protein DNA binding reaction set up as in the EMSA,

followed by immunoprecipition with an antibody specific for the HA tag on the SRY and SOX9 proteins and PCR for DNA of interest could also be used. This would allow the detection of bound DNA with out the interference of non specific shifting seen with the standard EMSA protocol. Lastly, a plasmid immunoprecipitation could be used. In this procedure co-transfection of E13 testis cells with SRY or SOX9 expression vectors and *Nt3* promoter plasmids followed by immunoprecipitation of protein with associated DNA (*Nt3* plasmid) and then PCR for DNA of interest. Each of these methods would allow the comparison of wild type and mutant SOX sites in the *Nt3* promoter and verify a site specific action. However, data will not necessarily reflect *Nt3* promoter regulation *in vivo*. To determine if SRY and SOX9 can activate the *Nt3* promoter *in vivo*, more studies such as ChIP assays will be required. An assay such as a ChIP to show SRY is bound *in vivo* will be required to identify Nt3 as the first known direct target of SRY action.

Together, the data obtained in the current study show that both SRY and SOX9 can activate mouse and rat *Nt3 in vitro* in a site-specific manner and that both proteins can act at a single SOX binding site suggesting that dimerization is not required. Therefore *Nt3* may be directly regulated by SRY or SOX9. However, more work is needed to determine if the promoter activation is through direct binding and occurs *in vivo*. Therefore Protein-DNA interaction assays are now underway including modified EMSAs and ChIP assays.

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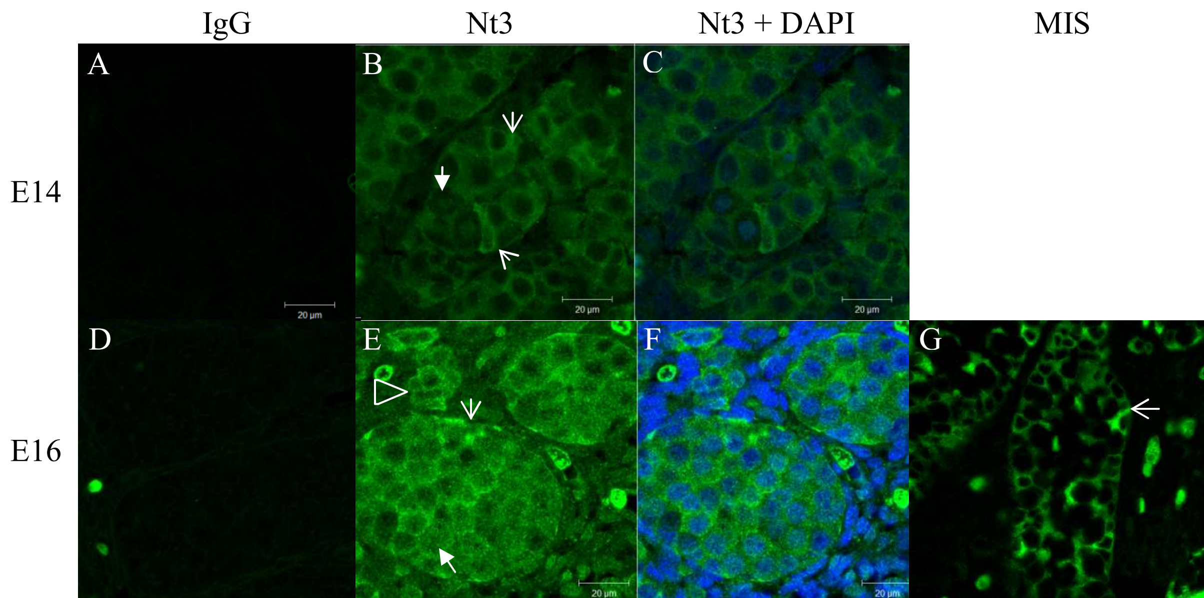


**Fig. 1.** Immunohistochemical localization of Neurotrophin 3 in embryonic day E14 and E16 testis. A. E14, IgG control. B. E14, Anti-Neurotrophin 3. C. E14, Anti-Neurotrophin 3 plus DAPI nuclear stain. D. E16, IgG control. E. E16, Anti-Neurotrophin 3. F. E16, Anti-Neurotrophin 3 plus DAPI nuclear stain. G. Anti-Mularian Inhibiting Substance, Sertoli cell marker not expressed at E14 (data not shown).

→ = Sertoli cells. ➝ = germ cells. ▷ = interstistial cells likely to be Leydig cells.

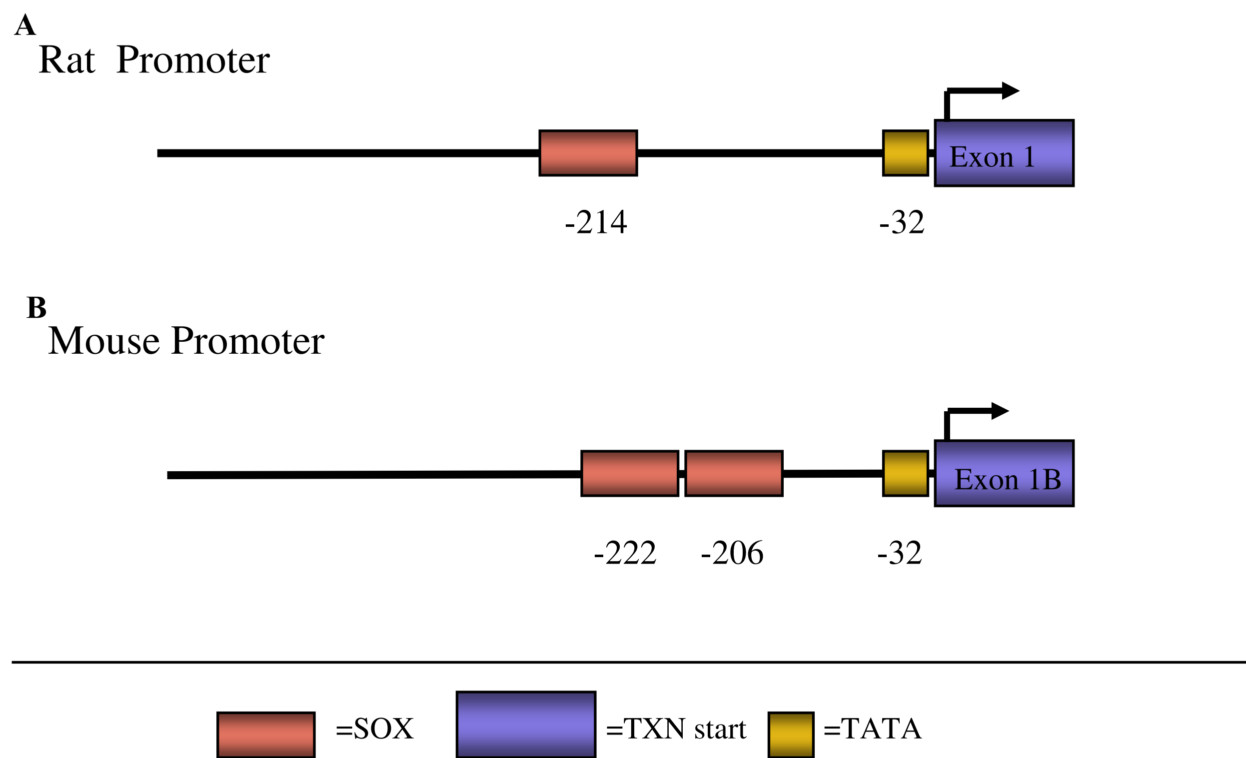


**Fig. 2**. *Nt3* promoter map indicating consensus SOX binding sites. A. Rat *Nt3* promoter with one SOX site 214 base pairs upstream of transcription start site. B. Mouse *Nt3* promoter with two SOX sites at 206 and 222 base pairs upstream of the transcription start site.

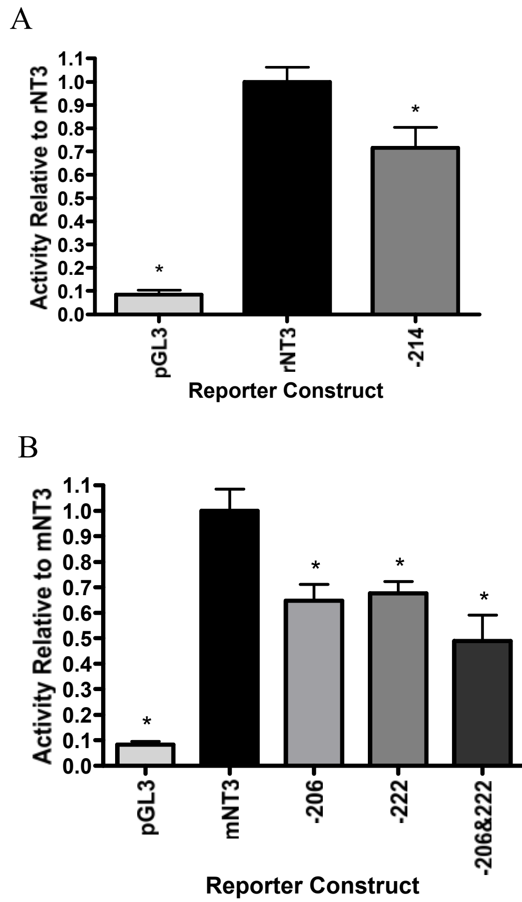


**Fig. 3**. *Nt3* promoter mutational assays. A. Rat *Nt3* promoter activity is compared to activity of a promoter with the 214 SOX site mutated. B. Mouse *Nt3* promoter activity is compared to activity of promoters with the 206, 222 or both 206 and 222 SOX sites mutated. Activity within assays was normalized to wild-type promoter activity and empty pGL3 vector activity shown represents background activity. Data is shown as the mean of a minimum of three replicate experiments each with triplicate samples for each condition. Error bars indicate SEM. * indicates statistical difference from wild type promoter activity with p-value $\geq 0.05$.

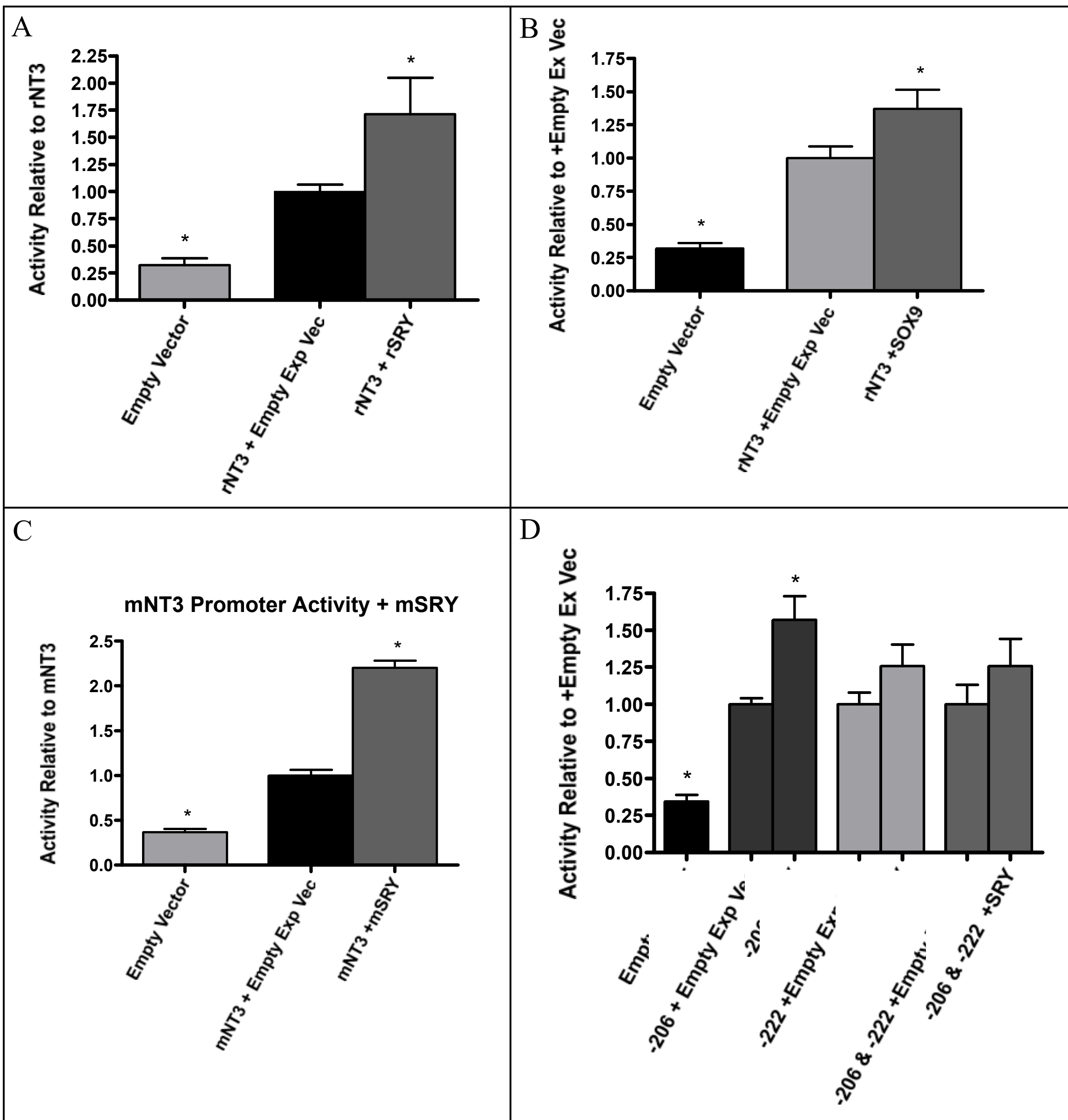


**Fig. 4**. Effects of SRY and SOX9 expression on *Nt3* promoter activity. A. Rat *Nt3* promoter activity with or without *Sry* co-transfection. B. Rat *Nt3* promoter activity stimulation with *Sox9*. C. Mouse *Nt3* promoter activity stimulation with *Sry*. D. Mutant mouse *Nt3* promoter activity stimulation with *Sry*. Activity within assays was normalized to wild-type promoter activity. Empty pGL3 vector activity represents background activity. Data represents mean +/- SEM from a minimum of three experiments. * indicates statistical difference from wild type promoter activity with p-value ≥ 0.05.

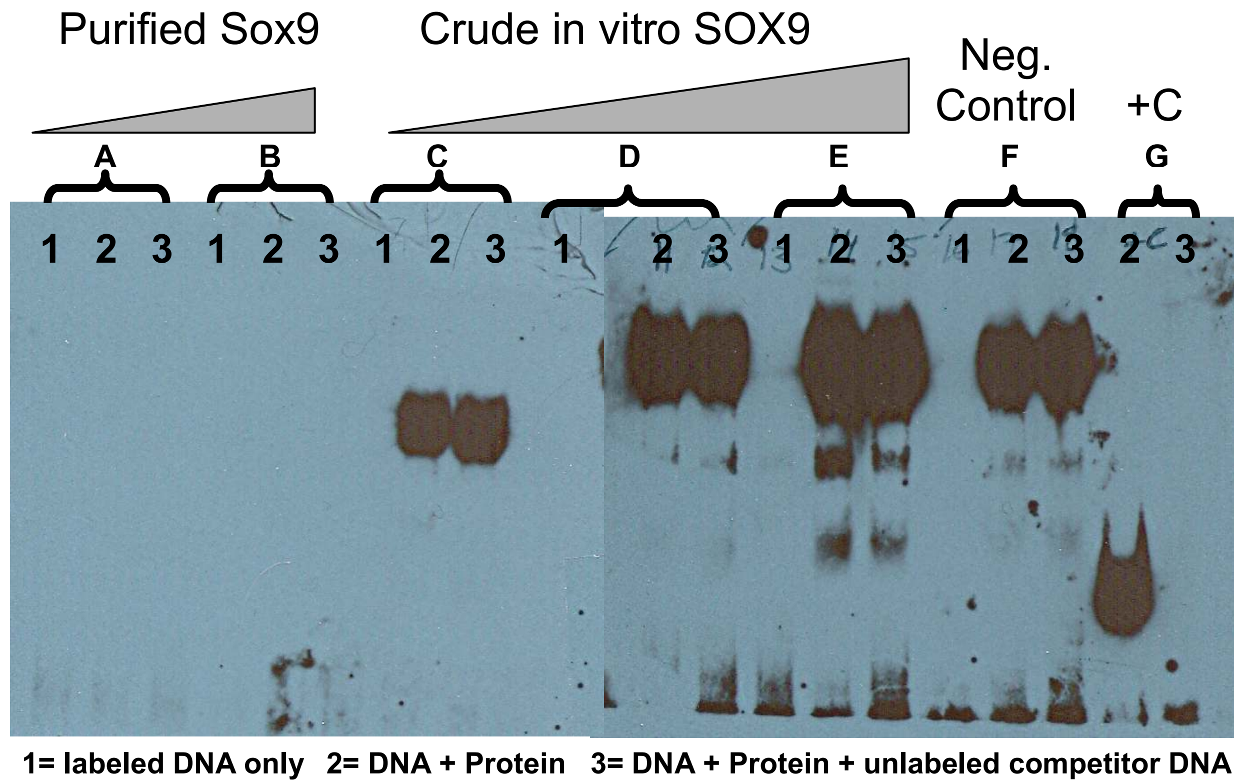


**Fig. 5.** Electrophoretic Mobility Shift Assay Trials. *In vitro* transcribed and translated HA tagged SOX9 protein was used to shift oligos with SOX binding sites. Each set of samples has three binding reactions labled 1, 2, 3. 1 = binding buffer and biotin labeled oligo. 2 = 1 plus SOX9 protein. 3 = 2 plus unlabeled oligo as a specific competitor. Sample sets A and B used low and high concentrations of anti-HA immunoprecipitation purified *in vitro* produced SOX9 protein respectively and oligos corresponding to the rat SOX binding site. Sample sets C, D, and E used increasing concentrations of *in vitro* produced SOX9 protein product with out purification and SOX site containing oligos. Sample set F used the same amount of unpurified SOX9 protein as in sample set D but in a reaction with oligos containing a mutated SOX binding site. Sample set G is a binding reaction positive control using EBNA protein and an oligo containing the appropriate binding site.

# CHAPTER 5

# SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

The body of work described in this dissertation has been aimed at understanding gonadal development and has been divided into two main questions. First, what is the normal transcriptional program of gonadal differentiation and testis development? To investigate this, previously unknown downstream genes critical in the mammalian SRY directed program of male sex determination and differentiation were identified using a genomic microarray approach and *Nt3* transcriptional activation was investigated as a direct target of SRY action. The ability of SRY and SOX9 to activate the *Nt3* promoter in a site specific manner provides preliminary evidence that it may be one of the missing links directly downstream of *Sry* for induction of male sex determination.

The second question addressed by this thesis was what is the mechanism of action for vinclozolin disruption of sex determination and testis differentiation which may lead to observed adult onset disease? A genomic microarray approach was again taken, this time to identify vinclozolin altered gene expression that may point to a mechanism of action. The genes and processes affected suggest alteration of apoptosis and vascular formation may be affected by vinclozolin treatment and transcriptional and epigenetic mechanisms may be important for mediation of affects seen with vinclozolin treatment.

The significance of the sum of work in this thesis centers on its contribution to the study of testis differentiation to better understand the mechanisms of functional and disrupted testis development. Identification of many new candidate genes for involvement in testis development and endocrine disruption of testis development have been identified, the known functions of which suggest roles that may help to fill in gaps in the understanding of testis development and its disruption leading to adult-onset trans-generational disease.

**Investigations into the Regulation of Sexual Differentiation**

For mammalian sex determination SRY acts as the master regulator to induce male sexual differentiation. However since the discovery of SRY as the Y chromosome linked inducer of maleness over 18 years ago, progress in understanding the mechanisms by which SRY induces the complex morphogenesis of the testis has been slow. A direct target for SRY in this process has yet to be identified. Furthermore the formation of a functional testis during embryogenesis is complex and relatively little is understood about the regulatory mechanisms required for orchestration of the cellular and morphological events that must occur.

To identify new candidate genes a microarray approach was used to investigate gene regulation during sex determination in rat and is detailed in chapter two. This study characterizes transcriptional regulation of sex determination in the rat on a genomic scale and compares regulated genes in both the mouse and rat. It has provided a resource for identifying candidate genes required for mammalian sex determination. Results suggest that both ovary and testis differentiation are highly transcriptionaly active processes with about half of the genome being expressed, however female transcript regulation is delayed compared to the male. Furthermore, several male specific regulated transcripts were identified but female regulated transcripts identified in this analysis all appeared to also be expressed in the male during sex determination. This is consistent with the dogma that the female pathway is the default in the absence of unique events initiated in the developing testis. Observations from chapter two suggest that a smaller group of genes regulated during sex determination may have a role in influencing a large number of different cellular processes, rather than larger groups of genes specific to selected pathways. Cellular processes may be affected by the independent actions of several genes or by gene-gene interactions. Cellular processes affected by genes regulated in

testis differentiation include cellular differentiation, proliferation, focal contact, RNA localization, and development suggesting that these are major processes critical for testis differentiation.

This genomic analysis of the gonadal transcriptome during sex determination has provided a global assessment of genes and cellular processes potentially involved in sex determination and gonad development. Microarray approaches to identify candidate genes in complex biological processes like gonadal differentiation may be the best way to obtain multiple novel candidates in species with annotated genomes. Results from this study include profiles of expression for over 8000 genes present between E13 and E16 in the rat testis and ovary including those regulated through time and in a sex specific manner. Interesting future studies could investigate the regulated cellular processes and follow up with functional analysis for specific candidate genes that were identified. This will help elucidate potential significance of identified candidates for gonadal sex determination and differentiation leading to a better understanding of these processes as a whole.

A few signaling pathways of interest including Wnt signaling were identified in the genomic microarray study. WNTs are secreted proteins implicated in cell growth, migration, and differentiation. Previously, a role for Wnt signaling was established in developing ovary but a role in testis had not been identified. In chapter two, two genes involved in WNT signaling were found to be regulated during testis development. Secreted frizzled related protein 4 (SFRP4) is a secreted lipoprotein receptor complex similar to the frizzled receptors in the WNT signaling cascade, but without the transmembrane activation domain [1]. SFRP4 is thought to antagonize cell survival and inhibit WNT signaling by binding WNT without activating its signaling cascade. This suggests that SFRP4 may be important in the inhibition of WNT signaling in the

male, whereas default WNT signaling in the female would promote ovarian differentiation. *Wnt5a* also appears on the testis regulated candidate gene list in chapter two. WNT5A has been shown to activate signaling cascades in a manner dependent on the receptor to which it binds [2]. A potential role for WNT5A and SFRP4 in gonadal development is yet to be determined and these studies suggest a critical inhibition of canonical WNT signaling in the developing testis. Interestingly in the time since this work was published three separate papers have been published, independently suggesting WNT signaling must be inhibited for male sex determination and development [3-5]. Together these data strongly suggest a potential role for inhibitors of WNT signaling such as SFRP4 in sex determination, and merit further investigation of this signaling pathway. One experiment to determine if *Sfrp4* can antagonize WNT signaling and promote testis differentiation would be to test the effects of overexpression or knock down of SFRP4 in cultured E13 gonadal cells by measuring the activation of a reporter with beta catenin response elements. If Sfrp4 can affect WNT signaling you would expect the accumulation of beta catenin to change and thus the expression level of the reporter gene in the reporter vector would also change. Another experiment which could test if Sfrp4 can antagonize WNT signaling and promote testis development would be to knock down Sfrp4 in an E13 testis in culture and determine if testis cord development is reduced and if markers of ovary development are induced. We know from recent studies that down regulation of WNT signaling must occur for testis development. If knockdown of Sfrp4 in testis cultures can inhibit testis development or induce markers of female development, than it could be identified as the negative regulator of WNT signaling likely to play this critical role in testis development

Studies on new candidate genes will be needed such as suggested above for *Sfrp4* and WNT signaling to link gene expression to function and cellular and morphological processes

critical to testis differentiation. However, to connect the genes and processes in the sex determination cascade to one another, transcriptional regulation of these targets will also need to be studied. In Chapter four potential regulation of Neurotrophin 3 (*Nt3*) by SRY and SOX9 was investigated. Since NT3 is known to play a critical role in testis differentiation and is expressed early in sex determination in Sertoli cells only, it is a potential candidate for direct regulation by SRY or SOX9. A search of the *Nt3* promoter as outlined in chapter four identified consensus SOX binding sites. Promoter mutation and activation analysis suggests that these sites are important for *Nt3* promoter activity, and that SRY and SOX9 can activate *Nt3* in a site specific manner. *Pdgs*, a previous candidate for SRY and SOX9 regulation, was only activated by SOX9 and that activation was dependent on dimerization at paired SOX sites. Together these data suggest that *Nt3* can be regulated by both SRY and SOX9 *in vitro*. However these data are not necessarily reflective of *Nt3* regulation *in vivo* and do not establish a direct interaction of SRY or SOX9 with the SOX sites of the *Nt3* promoter.

To show the potential for a direct interaction of SRY or SOX9 with the *Nt3* promoter and verify that this occurs only at consensus SOX binding sites, EMSA assays using i*n vitro* transcription/translation produced SRY and SOX9 proteins in reticulocyte lysates. However, results are inconclusive due to non-specific shifts that may or may not conceal specific DNA-protein interaction. SRY and SOX9 proteins further purified through immunoprecipitation using the HA tag did not cause a shift, but in the absence of a functional assay for these proteins it is difficult to determine if the proteins were functionally active. It will now be necessary to proceed with modified EMSAs or an alternative approach. A radio labeled oligo can replace the biotin labeled ones to address possible sensitivity issues. These experiments are underway but no results have been obtained thus far. A hybrid EMSA/ChIP assay is also being developed. For

this approach a protein DNA binding reaction is set up as in the EMSA, then immunoprecipitated with an antibody specific for the HA tag on the SRY and SOX9 proteins to pull down associated DNA on which a PCR is run to detect DNA of interest. Preliminary results with this method suggests that contamination of *Nt3* DNA from the binding reaction in the final immunoprecipitated DNA pool may be problematic as results are inconsistent and *Nt3* is often detected in non-specific IgG negative control reactions. Another assay which may be done in the future is a plasmid immunoprecipitation. In this method co-transfection of E13 testis cells with SRY or SOX9 expression vectors and *Nt3* promoter plasmids as described in chapter four is followed by immunoprecipitation of protein with associated DNA (*Nt3* plasmid) and then PCR for DNA of interest.

Comparison of wild type and mutated SOX sites in the *Nt3* promoter oligos/plasmids using any of the three methods described above would allow for detection of site specific binding by SRY. The first two methods are cell free and therefore have the advantage of being able to say binding interactions are direct. The plasmid immunoprecipitation method will show if SRY can act site specifically, however, since binding reactions occurred in a cellular environment the possibility exists that other factors mediate the interaction. Plasmid immunoprecipitation does have the advantage of being able to detect SRY or SOX9 association with the *Nt3* promoter rather then short oligos. This would show that SRY and SOX9 mediate up-regulation of *Nt3* in transfection assays directly by association with the promoter and not through indirect regulation of other cellular factors. Furthermore, if a shift is seen with plasmid immunoprecipitation but not in the cell free systems, it may suggest that there is another cellular factor that needs to be in a protein binding complex with SRY or SOX9 for them to associate with the SOX sites.

Results from the above studies may not necessarily reflect *Nt3* regulation *in vivo*. To determine if SRY or SOX9 associate with the *Nt3* promoter *in vivo*, a ChIP assay will need to be performed on embryonic gonads. This work is also currently underway but with out conclusive results. Preliminary data is inconsistent showing a need for procedural optimization. *Nt3* is detected after pulldown of SRY from male and intermittently from female gonads as well as from non-specific IgG pulldowns. Before work continues it will be necessary to determine the specificity of the antibody to SRY and optimize the assays to remove nonspecific background detection of *Nt3*.

Since connecting the genes and processes in the sex determination cascade to one another is important in the understanding of sex determination and gonadal developmet, new candidates of for transcriptional regulation identified in the chapter 2 developmental microarray study can be studied in a manor similar to potential SRY or SOX9 regulation of *Nt3*. A gene with unknown function, *A5d3*, is among genes of interest for future studies in sex determination. *A5d3* contains potential leucine zipper and phosphorylation sites [6]. The expression profile in embryonic testis identified in chapter two shows that it is initially expressed in E13 ovary and testis then strongly up-regulated only in testis. Although this gene currently has unknown functions, this expression pattern in developing testis, as well as its identification in vitamin A deficient synchronized testis where it was called VAD4 [7], make it an interesting candidate gene for involvement in testis differentiation. Furthermore, the leucine zipper motifs seem to suggest that *A5d3* may act as a transcriptional regulator. If this is the case, repeated cycles of A5D3 protein oligo interaction followed by oligo amplification starting with random oligos could be used to identify the potential concensus binding sites for A5D3 in target genes. Localization of *A5d3* to specific cell types in the developing testis may also lend further insight

into its role in testis development. With this combined information, the promoters of genes known to be important in sex determination that are expressed in the same cell types as A5D3 could be searched for identified combined binding sites. A potential regulatory control of these candidates by A5D3 could then be investigated in a manor similar to SRY regulation of *Nt3* as described above.

The studies in chapters two and four have added to the collective knowledge about the regulation of sex determination and testis differentiation. A host of new candidaes for involvement in sex determination and gonadal development such as *Sfrp4* and *A5d3* have been identified. While much remains to be investigated, these reports provide important preliminary evidence showing a role for Wnt and NT3 signaling downstream of SRY in normal male gonadal sex differentiation and that *Nt3* up-regulation may be through a direct interaction with SRY or SOX9.

**Mechanisms of Vinclozolin Disruption of Gonadal Development**

Embryonic vinclozolin exposure during the period of gonadal development and testis differentiation leads to adult onset trans-generational disease. Specifically, vinclozolin treatment embryonically leads to increased testis cell apoptosis in pubertal and adult rats, and reduced sperm count and motility. This adult phenotype occurs without any transparent disruption of the embryonic testis. To investigate the mechanism of vinclozolin action on the embryonic testis some of which ultimately must lead to the observed adult disease phenotypes, a microarray comparison of control and treated testis at E13, E14, and E16 was carried out as presented in chapter four. Only three genes previously found to be regulated during typical testis differentiation were altered by vinclozolin treatment. Thus the processes of normal testis development are not likely involved in the mechanism of vinclozolin action leading to adult

onset disease. This was not unexpected since the embryonic phenotype is not altered by these vinclozolin treatments. The know functions of genes that were altered by vinclozolin treatment suggest that vinclozolin may affect transcriptional regulation and possibly translation. Several other processes implicated by the functional roles of vincolozolin altered genes include epigenetics, apoptosis, vascular development, regulation of RNAs, and insulin signaling. Interesting future studies could investigate the functional roles of genes that could mediate vinclozolin action through these processes and link vinclozolin altered process together.

One candidate gene that may be particularly interesting to investigate further is calcium dependant *Camkd2*. This gene phosphorylates histones to affect transcription epigenetically and is down regulated early in the gonad at E13. Since histone phosphorylation can cause increased transcriptional activity, a decrease in expression of *Camkd2* is one explanation for the increase of transcript abundance seen after vinclozolin treatment. *Camkd2* is also known to play a role in vascular smooth muscle proliferation and migration. Vascularization is known to play a critical role in testis morphogenesis and in creating the germ cell stem cell niche. Future studies to investigate the role of *Camkd2* in embryonic germ cell stem cell fate decisions during testis morphogenesis would be interesting and may link increased testicular cell apoptosis in adult life to vinclozolin action.

Another gene worthy of further investigation is *Nedd4*. *Nedd4* is known to increase insulin signaling by IGF1 which is required for male sex determination. *Nedd4* is also a downstream mediator of insulin signaling working by forming a positive regulatory feedback loop. IGFR signaling in mitochondria causes an internalization of NEDD4 which leads to apoptotic protection. A decrease of *Nedd4* as seen after vinclozolin treatment at E14 may cause a decrease in insulin signaling disruptive to male sex determination, or a loss of apoptotic

protection which after puberty results in increased testis cell death. Investigation of the role of Nedd4 in apoptotic protection and insulin signaling in developing testis and its alteration by vinclozolin would be interesting future studies.

PMRT1 is a methyl transferase which has also been linked to insulin signaling. PMRT1 methylates ribonucleoprotein complexes one effect of which is insulin receptor internalization. Therefore a role for PMRT1 in insulin signaling for sex determination could also be investigated. A more likely role for PMRT1 is to mediate vinclozolin action on developing testis through epigenetic regulation of the embryonic germ cells. PMRT1 is known to be expressed in embryonic germ cells and may play a role in the epigenetic reprogramming of the germ line that occurs in the embryonic gonad during the time of vinclozolin treatment. The potential for the increased PMRT1 expression seen after vinclozolin treatment to alter the epigenetic status in the germ line may explain both the adult onset and trans-generational aspects of sperm count and motility reductions following of vinclozolin treatment.

*Gtl2* is a maternally imprinted RNA stabilizer that is methylated in males. It has been shown that these methylation patterns persist in the germ line through spermatogonia and spermatocyte stages. Furthermore, regulation of RNAs is known to be important in spermatogenesis. An altered methylation state of *Gtl2* in the male germ line, possibly by a methyltransferase such as PMRT1 could lead to altered RNA transcript stability in the adult testis leading to altered gametogenesis and the phenotypes associated with vinclozolin treatment. The role of *Gtl2* in these phenomena and the potential alteration of *Gtl2* imprinted status by methyl transferases such as PMRT1 could now be investigated. Specifically, differences in methylation states of CpG sites in and around the Gtl2 gene and imprinted region could be monitored in control and treated, embryonic and adult testis using bisulfite sequencing to see if

there are differences in methylation patterns in control and treated animals and if the patterns are similar in embryos and adults. The levels of expression of Gtl2 could also be checked to verify if expression levels of *Gtl2* are changed with vinclozolin treatment and if this correlates to methylation status of *Gtl2*. The methylation patterns specifically in sperm and in subsequent generations could also be checked to see if epigenetic changes in *Gtl2* can be passed between generations. Combined these studies would indicate if vinclozolin can modify the methylation status of *Glt2* if this modification is maintained in the adult and passed to subsequent generations and if the expression of *Gtl2* correlates to the methylation status of the gene region. Since *Gtl2* is known to play a role in RNA stabilization and small RNAs are known to be critical in regulating testis function by regulating the progression of spermatogonia through meiosis and spermatogenesis, it would also be interesting to investigate if Gtl2 expression affects small RNA levels. Specifically, if *Gtl2* levels are found to be different in control and treated adult animals, then the total expression of piRNAs in testis could be measured to determine if piRNA accumulation is affected. Levels and localization of PIWI proteins such as MIWI and MILLI which are required for piRNA accumulation could also be determined.

The investigation of other candidates identified in chapter 3 could be done in a similar way as that described for *Gtl2* above. In general the expression and methylation status of DNA near genes of interest could be determined in control and treated embryos and adults to if a candidate gene is epigenetically altered with correlated changes in gene expression and if this epigenetic change is carried through to the adult. If this turns out to be the case for a particular gene then the methylation near the gene of interest in sperm and subsequent generations could be investigated to determine if altered methylation states are passes to subsequent generations. This sort of general investigation can be combined with functional studies tailored to each candidate

(such as potential regulation of transcription by A5D3, down regulation of WNT signaling by SFRP4, or altered small RNA abundance by GTL2) to help determine how vinclozolin acts on the embryonic testis ultimately leading to reduced sperm count and motility persisting for three subsequent generations. Elucidation of the mechanisms of action of vinclozolin in the embryo may also lend insight to more general mechanisms of adult onset disease induction and transmission of disease states to subsequent generations. Therefore further investigation of vinclozolin altered genes and processes identified in this thesis may impact our understanding of several processes which are currently not well understood.

This thesis has contributed to the understanding of functional and disrupted sex determination and testis development. A large scale analysis of transcriptional regulation during testis differentiation using a genomic microarray approach has identified a number of gene candidates. Many of these candidates have known functions that implicate particular pathways or cellular processes in sex determination and testis development. A similar approach investigating disruption of testis development by vinclozolin on a large scale has also identified many gene candidates. Genes identified in this analysis have known functions that suggest mechanisms such as transcriptional regulation and epigenetics may play a role in vinclozolin action on differentiating testis. These studies have significantly contributed to information on gene regulation and disruption during sex determination and provide multiple new candidate genes. Further characterization of these candidate genes provides a number of potential future studies contributing to understanding of testis differentiation and its disruption that I hope will be pursued.

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# APPENDIX

## Supplemental Table S1

| Genes Removed by Culture Comparison | | | | | | |
|---|---|---|---|---|---|---|
| Common | E13 | E14 | E16 | Affy-ID | Genbank | Description |
| Col1a2 | 735 | 1040 | 1525 | 1370155_at | BM388837 | procollagen, type I, alpha 2 |
| Pgam1 | 2071 | 1600 | 1318 | 1386864_at | NM_053290 | phosphoglycerate mutase 1 |
| Cbx1 | 483 | 738 | 624 | 1374695_at | BF564593 | Chromobox homolog 1 (predicted) |
| Gstp2 | 973 | 763 | 557 | 1388122_at | X02904 | glutathione S-transferase, pi 2 |
| Cdh22 | 443 | 261 | 375 | 1388357_at | BI282972 | similarity to H.sapiens protein P518_HUMAN |
| Tubb2b | 668 | 513 | 356 | 1388131_at | X03369 | Tubulin, beta 2b |
| Emilin1 | 190 | 255 | 297 | 1372325_at | BI303596 | elastin microfibril interfacer 1 (predicted) |
| | 426 | 413 | 283 | 1371816_at | AI407560 | Rattus norvegicus transcribed sequences |
| | 195 | 286 | 263 | 1388527_at | BI283685 | Rattus norvegicus transcribed sequences |
| Tm9sf1 | 168 | 203 | 254 | 1390125_at | BE098998 | transmembrane 9 superfamily member 1 |
| Ing4 | 321 | 230 | 201 | 1373817_at | BM391761 | inhibitor of growth family, member 4 |
| Casp7 | 121 | 164 | 195 | 1389170_at | BF283754 | caspase 7 |
| Asna1 | 112 | 136 | 180 | 1372142_at | AI406558 | Arsenical pump-driving ATPase |
| Tuba4 | 83 | 119 | 175 | 1371542_at | BI284599 | Tubulin alpha-4 chain(LOC316531) |
| Pbx2 | 292 | 235 | 165 | 1389432_at | BE101096 | pre-B-cell leukemia transcription factor 2 |
| | 110 | 165 | 163 | 1398998_at | AI407273 | similarity to E. coli protein Beta-galactosidase |
| Hsd17b8 | 68 | 115 | 156 | 1382492_a_at | AA866404 | hydroxysteroid (17-beta) dehydrogenase 8 |
| Sav1 | 104 | 159 | 145 | 1373284_at | AI176607 | salvador homolog 1 (Drosophila) (predicted) |
| Bnip1 | 190 | 126 | 139 | 1367863_at | NM_080897 | BCL2/adenovirus E1B 19kD interacting protein 1 |
| Tmed4 | 168 | 103 | 133 | 1376161_at | AI235294 | transmembrane emp24 domain containing 4 (predicted) |
| Rtp801 | 214 | 133 | 132 | 1368025_at | NM_080906 | HIF-1 responsive RTP801 |
| | 205 | 156 | 127 | 1371817_at | BI285489 | similarity to protein pir:T46317 (H.sapiens) |
| Eraf | 60 | 147 | 126 | 1389160_at | AI230287 | erythroid associated factor (predicted) |
| | 166 | 90 | 116 | 1373559_at | AI228623 | Rattus norvegicus LOC360765 (LOC360765), mRNA |
| | 152 | 93 | 109 | 1376568_at | AI144997 | Rattus norvegicus cDNA clone UI-R-BT0-pr-a-07-0-UI 3' |
| | 65 | 91 | 100 | 1376681_at | AI177038 | similarity to E. coli protein Beta-galactosidase |
| | 59 | 85 | 97 | 1376804_at | BM386777 | Rattus norvegicus transcribed sequences |
| Ranbp1 | 151 | 103 | 97 | 1375427_at | AI411580 | RAN binding protein 1 (predicted) |
| | 60 | 69 | 95 | 1389059_at | BI278651 | Rattus norvegicus transcribed sequences |
| Sardh | 61 | 63 | 93 | 1372323_at | AI103641 | Sarcosine dehydrogenase |
| Igsf4a | 150 | 87 | 92 | 1384132_at | H31111 | immunoglobulin superfamily, member 4A |
| | 58 | 66 | 91 | 1373962_at | AI178556 | Rattus norvegicus transcribed sequences |
| Reep6 | 105 | 63 | 91 | 1372841_at | BG376982 | receptor accessory protein 6 |
| Trp53i13 | 46 | 87 | 85 | 1388812_at | AI230362 | tumor protein p53 inducible protein 13 (predicted) |
| Slc9a3r2 | 52 | 67 | 83 | 1388831_at | BI277485 | Solute carrier family 9, isoform 3 regulator 2 |
| | 144 | 109 | 81 | 1375845_at | BI290029 | similar to androgen-induced 1 (LOC292487) |
| Ier3 | 150 | 84 | 79 | 1388587_at | AI176519 | immediate early response 3 |
| Agtr2 | 51 | 100 | 78 | 1369711_at | NM_012494 | Rattus norvegicus angiotensin II receptor, type 2 |
| Slc30a2 | 97 | 60 | 77 | 1398264_at | NM_012890 | solute carrier family 30, member 2 |
| | 49 | 53 | 76 | 1385697_at | BI302799 | Rattus norvegicus transcribed sequences |
| | 31 | 51 | 75 | 1388546_at | AI013328 | Rattus norvegicus transcribed sequences |
| | 121 | 100 | 74 | 1378421_at | BG671734 | similarity to E. coli protein Beta-galactosidase |
| Chac1 | 92 | 61 | 74 | 1389573_at | AI170665 | cation transport regulator-like 1 (E. coli) (predicted) |
| Tpm5 | 53 | 86 | 74 | 1371184_x_at | AF053359 | nonmuscle tropomyosin 5 isoforms NM 5 and NM 6 |
| Acat2 | 36 | 78 | 74 | 1376226_at | BG377636 | Acetyl-Coenzyme A acetyltransferase 2 |
| Atp2c1 | 110 | 74 | 73 | 1387126_at | NM_131907 | ATPase, Ca++-sequestering |
| | 101 | 64 | 66 | 1381467_at | AI598594 | Rattus norvegicus transcribed sequences |
| | 45 | 93 | 64 | 1373522_at | BM385074 | Rattus norvegicus transcribed sequences |
| Clcn3 | 101 | 73 | 61 | 1380547_at | BI288519 | chloride channel 3 |
| Pdk1 | 84 | 53 | 40 | 1368079_at | NM_053826 | pyruvate dehydrogenase kinase 1 |
| Wdr19 | 77 | 42 | 39 | 1390219_at | AI412437 | WD repeat domain 19 (predicted) |

## Supplemental Table S2

| Gender Enriched Genes Above a Signal Cut-off Value of 100 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Male Enriched** | | | | | | | | | |
| Name | E13 ovary | E13 testis | E14 ovary | E14 testis | E16 ovary | E16 testis | Affy-ID | Genbank | Description |
| *Htr7* | 58 | 63 | 53 | 60 | 59 | 123 | 1369119_a_at | X69663 | 5-hydroxytryptamine (serotonin) receptor 7 |
| *Ada* | 42 | 63 | 48 | 67 | 46 | 149 | 1370071_at | NM_130399 | adenosine deaminase |
| *Afar* | 33 | 55 | 59 | 76 | 50 | 112 | 1368121_at | NM_013215 | aflatoxin B1 aldehyde reductase |
| *Alb* | 35 | 46 | 59 | 153 | 48 | 65 | 1367555_at | NM_134326 | albumin |
| *Aldh1a1* | 53 | 83 | 69 | 388 | 67 | 687 | 1387022_at | NM_022407 | aldehyde dehydrogenase family 1, member A1 |
| *Afp* | 36 | 33 | 57 | 106 | 35 | 49 | 1367758_at | NM_012493 | alpha-fetoprotein |
| *Cpa1* | 68 | 73 | 40 | 31 | 22 | 2357 | 1369657_at | NM_016998 | carboxypeptidase A1 |
| *Cxcl12* | 58 | 110 | 52 | 77 | 6 | 88 | 1387655_at | AF189724 | chemokine (C-X-C motif) ligand 12 |
| *Rdc1* | 55 | 86 | 63 | 92 | 41 | 103 | 1367940_at | NM_053352 | chemokine orphan receptor 1 |
| *Cfl1* | 75 | 85 | 56 | 101 | 63 | 113 | 1371339_at | AI599017 | cofilin 1 |
| *Cst8* | 35 | 66 | 30 | 185 | 38 | 182 | 1368767_at | NM_019258 | cystatin 8 |
| *Cyp11a* | 40 | 40 | 35 | 42 | 38 | 833 | 1368468_at | NM_017286 | cytochrome P450, subfamily 11A |
| *Cyp17* | 12 | 9 | 17 | 4 | 17 | 1899 | 1387123_at | NM_012753 | cytochrome P450, subfamily 17 |
| *Dkk3* | 63 | 76 | 72 | 74 | 67 | 102 | 1370328_at | AF245040 | dickkopf homolog 3 (Xenopus laevis) |
| *Fhl2* | 57 | 66 | 39 | 66 | 41 | 105 | 1371951_at | AA800031 | four and a half LIM domains 2 |
| *Glrx1* | 66 | 99 | 54 | 102 | 55 | 67 | 1367705_at | AF319950 | glutaredoxin 1 (thioltransferase) |
| *Gatm* | 39 | 59 | 35 | 84 | 35 | 134 | 1367627_at | NM_031031 | glycine amidinotransferase |
| *Hsd17b3* | 22 | 36 | 28 | 71 | 25 | 152 | 1369553_at | NM_054007 | hydroxysteroid 17-beta dehydrogenase 3 |
| *Insl3* | 30 | 28 | 34 | 26 | 28 | 1099 | 1388241_at | AF139918 | insulin-like 3 |
| *Ireb2* | 66 | 93 | 70 | 116 | 43 | 110 | 1387440_at | NM_022863 | iron-regulatory protein 2 |
| *Jag1* | 37 | 21 | 31 | 25 | 21 | 118 | 1368725_at | NM_019147 | jagged 1 |
| *Kidins220* | 71 | 115 | 72 | 107 | 68 | 94 | 1398311_a_at | AF313464 | kinase D-interacting substance of 220 kDa |
| *Lcn2* | 33 | 34 | 34 | 93 | 31 | 152 | 1387011_at | NM_130741 | lipocalin 2 |
| | 60 | 62 | 71 | 77 | 67 | 106 | 1387757_at | NM_139189 | liver regeneration p-53 related protein |
| *Lhcgr* | 30 | 46 | 25 | 46 | 29 | 143 | 1387423_at | NM_012978 | luteinizing hormone/choriogonadotropin |
| *Lox* | 31 | 46 | 39 | 67 | 54 | 125 | 1368171_at | NM_017061 | lysyl oxidase |
| *Msg1* | 32 | 107 | 42 | 334 | 66 | 464 | 1370805_at | AF104399 | melanocyte-specific gene 1 protein |
| *Myh6* | 23 | 39 | 37 | 21 | 22 | 135 | 1368093_at | NM_017239 | myosin heavy chain, polypeptide 6 |
| *Nppc* | 27 | 45 | 26 | 34 | 15 | 184 | 1387744_at | NM_053750 | natriuretic peptide precursor C |
| *Sry* | 24 | 174 | 19 | 42 | 20 | 23 | 1388279_at | AF275682 | Rattus norvegicus sex-determining region Y |
| *Ril* | 62 | 53 | 72 | 80 | 56 | 104 | 1387153_at | NM_017062 | reversion induced LIM gene |
| *Rgc32* | 73 | 151 | 60 | 186 | 57 | 108 | 1368080_at | NM_054008 | Rgc32 protein |
| *Scarb1* | 44 | 18 | 49 | 41 | 67 | 226 | 1367855_at | AF071495 | scavenger receptor class B, member 1 |
| *Scarb1* | 45 | 40 | 37 | 28 | 50 | 253 | 1386956_at | NM_031541 | scavenger receptor class B, member 1 |
| *Sfrp4* | 59 | 45 | 68 | 45 | 69 | 241 | 1368394_at | AF140346 | secreted frizzled-related protein 4 |
| *Svs5* | 10 | 17 | 18 | 28 | 11 | 211 | 1367615_at | NM_133516 | seminal vesicle secretion 5 |
| *Sh3kbp1* | 44 | 62 | 42 | 95 | 48 | 118 | 1370419_a_at | AF230520 | SH3-domain kinase binding protein 1 |
| *Star* | 47 | 31 | 34 | 10 | 9 | 231 | 1387174_a_at | AB006007 | steroidogenic acute regulatory protein |
| *Star* | 66 | 38 | 30 | 24 | 28 | 274 | 1368406_at | NM_031558 | steroidogenic acute regulatory protein |
| *Sod3* | 43 | 63 | 58 | 71 | 56 | 140 | 1368322_at | NM_012880 | superoxide dismutase 3 |
| *Srpx* | 55 | 59 | 65 | 100 | 71 | 121 | 1368671_at | NM_022524 | sushi-repeat-containing protein |
| *Tsx* | 20 | 59 | 22 | 278 | 24 | 656 | 1368736_at | NM_019203 | testis specific X-linked gene |
| *Hsd3b1* | 5 | 35 | 5 | 57 | 4 | 1817 | 1368578_at | NM_017265 | hydroxysteroid dehydrogenase-6, delta<5>-3- |
| *Tgfb3* | 41 | 37 | 59 | 59 | 71 | 120 | 1367859_at | NM_013174 | transforming growth factor, beta 3 |
| *Tagln* | 55 | 49 | 12 | 24 | 26 | 148 | 1367570_at | NM_031549 | transgelin |
| *Tmeff1* | 70 | 115 | 58 | 91 | 32 | 69 | 1387850_at | NM_023020 | transmembrane protein with EGF-like and two |
| *Tspan-2* | 51 | 103 | 53 | 67 | 36 | 91 | 1368105_at | AI228231 | Tspan-2 protein |
| *Amh* | 32 | 72 | 21 | 618 | 19 | 953 | 1393888_at | AI059285 | UI-R-C1-lb-f-01-0-UI.s1 UI-R-C1 Rattus |
| | 56 | 81 | 63 | 81 | 74 | 183 | 1393397_at | AI576488 | UI-R-G0-us-g-05-0-UI.s1 UI-R-G0 Rattus |
| *Usag1* | 59 | 46 | 60 | 70 | 54 | 108 | 1379281_at | AA892798 | uterine sensitization-associated gene 1 protein |
| *Jun* | 75 | 118 | 72 | 104 | 57 | 89 | 1374404_at | BI288619 | v-jun sarcoma virus 17 oncogene homolog |
| *Wnt5a* | 38 | 50 | 58 | 82 | 26 | 134 | 1369263_at | NM_022631 | wingless-type MMTV integration site 5A |
| *Calm1* | 18 | 39 | 25 | 72 | 26 | 116 | 1375905_at | AI412746 | similarity to tweety homolog 1 (Drosophila) |
| | 9 | 16 | 3 | 18 | 5 | 116 | 1389404_at | AI008883 | similar to forkhead-like 18; forkhead-related |
| | 27 | 38 | 26 | 71 | 32 | 120 | 1389236_at | AI104915 | similar to Myosin-binding protein H (MyBP-H) |
| | 62 | 73 | 54 | 95 | 58 | 115 | 1371441_at | BG662875 | similar to phosphoprotein enriched in |
| | 61 | 65 | 69 | 98 | 60 | 109 | 1390180_at | AI575012 | similar to PR-domain protein 8 (LOC305198), |
| | 72 | 80 | 71 | 117 | 63 | 88 | 1373663_at | AI102482 | similar to RIKEN cDNA 1110014F24 |
| | 55 | 111 | 54 | 72 | 65 | 69 | 1390056_at | BE111525 | similar to SH2/SH3 adaptor protein |
| | 56 | 78 | 49 | 105 | 42 | 62 | 1375819_at | BI299678 | similarity to (H.sapiens) lipopolysaccharide |

| Name | E13 ovary | E13 testis | E14 ovary | E14 testis | E16 ovary | E16 testis | Affy-ID | Genbank | Description |
|---|---|---|---|---|---|---|---|---|---|
| | 51 | 72 | 57 | 98 | 58 | 107 | 1390449_at | BI289132 | similarity to 1BGM (E. coli) O Chain O, Beta |
| | 61 | 103 | 69 | 80 | 66 | 86 | 1389302_at | BI289482 | similarity to 1LBG (E. coli) B Chain B, Lactose |
| | 54 | 61 | 44 | 78 | 47 | 102 | 1374228_at | BE113215 | similarity to 1LBG (E. coli) B Chain B, Lactose |
| | 29 | 22 | 20 | 45 | 32 | 117 | 1383263_at | BG664221 | similarity to B35272 osteoinductive factor - |
| | 69 | 113 | 69 | 102 | 51 | 72 | 1389659_at | AI230591 | similarity to CSL2_HUMAN Cathepsin L2 |
| | 71 | 87 | 68 | 109 | 58 | 91 | 1390174_at | BI289762 | similarity to EML1_HUMAN Echinoderm |
| | 49 | 46 | 71 | 71 | 70 | 101 | 1374309_at | BF406637 | similarity to hypothetical protein FLJ10324 |
| | 39 | 43 | 45 | 38 | 32 | 139 | 1373175_at | BI285951 | similarity to hypothetical protein FLJ22662 |
| | 65 | 102 | 46 | 57 | 51 | 65 | 1398431_at | BI294910 | similarity to JN0576 (H.sapiens) carbonate |
| | 27 | 23 | 46 | 64 | 59 | 138 | 1389096_at | BI274243 | similarity to KIAA0750 gene product |
| | 69 | 49 | 68 | 115 | 75 | 147 | 1388902_at | AI599031 | similarity to LOL1_HUMAN Lysyl oxidase |
| | 73 | 123 | 73 | 83 | 66 | 79 | 1372054_at | BG380399 | similarity to NP_003066.1 (H.sapiens) |
| | 56 | 103 | 65 | 60 | 54 | 59 | 1390130_at | BM392225 | similarity to NP_060453.1 (H.sapiens) |
| | 54 | 115 | 58 | 82 | 55 | 82 | 1375754_at | AA850890 | similarity to NP_060909.1 (H.sapiens) |
| | 66 | 100 | 74 | 73 | 66 | 53 | 1375844_at | AI406370 | similarity to NP_067013.1 (H.sapiens) |
| | 70 | 101 | 63 | 84 | 71 | 78 | 1385839_x_at | BF287928 | similarity to NP_114416.1 (H.sapiens) beta- |
| | 69 | 112 | 69 | 83 | 54 | 53 | 1373885_at | BF398015 | similarity to P45973 (H.sapiens) |
| | 25 | 40 | 53 | 78 | 53 | 103 | 1372579_at | BF288089 | similarity to S50831 (H.sapiens) S50831 |
| | 35 | 34 | 53 | 49 | 33 | 109 | 1373960_at | AI235631 | similarity to transmembrane protein induced by |
| | 53 | 39 | 48 | 57 | 40 | 122 | 1372219_at | AA012755 | similarity to Tropomyosin beta chain, fibroblast |
| | 33 | 43 | 42 | 88 | 65 | 169 | 1389253_at | BI289085 | similarity to VNN1_HUMAN Pantetheinase |
| | 56 | 82 | 51 | 105 | 39 | 125 | 1377821_at | BI284288 | Rattus norvegicus transcribed sequences |
| | 45 | 44 | 73 | 101 | 33 | 146 | 1374529_at | AI406660 | Rattus norvegicus transcribed sequences |
| | 59 | 51 | 64 | 106 | 69 | 175 | 1373590_at | BI295949 | Rattus norvegicus transcribed sequences |
| | 38 | 67 | 70 | 135 | 66 | 103 | 1398288_at | BF552873 | Rattus norvegicus transcribed sequences |
| | 15 | 16 | 21 | 38 | 31 | 156 | 1373596_at | AI230766 | Rattus norvegicus transcribed sequences |
| | 30 | 45 | 36 | 63 | 40 | 142 | 1389221_at | BI296275 | Rattus norvegicus transcribed sequences |
| | 32 | 42 | 27 | 73 | 12 | 101 | 1379598_at | AI071649 | Rattus norvegicus transcribed sequences |
| | 63 | 69 | 64 | 57 | 74 | 123 | 1373872_at | BE096535 | Rattus norvegicus transcribed sequences |
| | 69 | 85 | 57 | 74 | 61 | 108 | 1373035_at | AI031032 | Rattus norvegicus transcribed sequences |
| | 73 | 78 | 63 | 98 | 39 | 104 | 1375655_at | BE107208 | Rattus norvegicus transcribed sequences |
| | 69 | 79 | 69 | 55 | 62 | 103 | 1399109_at | BI281673 | Rattus norvegicus transcribed sequences |
| | 66 | 82 | 60 | 93 | 72 | 116 | 1390731_at | BI288533 | Rattus norvegicus transcribed sequences |
| | 73 | 81 | 69 | 97 | 73 | 108 | 1374490_at | AI177845 | Rattus norvegicus transcribed sequences |
| | 51 | 106 | 70 | 65 | 31 | 81 | 1375130_at | AI410230 | Rattus norvegicus transcribed sequences |
| | 74 | 100 | 69 | 74 | 52 | 72 | 1376594_at | AW524517 | Rattus norvegicus transcribed sequences |
| | 61 | 101 | 63 | 64 | 60 | 66 | 1381467_at | AI598594 | Rattus norvegicus transcribed sequences |
| | 74 | 103 | 72 | 80 | 73 | 90 | 1388543_at | BI291257 | Rattus norvegicus transcribed sequences |
| | 48 | 49 | 49 | 86 | 55 | 106 | 1374970_at | BF392911 | Rattus norvegicus transcribed sequences |
| | 60 | 73 | 67 | 75 | 68 | 102 | 1389251_at | AA944380 | Rattus norvegicus transcribed sequences |
| | 49 | 60 | 46 | 139 | 28 | 97 | 1392785_at | AA800908 | Rattus norvegicus transcribed sequences |
| | 61 | 67 | 66 | 108 | 58 | 76 | 1376907_at | AW521452 | Rattus norvegicus transcribed sequences |
| | 70 | 68 | 75 | 107 | 69 | 62 | 1388688_at | BI296599 | Rattus norvegicus transcribed sequences |

**Female Enriched**

| Name | E13 ovary | E13 testis | E14 ovary | E14 testis | E16 ovary | E16 testis | Affy-ID | Genbank | Description |
|---|---|---|---|---|---|---|---|---|---|
| *Bmp2* | 149 | 75 | 221 | 73 | 99 | 70 | 1398270_at | AA944827 | bone morphogenetic protein 2 |
| *Cdh2* | 98 | 68 | 84 | 58 | 140 | 61 | 1368642_at | NM_031333 | cadherin 2 |
| *Cnr1* | 32 | 32 | 103 | 19 | 110 | 11 | 1369677_at | X55812 | cannabinoid receptor 1 |
| *Eln* | 35 | 23 | 42 | 27 | 152 | 51 | 1388111_at | J04035 | elastin |
| *Grin3b* | 107 | 58 | 98 | 67 | 121 | 73 | 1388905_at | AI230770 | glutamate receptor, ionotropic, NMDA3B |
| *Gmpr* | 59 | 54 | 76 | 52 | 157 | 51 | 1386914_at | NM_057188 | guanosine monophosphate reductase |
| | 54 | 48 | 71 | 40 | 100 | 60 | 1375911_at | AI171772 | hypothetical protein LK44 |
| *Klk1* | 219 | 70 | 158 | 47 | 49 | 39 | 1387820_at | NM_012593 | kallikrein 1 |
| *Kai1* | 52 | 45 | 71 | 62 | 103 | 74 | 1386976_at | NM_031797 | kangai 1 |
| *Ncdn* | 81 | 55 | 89 | 60 | 114 | 60 | 1367956_at | NM_053543 | neurochondrin |
| *Pom210* | 48 | 47 | 65 | 43 | 118 | 66 | 1367919_at | NM_053322 | nuclear pore membrane glycoprotein 210 |
| *Ppicap* | 42 | 48 | 70 | 72 | 106 | 66 | 1387946_at | AF065438 | peptidylprolyl isomerase C-associated protein |
| *Ppp1r1a* | 80 | 75 | 169 | 69 | 167 | 48 | 1386968_at | NM_022676 | protein phosphatase 1, regulatory (inhibitor) |
| *RT1Aw2* | 39 | 40 | 68 | 37 | 191 | 29 | 1370428_x_at | AJ249701 | RT1 class Ib gene(Aw2) |
| *Slc21a7* | 39 | 37 | 70 | 44 | 124 | 39 | 1368606_at | NM_030838 | solute carrier family 21 (fatty acid transporter), |
| *Sp17* | 53 | 58 | 57 | 65 | 127 | 63 | 1370088_at | NM_053482 | sperm autoantigenic protein 17 |
| *Skd3* | 76 | 56 | 82 | 53 | 101 | 71 | 1367997_at | NM_022947 | suppressor of K+ transport defect 3 |
| | 89 | 58 | 101 | 69 | 72 | 56 | 1370374_at | AF335281 | tumor suppressor pHyde |
| *Ugcg* | 114 | 65 | 87 | 62 | 68 | 74 | 1387975_at | AF047707 | UDP-glucose:ceramide glycosyltransferase |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Anxa4* | 34 | 32 | 57 | 51 | 104 | 72 | 1389305_at | BM385237 | ZAP 36/annexin IV |
| | 111 | 61 | 61 | 56 | 41 | 64 | 1374786_at | BI298817 | Rattus norvegicus cDNA clone UI-R-CV2-chv- |
| | 66 | 39 | 98 | 66 | 149 | 68 | 1376029_at | BI295991 | Rattus norvegicus cDNA clone UI-R-DK0-cfd- |
| | 107 | 72 | 78 | 55 | 86 | 75 | 1389488_at | AA800750 | EST190247 Normalized rat lung |
| | 130 | 66 | 79 | 50 | 102 | 33 | 1375382_at | BE120346 | similar to KIAA0170 gene product; |
| | 107 | 63 | 69 | 69 | 53 | 66 | 1374618_at | BI279751 | similarity to protein pdb:1LBG (E. coli) |
| | 47 | 40 | 66 | 41 | 109 | 67 | 1374495_at | BM388121 | similarity to protein pdb:1LBG (E. coli) |
| | 69 | 65 | 78 | 74 | 104 | 65 | 1388463_at | AW252660 | similarity to protein ref:NP_057010.1 |
| | 65 | 53 | 96 | 50 | 115 | 58 | 1373312_at | BI295064 | similarity to protein ref:NP_064383.1 |
| | 103 | 72 | 86 | 47 | 72 | 62 | 1377138_at | AI709547 | similarity to protein sp:P00722 (E. coli) |
| | 48 | 58 | 57 | 53 | 101 | 57 | 1373900_at | BI284344 | similarity to protein sp:P08729 (H.sapiens) |
| | 64 | 60 | 58 | 44 | 107 | 59 | 1380568_a_at | AI575274 | similarity to protein sp:Q92466 (H.sapiens) |
| | 72 | 73 | 94 | 63 | 100 | 72 | 1374034_at | BG379410 | similarity to protein ref:NP_001742.1 |
| | 59 | 63 | 101 | 68 | 85 | 71 | 1375552_at | BG372976 | similarity to protein ref:NP_008878.1 |
| | 89 | 62 | 103 | 69 | 79 | 72 | 1373394_at | BM391986 | similarity to protein ref:NP_060221.1 |
| | 84 | 74 | 76 | 64 | 102 | 67 | 1373270_at | AI233288 | similarity to protein ref:NP_060453.1 |
| | 103 | 56 | 136 | 26 | 90 | 12 | 1377058_at | BI291872 | similarity to protein sp:P00722 (E. coli) |
| | 110 | 64 | 54 | 68 | 65 | 63 | 1374477_at | BE113700 | similarity to protein sp:Q99811 (H.sapiens) |
| | 87 | 51 | 90 | 55 | 126 | 49 | 1372968_at | BM385950 | similarity to protein pir:S37032 (R.norvegicus) |
| | 150 | 68 | 140 | 69 | 100 | 73 | 1375869_at | AI603439 | similarity to protein ref:NP_003556.1 |
| | 48 | 48 | 68 | 48 | 102 | 48 | 1372296_at | AA800892 | similarity to protein sp:P55822 (H.sapiens) |
| | 90 | 47 | 109 | 54 | 156 | 52 | 1375992_at | BM389934 | Rattus norvegicus transcribed sequences |
| | 89 | 66 | 118 | 64 | 173 | 49 | 1373696_at | AI171656 | Rattus norvegicus transcribed sequences |
| | 113 | 71 | 39 | 36 | 22 | 35 | 1376742_at | BF396436 | Rattus norvegicus transcribed sequences |
| | 122 | 67 | 100 | 50 | 76 | 32 | 1393349_x_at | BI288992 | Rattus norvegicus transcribed sequences |
| | 104 | 54 | 52 | 39 | 24 | 59 | 1376080_at | BF409812 | Rattus norvegicus transcribed sequences |
| | 106 | 66 | 73 | 74 | 74 | 62 | 1375363_at | BG374402 | Rattus norvegicus transcribed sequences |
| | 101 | 56 | 42 | 38 | 22 | 70 | 1377193_at | BF405021 | Rattus norvegicus transcribed sequences |
| | 104 | 59 | 77 | 52 | 59 | 65 | 1381210_at | AI639521 | Rattus norvegicus transcribed sequences |
| | 51 | 58 | 61 | 50 | 148 | 47 | 1372479_at | AI175666 | Rattus norvegicus transcribed sequences |
| | 125 | 68 | 69 | 42 | 47 | 41 | 1378367_at | BF389087 | Rattus norvegicus transcribed sequences |
| | 102 | 65 | 51 | 62 | 53 | 62 | 1375392_at | BF405277 | Rattus norvegicus transcribed sequences |
| | 46 | 42 | 72 | 47 | 113 | 52 | 1379371_at | BF284791 | Rattus norvegicus transcribed sequences |
| | 53 | 19 | 105 | 31 | 82 | 30 | 1374678_at | BE109578 | Rattus norvegicus transcribed sequences |
| | 84 | 57 | 75 | 66 | 104 | 71 | 1372354_at | BM389898 | Rattus norvegicus transcribed sequences |
| | 93 | 65 | 85 | 46 | 101 | 57 | 1398415_at | BG380708 | Rattus norvegicus transcribed sequences |
| | 77 | 68 | 91 | 52 | 103 | 69 | 1383935_at | AW252428 | Rattus norvegicus transcribed sequences |

Supplemental Table S2: Gender enriched gene lists. Lists of testis and ovary genes expressed above a signal value of 100 in one sex but not above 75 in the other sex at E13, E14 or E16, are listed for each sex separately.

**Supplemental Table S3**

| 316 Gender Enhanced Genes | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Common | E13 ovary | E13 testis | E14 ovary | E14 testis | E16 ovary | E16 testis | Affy-ID | Genbank | Description |
| *A5D3* | 200 | 258 | 262 | 877 | 509 | 1514 | 1370459_at | AY007690 | A5D3 protein |
| *Abcc6* | 40 | 77 | 50 | 54 | 56 | 57 | 1368452_at | NM_031013 | liver multidrug resistance-associated protein 6 |
| *Abcd3* | 144 | 127 | 141 | 117 | 219 | 115 | 1368057_at | NM_012804 | ATP-binding cassette, sub-family D (ALD), member 3 |
| *Acta2* | 121 | 203 | 145 | 249 | 149 | 506 | 1370857_at | BI282702 | smooth muscle alpha-actin |
| *Actn1* | 371 | 238 | 262 | 303 | 228 | 313 | 1389189_at | BF555956 | actinin, alpha 1 |
| *Adamts1* | 87 | 71 | 60 | 83 | 47 | 83 | 1368223_at | NM_024400 | a disintegrin and metalloproteinase with thrombospondin |
| *Agtr2* | 30 | 51 | 52 | 100 | 31 | 78 | 1369711_at | NM_012494 | Angiotensin receptor 2; go_component: membrane [goid |
| *Alas1* | 243 | 132 | 143 | 148 | 199 | 268 | 1367982_at | NM_024484 | aminolevulinic acid synthase 1 |
| *Aldh2* | 184 | 143 | 298 | 143 | 353 | 133 | 1367999_at | NM_032416 | aldehyde dehydrogenase 2 |
| *Amhr2* | 577 | 457 | 964 | 300 | 847 | 172 | 1368243_at | NM_030998 | anti-Mullerian hormone type 2 receptor |
| *Apoc1* | 93 | 106 | 84 | 38 | 124 | 37 | 1368587_at | NM_012824 | apolipoprotein C-I |
| *Ar* | 61 | 73 | 55 | 83 | 62 | 75 | 1369159_at | NM_012502 | androgen receptor |
| *Axin2* | 92 | 104 | 120 | 100 | 217 | 93 | 1390429_at | BF398114 | axin2 |
| *Bmp2* | 149 | 75 | 221 | 73 | 99 | 70 | 1398270_at | AA944827 | bone morphogenetic protein 2 |
| *C3* | 167 | 183 | 389 | 316 | 744 | 374 | 1371703_at | AI407114 | complement component 3 |
| *Catnb* | 2906 | 3152 | 3117 | 3685 | 1629 | 2777 | 1373067_at | AI102738 | beta-catenin |
| *Ccnd2* | 318 | 303 | 393 | 287 | 548 | 324 | 1375266_at | BG380633 | Rattus norvegicus transcribed sequence with strong |
| *Cd74* | 92 | 108 | 130 | 24 | 186 | 51 | 1367679_at | NM_013069 | CD74 antigen (invariant polpypeptide of major |
| *Cdh2* | 98 | 68 | 84 | 58 | 140 | 61 | 1368642_at | NM_031333 | cadherin 2 |
| *Cdh22* | 239 | 118 | 129 | 66 | 112 | 96 | 1377008_at | BI296868 | Rattus norvegicus transcribed sequence with weak |
| *Cdkn1b* | 304 | 234 | 334 | 179 | 262 | 137 | 1373812_at | BE110915 | cyclin-dependent kinase inhibitor 1B |
| *Cfl1* | 75 | 85 | 56 | 101 | 63 | 113 | 1371339_at | AI599017 | cofilin 1 |
| *Cldn11* | 228 | 135 | 305 | 146 | 212 | 414 | 1376711_at | BG673439 | DRNBVE04 Rat DRG Library Rattus norvegicus cDNA |
| *Clta* | 140 | 77 | 152 | 70 | 186 | 106 | 1389567_at | BE329208 | Rattus norvegicus transcribed sequence with moderate |
| *Cnr1* | 32 | 32 | 103 | 19 | 110 | 11 | 1369677_at | X55812 | cannabinoid receptor 1 |
| *Cox4b* | 84 | 128 | 74 | 88 | 70 | 113 | 1368648_at | NM_053472 | cytochrome c oxidase, subunit 4b |
| *Csrp1* | 245 | 283 | 164 | 262 | 164 | 352 | 1370057_at | NM_017148 | cysteine and glycine-rich protein 1 |
| *Csrp2* | 360 | 588 | 223 | 736 | 125 | 455 | 1370282_at | U44948 | cysteine rich protein 2 |
| *Ctsh* | 240 | 243 | 405 | 294 | 551 | 344 | 1386899_at | NM_012939 | cathepsin H |
| *Cttnb* | 81 | 99 | 65 | 84 | 39 | 84 | 1370123_a_at | AF054618 | cortactin isoform B |
| *Cxcl12* | 386 | 350 | 296 | 349 | 159 | 359 | 1369633_at | AI171777 | chemokine (C-X-C motif) ligand 12 |
| *Cxcr4* | 111 | 128 | 156 | 82 | 178 | 49 | 1373661_a_at | AA945737 | Chemokine receptor (LCR1) |
| *Cxcr4* | 151 | 141 | 201 | 119 | 187 | 72 | 1389244_x_at | AA945737 | Chemokine receptor (LCR1) |
| *Cyp1b1* | 513 | 270 | 932 | 224 | 788 | 188 | 1368990_at | NM_012940 | cytochrome P450, subfamily 1B, polypeptide 1 |
| *Dia1* | 318 | 212 | 334 | 190 | 281 | 218 | 1370808_at | J03867 | diaphorase 1 |
| *Dig1* | 565 | 423 | 554 | 309 | 660 | 367 | 1388102_at | U66322 | dithiolethione-inducible gene-1 |
| *Dlc2* | 572 | 360 | 560 | 385 | 593 | 362 | 1372612_at | BG372973 | dynein light chain-2 |
| *Dlgh1* | 61 | 58 | 84 | 53 | 65 | 72 | 1368944_at | NM_012788 | discs, large homolog 1 (Drosophila) |
| *Echs1* | 200 | 164 | 212 | 199 | 247 | 160 | 1367829_at | NM_078623 | enoyl Coenzyme A hydratase, short chain 1 |
| *Ednra* | 63 | 100 | 78 | 100 | 51 | 126 | 1369511_at | NM_012550 | endothelin receptor type A |
| *Egln1* | 244 | 195 | 194 | 167 | 212 | 130 | 1389207_at | BI282122 | EGL nine homolog 1 (C. elegans) |
| *Egr1* | 94 | 171 | 93 | 69 | 182 | 129 | 1368321_at | NM_012551 | early growth response 1 |
| *Eln* | 35 | 23 | 42 | 27 | 152 | 51 | 1388111_at | J04035 | elastin |
| *Enpp2* | 102 | 86 | 179 | 94 | 233 | 89 | 1368536_at | NM_057104 | ectonucleotide pyrophosphatase/phosphodiesterase 2 |
| *Epb4.1l3* | 183 | 142 | 77 | 82 | 156 | 74 | 1368515_at | NM_053927 | erythrocyte protein band 4.1-like 3 |
| *Fads3* | 126 | 74 | 93 | 91 | 92 | 87 | 1372476_at | AW533321 | putative fatty acid desaturase |
| *Fgf13* | 332 | 217 | 378 | 130 | 454 | 116 | 1368114_at | NM_053428 | fibroblast growth factor 13 |
| *Fst* | 89 | 27 | 337 | 34 | 406 | 23 | 1387843_at | NM_012561 | follistatin |
| *Galnt1* | 112 | 180 | 150 | 227 | 143 | 195 | 1369269_at | NM_024373 | polypeptide GalNAc transferase T1 |
| *Gamt* | 294 | 245 | 365 | 216 | 420 | 274 | 1368253_at | NM_012793 | guanidinoacetate methyltransferase |
| *Gatm* | 39 | 59 | 35 | 84 | 35 | 134 | 1367627_at | NM_031031 | glycine amidinotransferase |
| *Gfer* | 172 | 140 | 181 | 142 | 204 | 132 | 1367867_at | NM_013222 | growth factor, erv1 -like |
| *Gfra2* | 86 | 114 | 104 | 202 | 91 | 218 | 1369167_at | NM_012750 | glial cell line derived neurotrophic factor family receptor |
| *Glrx1* | 66 | 99 | 54 | 102 | 55 | 67 | 1367705_at | AF319950 | glutaredoxin 1 (thioltransferase) |
| *Glud1* | 218 | 174 | 227 | 178 | 254 | 147 | 1370200_at | BI284411 | glutamate dehydrogenase 1 |
| *Gmpr* | 59 | 54 | 76 | 52 | 157 | 51 | 1386914_at | NM_057188 | guanosine monophosphate reductase |
| *Gp38* | 132 | 95 | 217 | 118 | 190 | 96 | 1386913_at | NM_019358 | glycoprotein 38 |
| *Gpr56* | 310 | 252 | 354 | 226 | 385 | 200 | 1371696_at | AI412938 | G protein-coupled receptor 56 |
| *Grn* | 559 | 366 | 574 | 324 | 507 | 401 | 1386893_at | NM_017113 | granulin |
| *Gucy1b3* | 119 | 232 | 70 | 158 | 35 | 88 | 1374389_at | BF399387 | guanylate cyclase 1, soluble, beta 3 |
| *H2a* | 267 | 249 | 249 | 197 | 292 | 183 | 1374293_at | AI137495 | Rattus norvegicus transcribed sequence with strong |
| *Hnk1st* | 53 | 91 | 52 | 65 | 72 | 67 | 1367944_at | NM_080397 | HNK-1 sulfotransferase |
| *Hrsp12* | 229 | 337 | 284 | 581 | 209 | 586 | 1368060_at | NM_031714 | heat-responsive protein 12 |
| *Hsd17b3* | 22 | 36 | 28 | 71 | 25 | 152 | 1369553_at | NM_054007 | hydroxysteroid 17-beta dehydrogenase 3 |
| *Igfbp2* | 960 | 1018 | 1471 | 615 | 1478 | 502 | 1367648_at | NM_013122 | insulin-like growth factor binding protein 2 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Igfbp3 | 328 | 217 | 125 | 226 | 166 | 410 | 1386881_at | NM_012588 | insulin-like growth factor binding protein 3 |
| Il13ra2 | 32 | 36 | 55 | 89 | 81 | 100 | 1369266_at | NM_133538 | interleukin 13 receptor, alpha 2 |
| Itpr1 | 247 | 185 | 239 | 135 | 220 | 105 | 1387907_at | J05510 | |
| Itpr3 | 87 | 78 | 93 | 61 | 99 | 52 | 1368005_at | NM_013138 | inositol 1, 4, 5-triphosphate receptor 3 |
| Itsn | 50 | 80 | 61 | 67 | 44 | 66 | 1397161_a_at | AF132672 | intersectin 1 |
| Ivd | 106 | 88 | 109 | 85 | 113 | 70 | 1370232_at | AI102838 | isovaleryl coenzyme A dehydrogenase |
| Jag1 | 37 | 21 | 31 | 25 | 21 | 118 | 1368725_at | NM_019147 | jagged 1 |
| Klf4 | 39 | 33 | 48 | 51 | 87 | 48 | 1387260_at | NM_053713 | Kruppel-like factor 4 (gut) |
| Klk1 | 219 | 70 | 158 | 47 | 49 | 39 | 1387820_at | NM_012593 | kallikrein 1 |
| Krt118 | 78 | 124 | 72 | 137 | 57 | 119 | 1388155_at | BI286012 | keratin complex 1, acidic, gene 18 |
| Lamb2 | 173 | 103 | 163 | 91 | 178 | 109 | 1367880_at | NM_012974 | laminin, beta 2 |
| Lbr | 541 | 469 | 512 | 444 | 599 | 388 | 1373439_at | AI178491 | lamin B receptor |
| Lcn2 | 33 | 34 | 34 | 93 | 31 | 152 | 1387011_at | NM_130741 | lipocalin 2 |
| LOC246255 | 253 | 207 | 212 | 196 | 286 | 187 | 1388107_at | BF282131 | protein phosphatase 2A B regulatory subunit delta |
| Marcks | 745 | 836 | 868 | 1263 | 537 | 1271 | 1373432_at | BE111604 | myristoylated alanine rich protein kinase C substrate |
| Marcks | 929 | 731 | 719 | 892 | 401 | 918 | 1375523_at | BE108178 | myristoylated alanine rich protein kinase C substrate |
| Marcks | 283 | 485 | 479 | 638 | 235 | 715 | 1370948_a_at | M59859 | myristoylated alanine rich protein kinase C substrate |
| Marcks | 64 | 115 | 94 | 142 | 30 | 180 | 1388157_at | BE111706 | myristoylated alanine rich protein kinase C substrate |
| Mg87 | 216 | 176 | 309 | 214 | 398 | 214 | 1368071_at | NM_134410 | Mg87 protein |
| Mkln1 | 212 | 190 | 227 | 185 | 291 | 181 | 1368018_at | NM_031359 | muskelin |
| Msx1 | 47 | 30 | 74 | 43 | 130 | 57 | 1368302_at | NM_031059 | homeo box, msh-like 1 |
| Ndufa10 | 159 | 157 | 177 | 97 | 228 | 117 | 1389334_at | BI277594 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex |
| Ngfrap1 | 1213 | 1288 | 1339 | 1161 | 1499 | 826 | 1369948_at | NM_053401 | nerve growth factor receptor associated protein 1 |
| Nopp140 | 49 | 93 | 85 | 90 | 53 | 78 | 1368033_at | BI290791 | nucleolar phosphoprotein p130 |
| Nppc | 27 | 45 | 26 | 34 | 15 | 184 | 1387744_at | NM_053750 | natriuretic peptide precursor C |
| Npr1 | 124 | 115 | 133 | 117 | 139 | 73 | 1368201_at | NM_012613 | natriuretic peptide receptor 1 |
| Nrep | 1071 | 1078 | 714 | 1144 | 741 | 1182 | 1371412_a_at | BE107450 | neuronal regeneration related protein |
| Nrg1 | 35 | 78 | 31 | 28 | 16 | 44 | 1369783_a_at | U02319 | neuregulin 1 |
| NVP3 | 159 | 142 | 135 | 148 | 84 | 183 | 1369562_at | NM_017356 | neural visinin-like Ca2+-binding protein type 3 |
| Osi | 55 | 83 | 64 | 80 | 63 | 68 | 1371221_at | AF053095 | oxidative stress induced |
| Oxr1 | 170 | 146 | 136 | 155 | 280 | 159 | 1367869_at | NM_057153 | Rattus norvegicus oxidation resistance 1 (Oxr1), mRNA. |
| Pacsin2 | 100 | 85 | 116 | 59 | 112 | 85 | 1368068_a_at | NM_130740 | protein kinase C and casein kinase substrate in neurons |
| Pawr | 80 | 81 | 83 | 90 | 73 | 136 | 1368702_at | U05989 | PRKC, apoptosis, WT1, regulator |
| Pdi2 | 125 | 88 | 118 | 79 | 88 | 72 | 1387091_at | NM_017226 | peptidyl arginine deiminase, type 2 |
| Pfkl | 1954 | 1724 | 1213 | 1189 | 878 | 1398 | 1371731_at | AI408151 | phosphofructokinase, liver, B-type |
| pips | 382 | 281 | 427 | 289 | 569 | 272 | 1387148_at | NM_134386 | Per1 interacting protein |
| Podxl | 530 | 468 | 679 | 378 | 818 | 211 | 1369895_s_at | AF109393 | podocalyxin-like |
| Ppif | 221 | 223 | 268 | 202 | 366 | 177 | 1370319_at | U68544 | peptidylprolyl isomerase F |
| Prkar2b | 155 | 163 | 169 | 179 | 155 | 315 | 1371133_a_at | M12492 | protein kinase, cAMP dependent regulatory, type II beta |
| Psdzip70 | 301 | 216 | 347 | 99 | 231 | 118 | 1377171_at | AA875041 | PSD-Zip70 |
| Ptprf | 389 | 253 | 424 | 231 | 443 | 241 | 1368036_at | M60103 | protein tyrosine phosphatase, receptor type, F |
| Pv1 | 243 | 132 | 111 | 53 | 36 | 26 | 1368284_at | NM_020086 | PV-1 |
| Rala | 225 | 277 | 214 | 312 | 136 | 235 | 1368405_at | NM_031093 | v-ral simian leukemia viral oncogene homolog A |
| Ramp1 | 81 | 127 | 93 | 132 | 80 | 138 | 1367791_at | NM_031645 | receptor (calcitonin) activity modifying protein 1 |
| Rdc1 | 55 | 86 | 63 | 92 | 41 | 103 | 1367940_at | NM_053352 | chemokine orphan receptor 1 |
| Rere | 373 | 191 | 349 | 198 | 315 | 201 | 1371890_at | AI172033 | arginine-glutamic acid dipeptide (RE) repeats |
| Rgc32 | 73 | 151 | 60 | 186 | 57 | 108 | 1368080_at | NM_054008 | Rgc32 protein |
| Rgs19ip1 | 93 | 89 | 98 | 77 | 121 | 65 | 1368065_at | NM_053341 | regulator of G-protein signaling 19 interacting protein 1 |
| Ril | 62 | 53 | 72 | 80 | 56 | 104 | 1387153_at | NM_017062 | reversion induced LIM gene |
| Scn4b | 425 | 313 | 368 | 220 | 337 | 173 | 1373188_at | AI137995 | sodium channel, voltage-gated, type IV, beta |
| Serpinb5 | 135 | 88 | 146 | 73 | 155 | 80 | 1369972_at | NM_057108 | serine (or cysteine) proteinase inhibitor, clade B, |
| Sfrp4 | 59 | 45 | 68 | 45 | 69 | 241 | 1368394_at | AF140346 | secreted frizzled-related protein 4 |
| Slc2a5 | 136 | 118 | 166 | 99 | 250 | 69 | 1368460_at | NM_031741 | solute carrier family 2 (facilitated glucose transporter), |
| Slc38a1 | 169 | 158 | 234 | 180 | 95 | 160 | 1370409_at | AF075704 | solute carrier family 38, member 1 |
| Slc5a6 | 103 | 54 | 73 | 73 | 67 | 80 | 1367815_at | NM_130746 | solute carrier family 5, member 6 |
| Tcf21 | 396 | 581 | 201 | 691 | 121 | 651 | 1388782_at | BE113336 | transcription factor 21 |
| Tfrc | 149 | 289 | 165 | 167 | 193 | 209 | 1371113_a_at | M58040 | transferrin receptor |
| Top2a | 169 | 109 | 173 | 142 | 191 | 159 | 1369990_at | NM_022183 | topoisomerase (DNA) 2 alpha |
| Tpm1 | 251 | 301 | 144 | 283 | 99 | 325 | 1370288_a_at | AF372216 | tropomyosin 1, alpha |
| Tpra40 | 115 | 61 | 87 | 56 | 88 | 58 | 1371635_at | AI599069 | Rattus norvegicus transcribed sequence with moderate |
| Tspan2 | 51 | 103 | 53 | 67 | 36 | 91 | 1368105_at | AI228231 | Tspan-2 protein |
| Tst | 210 | 181 | 384 | 136 | 453 | 108 | 1370881_at | AI411117 | thiosulfate sulfurtransferase |
| Ugcg | 114 | 65 | 87 | 62 | 68 | 74 | 1387975_at | AF047707 | UDP-glucose:ceramide glycosyltransferase |
| Uxs1 | 88 | 104 | 88 | 148 | 52 | 151 | 1376127_at | BE117201 | UDP-glucuronate decarboxylase 1 |
| Vsnl1 | 24 | 32 | 22 | 23 | 18 | 86 | 1368854_at | AI227991 | visinin-like 1 |
| Wnt2b | 89 | 159 | 135 | 125 | 165 | 112 | 1388174_at | AF204873 | wingless-type MMTV integration site family, member 2B |
| Wnt4 | 141 | 66 | 222 | 86 | 249 | 63 | 1368641_at | NM_053402 | wingless-type MMTV integration site family, member 4 |
| Wnt5a | 38 | 50 | 58 | 82 | 26 | 134 | 1369263_at | NM_022631 | wingless-type MMTV integration site 5A |
| Xtrp3 | 59 | 138 | 52 | 60 | 75 | 92 | 1369704_at | NM_133296 | X transporter protein 3 |
| Zrf2 | 137 | 187 | 139 | 181 | 217 | 141 | 1373004_at | AA964764 | zuotin related factor 2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 90 | 58 | 72 | 87 | 87 | 76 | 1398367_at | BF283504 | EST448095 Rat Gene Index, normalized rat, Rattus |
| 346 | 215 | 303 | 310 | 212 | 380 | 1374432_at | BE118251 | R.norvegicus mRNA for novel gene expressed in |
| 565 | 668 | 341 | 513 | 279 | 356 | 1388131_at | X03369 | Rat mRNA for beta-tubulin T beta15 |
| 120 | 151 | 114 | 181 | 96 | 292 | 1373718_at | BM384071 | Rat mRNA for beta-tubulin T beta15 |
| 199 | 179 | 127 | 210 | 72 | 210 | 1376066_at | AI103572 | Rattus norvegicus RHOE mRNA, complete cds |
| 275 | 177 | 208 | 153 | 178 | 175 | 1398927_at | BF406225 | Rattus norvegicus similar to 0610010K06Rik protein |
| 58 | 77 | 100 | 78 | 120 | 69 | 1372706_at | AA892845 | Rattus norvegicus similar to beta-hexosaminidase |
| 98 | 90 | 105 | 82 | 120 | 71 | 1388460_at | BM384693 | Rattus norvegicus similar to Capg protein |
| 88 | 67 | 71 | 58 | 95 | 58 | 1372157_at | AI232807 | Rattus norvegicus similar to CGI-143 protein |
| 265 | 282 | 261 | 343 | 423 | 222 | 1373822_at | BI275708 | Rattus norvegicus similar to Chain A, Solution Structure |
| 54 | 40 | 46 | 45 | 79 | 32 | 1390937_at | AW523875 | Rattus norvegicus similar to chromosome 14 open |
| 158 | 132 | 162 | 115 | 182 | 121 | 1371609_at | AI232270 | Rattus norvegicus similar to DNA segment, Chr 10, |
| 77 | 92 | 167 | 130 | 238 | 155 | 1376858_at | BI281836 | Rattus norvegicus similar to Exocyst complex |
| 68 | 75 | 78 | 126 | 127 | 87 | 1389636_at | AI231088 | Rattus norvegicus similar to KIAA0833 protein |
| 104 | 103 | 115 | 74 | 185 | 97 | 1392926_at | BI281952 | Rattus norvegicus similar to laminin alpha-1 chain |
| 123 | 129 | 77 | 114 | 71 | 155 | 1373151_at | AW252169 | Rattus norvegicus similar to lipoma HMGIC fusion |
| 76 | 131 | 224 | 363 | 353 | 840 | 1371357_at | AI233246 | Rattus norvegicus similar to mac25 |
| 27 | 38 | 26 | 71 | 32 | 120 | 1389236_at | AI104915 | Rattus norvegicus similar to Myosin-binding protein H |
| 245 | 221 | 260 | 200 | 207 | 129 | 1372959_at | BI289467 | Rattus norvegicus similar to nucleoside diphosphate |
| 62 | 73 | 54 | 95 | 58 | 115 | 1371441_at | BG662875 | Rattus norvegicus similar to phosphoprotein enriched in |
| 20 | 5 | 33 | 76 | 13 | 91 | 1388339_at | BE112895 | Rattus norvegicus similar to phosphoprotein enriched in |
| 561 | 495 | 700 | 364 | 779 | 291 | 1371481_at | BI274372 | Rattus norvegicus similar to POLYPOSIS LOCUS |
| 88 | 97 | 77 | 116 | 79 | 132 | 1373532_at | BI276934 | Rattus norvegicus similar to RIKEN cDNA 1810013P09 |
| 266 | 176 | 244 | 212 | 287 | 228 | 1390380_at | BE116633 | Rattus norvegicus similar to Ski proto-oncogene |
| 45 | 85 | 47 | 60 | 45 | 65 | 1375596_at | BI291219 | Rattus norvegicus similar to transient receptor potential |
| 580 | 461 | 698 | 382 | 824 | 338 | 1373399_at | BI291997 | Rattus norvegicus similar to WD repeat protein WDR6 |
| 188 | 284 | 170 | 376 | 144 | 308 | 1383912_at | BM390524 | Rattus norvegicus transcribed sequence with moderate |
| 61 | 103 | 69 | 80 | 66 | 86 | 1389302_at | BI289482 | Rattus norvegicus transcribed sequence with moderate |
| 150 | 133 | 125 | 195 | 143 | 160 | 1374178_at | AI103954 | Rattus norvegicus transcribed sequence with moderate |
| 24 | 50 | 34 | 78 | 73 | 81 | 1372590_at | BM386449 | Rattus norvegicus transcribed sequence with moderate |
| 149 | 160 | 119 | 123 | 73 | 125 | 1372563_at | AA799488 | Rattus norvegicus transcribed sequence with moderate |
| 175 | 192 | 93 | 142 | 57 | 116 | 1372457_at | BF284182 | Rattus norvegicus transcribed sequence with moderate |
| 188 | 101 | 228 | 146 | 259 | 215 | 1374731_at | BI275929 | Rattus norvegicus transcribed sequence with moderate |
| 112 | 71 | 121 | 107 | 116 | 123 | 1371562_at | BI296640 | Rattus norvegicus transcribed sequence with moderate |
| 79 | 33 | 55 | 35 | 50 | 58 | 1377463_at | BE099244 | Rattus norvegicus transcribed sequence with moderate |
| 337 | 218 | 306 | 193 | 322 | 358 | 1371477_at | BG380735 | Rattus norvegicus transcribed sequence with moderate |
| 182 | 219 | 204 | 186 | 352 | 195 | 1372229_at | AI179119 | Rattus norvegicus transcribed sequence with moderate |
| 225 | 185 | 230 | 150 | 335 | 179 | 1372857_at | AI407930 | Rattus norvegicus transcribed sequence with moderate |
| 144 | 103 | 126 | 72 | 117 | 67 | 1388522_at | AI170820 | Rattus norvegicus transcribed sequence with moderate |
| 472 | 447 | 538 | 460 | 693 | 431 | 1372473_at | AW434048 | Rattus norvegicus transcribed sequence with moderate |
| 620 | 548 | 841 | 246 | 1382 | 254 | 1373911_at | BM389026 | Rattus norvegicus transcribed sequence with moderate |
| 395 | 351 | 488 | 345 | 599 | 399 | 1375146_at | BI282168 | Rattus norvegicus transcribed sequence with moderate |
| 224 | 133 | 199 | 93 | 223 | 103 | 1373393_at | BM384468 | Rattus norvegicus transcribed sequence with moderate |
| 49 | 61 | 64 | 97 | 90 | 178 | 1375638_at | AI009714 | Rattus norvegicus transcribed sequence with moderate |
| 150 | 79 | 123 | 104 | 95 | 127 | 1373246_at | BF397920 | Rattus norvegicus transcribed sequence with moderate |
| 93 | 30 | 95 | 106 | 94 | 133 | 1388511_at | AI412079 | Rattus norvegicus transcribed sequence with moderate |
| 173 | 169 | 223 | 176 | 221 | 143 | 1388636_at | BI280272 | Rattus norvegicus transcribed sequence with moderate |
| 240 | 210 | 245 | 176 | 261 | 151 | 1372232_at | AI170808 | Rattus norvegicus transcribed sequence with moderate |
| 117 | 77 | 104 | 91 | 112 | 100 | 1373694_at | H33486 | Rattus norvegicus transcribed sequence with moderate |
| 109 | 62 | 79 | 47 | 78 | 90 | 1372302_at | BF284897 | Rattus norvegicus transcribed sequence with moderate |
| 115 | 87 | 134 | 73 | 169 | 76 | 1374527_at | AI172274 | Rattus norvegicus transcribed sequence with moderate |
| 193 | 108 | 143 | 122 | 147 | 116 | 1372198_at | BG376471 | Rattus norvegicus transcribed sequence with moderate |
| 65 | 53 | 96 | 50 | 115 | 58 | 1373312_at | BI295064 | Rattus norvegicus transcribed sequence with moderate |
| 49 | 18 | 85 | 31 | 141 | 55 | 1388952_at | BI277039 | Rattus norvegicus transcribed sequence with moderate |
| 101 | 83 | 62 | 95 | 68 | 106 | 1376623_at | AI409186 | Rattus norvegicus transcribed sequence with moderate |
| 77 | 46 | 58 | 39 | 82 | 54 | 1373304_at | BM392002 | Rattus norvegicus transcribed sequence with moderate |
| 270 | 263 | 298 | 311 | 292 | 528 | 1373733_at | AI227742 | Rattus norvegicus transcribed sequence with moderate |
| 57 | 82 | 63 | 100 | 53 | 79 | 1376100_at | BI274903 | Rattus norvegicus transcribed sequence with moderate |
| 118 | 150 | 86 | 155 | 104 | 229 | 1372818_at | BI284441 | Rattus norvegicus transcribed sequence with moderate |
| 592 | 534 | 825 | 337 | 1430 | 169 | 1388271_at | BM383531 | Rattus norvegicus transcribed sequence with moderate |
| 97 | 71 | 111 | 73 | 112 | 108 | 1389571_at | BG666368 | Rattus norvegicus transcribed sequence with moderate |
| 276 | 171 | 300 | 155 | 221 | 150 | 1376025_at | BG670963 | Rattus norvegicus transcribed sequence with moderate |
| 83 | 81 | 101 | 158 | 71 | 239 | 1373882_at | AI170324 | Rattus norvegicus transcribed sequence with moderate |
| 70 | 59 | 66 | 56 | 50 | 82 | 1375182_at | BE109671 | Rattus norvegicus transcribed sequence with moderate |
| 407 | 343 | 439 | 290 | 520 | 355 | 1372291_at | BI274517 | Rattus norvegicus transcribed sequence with moderate |
| 849 | 513 | 978 | 379 | 803 | 276 | 1371908_at | AA891920 | Rattus norvegicus transcribed sequence with strong |
| 59 | 33 | 64 | 47 | 94 | 45 | 1398377_at | AI172116 | Rattus norvegicus transcribed sequence with strong |
| 293 | 231 | 307 | 228 | 373 | 211 | 1371584_at | BM390843 | Rattus norvegicus transcribed sequence with strong |
| 286 | 251 | 306 | 290 | 210 | 349 | 1374987_at | AI232974 | Rattus norvegicus transcribed sequence with strong |
| 151 | 114 | 141 | 109 | 193 | 102 | 1388965_at | AI144583 | Rattus norvegicus transcribed sequence with strong |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 214 | 137 | 225 | 146 | 214 | 194 | 1376052_at | AW525031 | Rattus norvegicus transcribed sequence with strong |
| 175 | 135 | 212 | 96 | 285 | 98 | 1389142_at | AI013361 | Rattus norvegicus transcribed sequence with strong |
| 127 | 109 | 158 | 129 | 112 | 203 | 1371976_at | AI102758 | Rattus norvegicus transcribed sequence with strong |
| 51 | 102 | 85 | 110 | 36 | 122 | 1393669_at | BM383757 | Rattus norvegicus transcribed sequence with strong |
| 370 | 218 | 297 | 248 | 254 | 217 | 1372274_at | AI009727 | Rattus norvegicus transcribed sequence with strong |
| 189 | 116 | 159 | 95 | 86 | 99 | 1389797_at | AI317854 | Rattus norvegicus transcribed sequence with strong |
| 103 | 56 | 136 | 26 | 90 | 12 | 1377058_at | BI291872 | Rattus norvegicus transcribed sequence with strong |
| 105 | 91 | 102 | 75 | 137 | 67 | 1377692_at | BF558563 | Rattus norvegicus transcribed sequence with strong |
| 42 | 92 | 59 | 66 | 42 | 32 | 1382672_a_at | AW918207 | Rattus norvegicus transcribed sequence with strong |
| 81 | 119 | 77 | 89 | 78 | 158 | 1373036_at | BF283621 | Rattus norvegicus transcribed sequence with strong |
| 240 | 152 | 144 | 102 | 140 | 71 | 1389409_at | AI177869 | Rattus norvegicus transcribed sequence with strong |
| 1274 | 960 | 2154 | 1231 | 2640 | 1632 | 1372293_at | AF023090 | Rattus norvegicus transcribed sequence with weak |
| 47 | 91 | 59 | 66 | 40 | 85 | 1375696_at | BF420426 | Rattus norvegicus transcribed sequence with weak |
| 68 | 81 | 63 | 64 | 43 | 81 | 1374830_at | AA946242 | Rattus norvegicus transcribed sequence with weak |
| 78 | 66 | 79 | 75 | 52 | 89 | 1388887_at | AI178222 | Rattus norvegicus transcribed sequence with weak |
| 114 | 82 | 94 | 80 | 102 | 67 | 1374006_at | BI295878 | Rattus norvegicus transcribed sequence with weak |
| 65 | 86 | 106 | 70 | 138 | 122 | 1371520_at | BI296732 | Rattus norvegicus transcribed sequence with weak |
| 73 | 102 | 92 | 159 | 83 | 161 | 1373881_at | BF285771 | Rattus norvegicus transcribed sequence with weak |
| 150 | 68 | 140 | 69 | 100 | 73 | 1375869_at | AI603439 | Rattus norvegicus transcribed sequence with weak |
| 80 | 108 | 77 | 116 | 96 | 120 | 1389657_at | AI175762 | Rattus norvegicus transcribed sequence with weak |
| 33 | 37 | 34 | 50 | 45 | 79 | 1378423_at | AI639060 | Rattus norvegicus transcribed sequence with weak |
| 87 | 94 | 65 | 105 | 76 | 78 | 1375023_at | BE104415 | Rattus norvegicus transcribed sequence with weak |
| 105 | 77 | 78 | 123 | 88 | 81 | 1373701_at | BI275605 | Rattus norvegicus transcribed sequence with weak |
| 191 | 115 | 231 | 126 | 256 | 135 | 1376144_at | AA819679 | Rattus norvegicus transcribed sequence with weak |
| 66 | 83 | 94 | 77 | 116 | 73 | 1372098_at | AI172092 | Rattus norvegicus transcribed sequence with weak |
| 73 | 83 | 60 | 98 | 134 | 217 | 1374458_at | AI600031 | Rattus norvegicus transcribed sequence with weak |
| 198 | 132 | 203 | 118 | 163 | 109 | 1376342_at | BI295185 | Rattus norvegicus transcribed sequence with weak |
| 55 | 57 | 73 | 77 | 111 | 57 | 1376253_at | AW434178 | Rattus norvegicus transcribed sequence with weak |
| 211 | 135 | 212 | 167 | 221 | 171 | 1371797_at | BM391890 | Rattus norvegicus transcribed sequence with weak |
| 48 | 48 | 68 | 48 | 102 | 48 | 1372296_at | AA800892 | Rattus norvegicus transcribed sequence with weak |
| 191 | 167 | 196 | 188 | 347 | 209 | 1399133_at | AW520767 | Rattus norvegicus transcribed sequence with weak |
| 233 | 350 | 278 | 496 | 176 | 487 | 1375538_at | AI230737 | Rattus norvegicus transcribed sequences |
| 129 | 233 | 145 | 165 | 134 | 168 | 1380158_at | BF284973 | Rattus norvegicus transcribed sequences |
| 86 | 129 | 91 | 110 | 88 | 109 | 1393268_at | AI071071 | Rattus norvegicus transcribed sequences |
| 61 | 101 | 63 | 64 | 60 | 66 | 1381467_at | AI598594 | Rattus norvegicus transcribed sequences |
| 62 | 98 | 69 | 85 | 65 | 60 | 1388928_at | BF399310 | Rattus norvegicus transcribed sequences |
| 52 | 85 | 76 | 89 | 76 | 117 | 1373298_at | BI288011 | Rattus norvegicus transcribed sequences |
| 289 | 343 | 309 | 649 | 288 | 771 | 1388936_at | BI296340 | Rattus norvegicus transcribed sequences |
| 272 | 302 | 144 | 318 | 149 | 349 | 1388763_at | AI230762 | Rattus norvegicus transcribed sequences |
| 38 | 67 | 70 | 135 | 66 | 103 | 1398288_at | BF552873 | Rattus norvegicus transcribed sequences |
| 67 | 88 | 75 | 131 | 93 | 151 | 1373114_at | AI408442 | Rattus norvegicus transcribed sequences |
| 55 | 71 | 57 | 93 | 54 | 54 | 1377092_at | BF389682 | Rattus norvegicus transcribed sequences |
| 408 | 527 | 529 | 751 | 475 | 812 | 1371942_at | BM390378 | Rattus norvegicus transcribed sequences |
| 176 | 193 | 154 | 242 | 95 | 436 | 1388666_at | AI179988 | Rattus norvegicus transcribed sequences |
| 285 | 299 | 273 | 333 | 205 | 383 | 1372905_at | AW433888 | Rattus norvegicus transcribed sequences |
| 188 | 245 | 162 | 312 | 91 | 340 | 1377151_at | AI102833 | Rattus norvegicus transcribed sequences |
| 64 | 57 | 78 | 143 | 61 | 183 | 1374817_at | BE103235 | Rattus norvegicus transcribed sequences |
| 30 | 45 | 36 | 63 | 40 | 142 | 1389221_at | BI296275 | Rattus norvegicus transcribed sequences |
| 107 | 103 | 110 | 97 | 86 | 140 | 1374831_at | AI172579 | Rattus norvegicus transcribed sequences |
| 70 | 82 | 92 | 87 | 68 | 128 | 1372708_at | BI296544 | Rattus norvegicus transcribed sequences |
| 85 | 102 | 75 | 110 | 76 | 126 | 1388942_at | BM390129 | Rattus norvegicus transcribed sequences |
| 124 | 129 | 68 | 121 | 51 | 123 | 1375053_at | BE329035 | Rattus norvegicus transcribed sequences |
| 238 | 246 | 143 | 165 | 67 | 122 | 1376457_at | AI175861 | Rattus norvegicus transcribed sequences |
| 79 | 91 | 83 | 71 | 62 | 101 | 1390134_at | AI411274 | Rattus norvegicus transcribed sequences |
| 32 | 42 | 27 | 73 | 12 | 101 | 1379598_at | AI071649 | Rattus norvegicus transcribed sequences |
| 55 | 82 | 61 | 78 | 49 | 99 | 1372805_at | BE328984 | Rattus norvegicus transcribed sequences |
| 49 | 60 | 46 | 139 | 28 | 97 | 1392785_at | AA800908 | Rattus norvegicus transcribed sequences |
| 77 | 88 | 93 | 99 | 61 | 94 | 1372623_at | BE101435 | Rattus norvegicus transcribed sequences |
| 87 | 93 | 84 | 113 | 54 | 92 | 1375194_at | AI231460 | Rattus norvegicus transcribed sequences |
| 49 | 60 | 59 | 56 | 45 | 81 | 1376991_at | BE095620 | Rattus norvegicus transcribed sequences |
| 48 | 67 | 50 | 73 | 48 | 79 | 1377310_at | BG374304 | Rattus norvegicus transcribed sequences |
| 1191 | 78 | 824 | 270 | 255 | 40 | 1375535_at | AI103917 | Rattus norvegicus transcribed sequences |
| 632 | 19 | 448 | 136 | 82 | 2 | 1374684_at | AI228978 | Rattus norvegicus transcribed sequences |
| 612 | 341 | 679 | 248 | 645 | 307 | 1371710_at | BM391283 | Rattus norvegicus transcribed sequences |
| 407 | 256 | 332 | 262 | 299 | 200 | 1373829_at | AI412658 | Rattus norvegicus transcribed sequences |
| 310 | 186 | 201 | 98 | 301 | 142 | 1372221_at | BM389079 | Rattus norvegicus transcribed sequences |
| 304 | 199 | 297 | 216 | 295 | 206 | 1399060_at | BE113005 | Rattus norvegicus transcribed sequences |
| 270 | 173 | 251 | 189 | 250 | 210 | 1374331_at | AI178068 | Rattus norvegicus transcribed sequences |
| 256 | 157 | 193 | 175 | 279 | 278 | 1377173_at | BE104535 | Rattus norvegicus transcribed sequences |
| 213 | 138 | 187 | 136 | 133 | 127 | 1375034_at | AI410383 | Rattus norvegicus transcribed sequences |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 206 | 129 | 169 | 135 | 172 | 137 | 1391484_at | BF284786 | Rattus norvegicus transcribed sequences |
| 127 | 76 | 139 | 115 | 131 | 121 | 1373001_at | AA817990 | Rattus norvegicus transcribed sequences |
| 113 | 66 | 110 | 76 | 161 | 106 | 1390647_at | AI410924 | Rattus norvegicus transcribed sequences |
| 98 | 58 | 78 | 45 | 63 | 38 | 1384169_a_at | AA900477 | Rattus norvegicus transcribed sequences |
| 97 | 62 | 82 | 86 | 77 | 117 | 1374971_at | AA818954 | Rattus norvegicus transcribed sequences |
| 90 | 46 | 87 | 71 | 66 | 67 | 1372855_at | BF287135 | Rattus norvegicus transcribed sequences |
| 82 | 41 | 87 | 49 | 95 | 72 | 1372348_at | AI104502 | Rattus norvegicus transcribed sequences |
| 379 | 253 | 500 | 287 | 655 | 292 | 1399053_at | BM391257 | Rattus norvegicus transcribed sequences |
| 121 | 76 | 158 | 98 | 279 | 90 | 1389216_at | AW523499 | Rattus norvegicus transcribed sequences |
| 78 | 92 | 153 | 101 | 204 | 97 | 1377161_at | BG378317 | Rattus norvegicus transcribed sequences |
| 64 | 46 | 91 | 41 | 64 | 51 | 1376937_at | BM389685 | Rattus norvegicus transcribed sequences |
| 50 | 60 | 89 | 55 | 52 | 59 | 1390661_at | BE115928 | Rattus norvegicus transcribed sequences |
| 78 | 59 | 83 | 45 | 91 | 63 | 1399064_at | AI009591 | Rattus norvegicus transcribed sequences |
| 242 | 139 | 444 | 128 | 512 | 152 | 1389353_at | BM386525 | Rattus norvegicus transcribed sequences |
| 370 | 322 | 400 | 279 | 433 | 266 | 1373548_at | BE107518 | Rattus norvegicus transcribed sequences |
| 135 | 47 | 375 | 80 | 408 | 52 | 1372750_at | AI102517 | Rattus norvegicus transcribed sequences |
| 256 | 143 | 340 | 62 | 327 | 88 | 1389697_at | AW529759 | Rattus norvegicus transcribed sequences |
| 204 | 142 | 300 | 155 | 309 | 165 | 1373291_at | AI176713 | Rattus norvegicus transcribed sequences |
| 229 | 228 | 226 | 196 | 231 | 122 | 1372921_at | AI073219 | Rattus norvegicus transcribed sequences |
| 110 | 78 | 132 | 161 | 224 | 117 | 1375932_at | BF284677 | Rattus norvegicus transcribed sequences |
| 254 | 222 | 249 | 120 | 178 | 88 | 1373786_at | AA924756 | Rattus norvegicus transcribed sequences |
| 83 | 84 | 98 | 88 | 142 | 74 | 1399108_at | BM383630 | Rattus norvegicus transcribed sequences |
| 96 | 127 | 126 | 107 | 129 | 84 | 1388584_at | AI598881 | Rattus norvegicus transcribed sequences |
| 121 | 115 | 104 | 91 | 128 | 47 | 1388891_at | BG374285 | Rattus norvegicus transcribed sequences |
| 99 | 91 | 79 | 69 | 118 | 75 | 1373923_at | BF283756 | Rattus norvegicus transcribed sequences |
| 66 | 68 | 86 | 80 | 115 | 73 | 1383308_a_at | AI071048 | Rattus norvegicus transcribed sequences |
| 112 | 95 | 99 | 89 | 109 | 70 | 1389790_at | AI410565 | Rattus norvegicus transcribed sequences |
| 53 | 40 | 66 | 29 | 105 | 25 | 1376074_at | AA942690 | Rattus norvegicus transcribed sequences |
| 31 | 32 | 56 | 35 | 89 | 32 | 1388632_at | BG665929 | Rattus norvegicus transcribed sequences |
| 104 | 87 | 82 | 45 | 88 | 50 | 1373580_at | BI289991 | Rattus norvegicus transcribed sequences |
| 73 | 54 | 87 | 24 | 86 | 24 | 1384420_at | AA859937 | Rattus norvegicus transcribed sequences |
| 77 | 59 | 81 | 36 | 86 | 35 | 1373886_at | BF284692 | Rattus norvegicus transcribed sequences |
| 51 | 58 | 54 | 48 | 76 | 48 | 1388818_at | AA946074 | Rattus norvegicus transcribed sequences |
| 151 | 90 | 106 | 60 | 117 | 81 | 1376620_at | AI137912 | UI-R-A1-dp-h-09-0-UI.s1 UI-R-A1 |
| 202 | 113 | 151 | 121 | 163 | 159 | 1373536_at | AW525196 | UI-R-BJ0p-aio-h-09-0-UI.s1 UI-R-BJ0p |
| 105 | 54 | 76 | 102 | 95 | 56 | 1385241_at | AI071210 | UI-R-C2-my-d-11-0-UI.s1 UI-R-C2 |
| 113 | 113 | 140 | 103 | 181 | 85 | 1389229_at | AI113146 | UI-R-C2p-np-a-08-0-UI.s1 UI-R-C2p |
| 85 | 66 | 61 | 100 | 51 | 90 | 1384967_at | BM386930 | UI-R-CN1-cjh-n-24-0-UI.s1 UI-R-CN1 |
| 77 | 143 | 96 | 128 | 112 | 167 | 1375908_at | BI282616 | UI-R-CW0s-ccd-b-05-0-UI.s1 UI-R-CW0s |
| 161 | 118 | 174 | 81 | 133 | 85 | 1375950_a_at | BI294235 | UI-R-DK0-cea-c-07-0-UI.s1 UI-R-DK0 |
| 42 | 80 | 53 | 36 | 41 | 85 | 1372385_at | AA957292 | UI-R-E1-fq-h-06-0-UI.s1 UI-R-E1 |

Supplemental Table S3: List of 316 sex enhanced genes. Genes in this list have a 1.5 fold expression difference between the sexes for any single time point.

Table 3-S1. E13 vinclozolin altered transcripts

| Category | Gene | V/C | Cross Listed* | Systematic |
|---|---|---|---|---|
| Translation & Protein Modification | Splicing factor 3b, subunit 1(Sf3b1) | 1.95 | A | 1382691_at |
| | PRP4 pre-mRNA processing factor 4 homolog B (Prpf4b) | 1.90 | | 1397203_at |
| | Importin 5 (Ipo5) | 1.88 | A | 1396742_at |
| | Eukaryotic translation initiation factor 3, subunit 10 (Eif3s10) | 1.76 | A | 1379485_at |
| | Glutamyl-prolyl-tRNA synthetase (Eprs) | 1.66 | E14, A | 1382040_at |
| | SLU7 splicing factor homolog (Slu7) | 1.65 | | 1398588_at |
| | RNA binding motif protein 7 (Rbm7) | 1.52 | | 1394155_at |
| Transcription | THO complex 2 (Thoc2) | 3.01 | | 1385493_at |
| | Zinc finger protein 68 (Zfp68) | 1.63 | E14 | 1395553_at |
| | Zinc finger protein 260 (Zfp260) | 1.55 | E14, A | 1369501_at |
| | Vav2 oncogene (Vav2) | 0.63 | | 1393349_x_at |
| Metabolism & Transport | Phosphoglucomutase 1 (Pgm1) | 1.62 | | 1369473_at |
| | 5'-3' exoribonuclease 2 (Xrn2) | 1.61 | A | 1393702_at |
| | Thymidine kinase 1 (Tk1) | 0.64 | | 1372631_at |
| | Rhomboid domain containing 3 (Rhbdd3) | 0.57 | | 1372049_at |
| Signaling | Calcium/calmodulin-dependent protein kinase II, delta (Camk2d) | 1.74 | | 1371263_a_at |
| | Rho guanine nucleotide exchange factor (GEF) 17 (Arhgef17) | 0.62 | | 1374907_at |
| Epigenetics | Similar to GTL2, imprinted maternally expressed untranslated | 3.00 | | 1368887_at |
| | Chromatin modifying protein 1B (Chmp1b) | 1.53 | E14 | 1382112_at |
| Cytoskeleton-ECM | Vesicle-associated membrane protein 2 (Vamp2) | 0.67 | | 1369974_at |
| | C-type lectin domain family 14, member a (Clec14a) | 1.61 | | 1390782_at |
| Development | Angel homolog 1 (Drosophila, Angel1) | 0.51 | E14 | 1393833_at |
| Receptor & Binding Proteins | Angiotensin II receptor, type 2 (Agtr2) | 2.40 | E14 | 1369711_at |
| Proteolysis | Ubiquinol cytochrome c reductase core protein 2 (Uqcrc2) | 1.80 | A | 1382280_at |
| Unknown | Similar to KIAA0368 | 1.89 | A | 1393683_at |
| | Similar to mKIAA0738 protein | 1.78 | A | 1377580_at |
| | WD repeat domain 75 (Wdr75) | 1.57 | A | 1395058_at |
| | Similar to KIAA0913 protein | 0.66 | | 1390315_a_at |
| EST's | --- | 2.71 | | 1379497_at |
| | --- | 2.27 | E16 | 1391886_at |
| | --- | 2.07 | E16 | 1375601_at |
| | --- | 1.84 | | 1393522_at |
| | --- | 1.80 | E16 | 1396596_at |
| | --- | 1.76 | | 1381817_at |
| | --- | 1.73 | | 1375422_at |
| | --- | 1.71 | | 1393371_at |
| | --- | 1.68 | A | 1395744_at |
| | --- | 1.67 | | 1379712_at |
| | --- | 1.66 | E16 | 1379866_at |
| | --- | 1.61 | | 1385925_at |
| | --- | 1.60 | | 1375604_at |
| | --- | 0.63 | | 1367517_at |
| | --- | 0.61 | | 1372560_at |
| | --- | 0.60 | | 1379244_at |
| | Similar to Riken cDNA C230021P08 | 0.27 | | 1385485_x_at |

*For any gene identified in multiple lists, the other lists in which it appears are indicated in the Cross Listed column. E14= altered by vinclozolin at E14, E16= altered by vinclozolin at E16, A= appeared in a list of F1 E16 vinclozolin altered transcripts published by Anway et al 2008

Table 3-S2. E14 vinclozolin altered transcripts

| Category | Gene | V/C | Cross Listed* | Affy ID |
|---|---|---|---|---|
| Transcription | Zinc finger protein 212 (Zfp212) | 2.36 | | 1380094_a_at |
| | Nuclear receptor co-repressor 1 (Ncor1) | 1.70 | A | 1372102_at |
| | Polymerase (DNA directed), lambda (Poll) | 1.67 | | 1389268_at |
| | Similar to zinc finger protein 385 /// zinc finger protein 385A | 1.65 | | 1382826_at |
| | GATA binding protein 4 (Gata4) | 1.63 | | 1387894_at |
| | E2F1-inducible gene | 1.59 | | 1383729_at |
| | DEP domain containing 5 (Depdc5) | 1.58 | | 1373752_at |
| | T-cell lymphoma invasion and metastasis 1 (Tiam1) | 1.57 | | 1392980_at |
| | Unc-5 homolog B (C. elegans, Unc5b) | 1.54 | | 1393799_at |
| | Transformation related protein 53 inducible protein 11 (Trp53i11) | 1.51 | | 1375420_at |
| | Zinc finger protein 68 (Zfp68) | 0.66 | | 1385153_at |
| | DEAH (Asp-Glu-Ala-His) box polypeptide 36 (Dhx36) | 0.65 | | 1385871_at |
| | F-box protein 30 (Fbxo30) | 0.64 | A | 1382059_at |
| | PHD finger protein 14 (Phf14) | 0.64 | | 1392452_at |
| | Serologically defined colon cancer antigen 1 (Sdccag1) | 0.63 | | 1375450_at |
| | Microrchidia 3 (Morc3) | 0.62 | | 1390142_at |
| | Zinc finger, MYM-type 2 (Zmym2) | 0.62 | A | 1393363_at |
| | Kelch-like 7 (Drosophila, Klhl7) | 0.62 | | 1388799_at |
| | Suppression of tumorigenicity 7-like (St7l) | 0.61 | | 1390977_at |
| | Proline-rich nuclear receptor coactivator 2 (Pnrc2) | 0.61 | A | 1397313_at |
| | Transcription factor CP2-like 1 (Tcfcp2l1) | 0.60 | | 1393337_at |
| | IKAROS family zinc finger 5 (Ikzf5) | 0.59 | | 1386550_at |
| | Serologically defined colon cancer antigen 1 (Sdccag1) | 0.57 | E16 | 1396116_at |
| | Coiled-coil domain containing 127 (Ccdc127) | 0.57 | | 1385102_at |
| | Hypothetical protein LOC679612 | 0.57 | A | 1375130_at |
| | DEAH (Asp-Glu-Ala-His) box polypeptide 40 (Dhx40) | 0.57 | | 1377785_at |
| | Zinc finger protein 260 (Zfp260) | 0.53 | E13, A | 1369501_at |
| | Coiled-coil domain containing 49 (Ccdc49) | 0.52 | | 1374888_at |
| | Transcription factor 12 (Tcf12) | 0.52 | A | 1387374_at |
| | Zinc finger protein 655 (Zfp655) | 0.49 | A | 1398536_at |
| | Zinc finger protein 157 (Zfp157) | 0.43 | | 1391709_at |
| | Myeloid/lymphoid leukemia (trithorax homolog, Drosophila, Mllt3) | 0.42 | | 1368279_at |
| Signaling | WAS/WASL interacting protein family, member 3 (Wipf3) | 2.29 | A | 1370648_a_at |
| | Sushi domain containing 3 (Susd3) | 2.24 | | 1377351_at |
| | Rap GTPase interactor (Radil) | 2.03 | | 1374309_at |
| | Similar to calmodulin-binding transcription activator 1 | 1.86 | | 1391030_at |
| | Pleckstrin homology domain, family A member 4 (Plekha4) | 1.85 | | 1384570_at |
| | Similar to nucleoredoxin | 1.80 | | 1391749_a_at |
| | Protein tyrosine phosphatase, receptor type, V (Ptprv) | 1.75 | | 1387136_at |
| | 2',3'-cyclic nucleotide 3' phosphodiesterase (Cnp) | 1.74 | A | 1387897_at |
| | Protein tyrosine phosphatase, non-receptor type 6 (Ptpn6) | 1.67 | | 1368010_at |
| | Rap1 GTPase-activating protein (Rap1gap) | 1.66 | | 1373631_at |
| | Protein tyrosine phosphatase, receptor, N polypeptide 2 (Ptprn2) | 1.60 | | 1370182_at |
| | Protein tyrosine phosphatase, non-receptor type 12 (Ptpn12) | 0.62 | A | 1369496_at |
| | Protein tyrosine phosphatase, receptor type, D (Ptprd) | 0.60 | | 1370488_a_at |
| | RAB5A, member RAS oncogene family (Rab5a) | 0.59 | A | 1387641_at |
| | Sorting nexin 16 (Snx16) | 0.58 | | 1387510_at |
| | NIMA (never in mitosis gene a)-related expressed kinase 7 (Nek7) | 0.56 | | 1380121_at |
| | A kinase (PRKA) anchor protein (gravin) 12 (Akap12) | 0.53 | | 1368869_at |
| | TAO kinase 1 (Taok1) | 0.51 | A | 1388002_at |
| | Protein phosphatase 1, regulatory (inhibitor) subunit 3C (Ppp1r3c) | 0.46 | A | 1373108_at |
| | Guanylate cyclase 1, soluble, alpha 3 (Gucy1a3) | 0.44 | A | 1387079_at |
| Cytoskeleton-ECM | Bassoon (Bsn) | 2.31 | | 1374192_at |
| | Espin (Espn) | 2.28 | | 1368774_a_at |
| | Collagen, type II, alpha 1 (Col2a1) | 1.80 | | 1371226_at |
| | C-type lectin domain family 16, member A (Clec16a) | 1.71 | | 1381686_at |
| | Sparc/osteonectin cwcv & kazal-like dom proteoglycan 2 (Spock2) | 1.68 | E16, C | 1388452_at |
| | Thrombomodulin (Thbd) | 1.66 | | 1375951_at |
| | Myosin, light chain 9, regulatory (Myl9) | 1.65 | | 1388298_at |
| | Nucleoporin 210 (Nup210) | 1.54 | | 1367919_at |
| | Leucine rich repeat & fibronectin III domain containing 3 (Lrfn3) | 1.52 | | 1384540_at |
| | Keratinocytes associated transmembrane protein 2 | 0.62 | | 1380407_at |
| | Dynamin 1-like (Dnm1l) | 0.61 | A | 1369220_at |
| | Vascular cell adhesion molecule 1 (Vcam1) | 0.56 | | 1368474_at |

| | | | | |
|---|---|---|---|---|
| | Cell cycle associated protein 1 (Caprin1) | 0.52 | A | 1395173_at |
| | Multimerin 1 (Mmrn1) | 0.48 | | 1392053_at |
| | Kinesin family member 11 (Kif11) | 0.48 | A | 1385619_at |
| | Fibronectin leucine rich transmembrane protein 3 (Flrt3) | 0.40 | | 1378057_at |
| | Osteoglycin (Ogn) | 0.35 | | 1376749_at |
| Metabolism | ATPase, Ca++ transporting, plasma membrane 3 (Atp2b3) | 2.66 | A | 1388037_at |
| | Carbohydrate sulfotransferase 2 (Chst2) | 1.67 | E16 | 1379300_at |
| | Solute carrier family 45, member 4 (Slc45a4) | 1.66 | | 1374120_at |
| | Solute carrier family 30 (zinc transporter), member 2 (Slc30a2) | 1.63 | | 1398264_at |
| | Rhomboid, veinlet-like 3 (Drosophila, Rhbdl3) | 1.61 | | 1379026_at |
| | Solute carrier family 33(acetyl-CoA transport) member 1 (Slc33a1) | 1.61 | | 1394304_at |
| | Glutathione S-transferase, alpha 4 (Gsta4) | 1.56 | | 1372297_at |
| | Selenoprotein P, plasma, 1 (sepp1) | 0.65 | | 1368806_at |
| | Collectin sub-family member 12 (Colec12) | 0.62 | | 1372818_at |
| | N-acetylneuraminic acid phosphatase (Nanp) | 0.62 | A | 1398746_at |
| | Sarcosine dehydrogenase (Sardh) | 0.60 | E16 | 1370573_at |
| | Mannosidase, alpha, class 1A, member 2 (Man1a2) | 0.57 | | 1394346_at |
| | Cysteine dioxygenase 1, cytosolic (Cdo1) | 0.48 | | 1367755_at |
| | Biliverdin reductase B (flavin reductase (NADPH)) | 0.46 | | 1392539_at |
| | Epoxide hydrolase 2, cytoplasmic (Ephx2) | 0.35 | E16 | 1369663_at |
| Development | DOM-3 homolog Z (C. elegans, Dom3z) | 1.76 | A | 1391052_at |
| | Optic atrophy 3 (human, Opa3) | 1.73 | | 1375448_at |
| | D4, zinc and double PHD fingers family 1 (Dpf1) | 1.70 | | 1370977_at |
| | SRY-box containing gene 7 (Sox7) | 1.66 | | 1384415_at |
| | Ataxin 2-like (Atxn2l) | 1.59 | | 1388826_at |
| | Angel homolog 1 (Drosophila, Angel1) | 1.55 | E13 | 1393833_at |
| | Discs, large homolog-associated protein 4 (Drosophila, Dlgap4) | 1.50 | | 1370507_at |
| | Similar to mirror-image polydactyly 1 | 0.65 | | 1392733_at |
| | Sarcoglycan, beta (dystrophin-associated glycoprotein) (Sgcb) | 0.64 | | 1374796_at |
| | Neural precursor exp. Developmentally down-reg. gene 4 (Nedd4) | 0.60 | A | 1383899_at |
| | Similar to muscleblind-like 2 isoform 1 | 0.58 | A | 1394931_at |
| | Neurofilament, light polypeptide (Nefl) | 0.54 | | 1370058_at |
| | Adenomatosis polyposis coli 2 (Apc2) | 0.33 | | 1397579_x_at |
| Translation & Protein Modification | Mitochondrial ribosomal protein L14 (Mrpl14) | 1.80 | | 1395063_at |
| | Mitochondrial translation optimization 1 (S. cerevisiae, Mto1) | 1.79 | | 1377493_at |
| | RNA binding motif protein 19 (Rbm19) | 1.69 | | 1390848_at |
| | Similar to gem (nuclear organelle) associated protein 5 | 1.63 | | 1378501_at |
| | Similar to cleavage stimulation factor, 3 pre-RNA subunit 2 | 1.58 | | 1392829_at |
| | Ribonuclease P 25 subunit (human, Rpp25) | 1.57 | | 1376110_at |
| | Pumilio 2 (Drosophila, Pum2) | 0.65 | A | 1382198_at |
| | Nuclear protein in the AT region (Npat) | 0.65 | A | 1396192_at |
| | Eukaryotic translation initiation factor 1A (Eif1a) | 0.64 | A | 1397892_at |
| | Eukaryotic translation initiation factor 2, sub 3, X-linked (Eif2s3x) | 0.62 | A | 1397693_at |
| | Glutamyl-prolyl-tRNA synthetase (Eprs) | 0.47 | E13, A | 1382040_at |
| Epigenetics | Hypermethylated in cancer 2 (Hic2) | 1.63 | | 1390030_at |
| | Chromatin modifying protein 1B (Chmp1b) | 1.56 | E13 | 1382112_at |
| | Enhancer of polycomb homolog 2 (Drosophila, Epc2) | 0.63 | A | 1383467_at |
| | GC-rich promoter binding protein 1 (Gpbp1) | 0.62 | A | 1396128_at |
| | SNF2 histone linker PHD RING helicase (Shprh) | 0.51 | A | 1379951_at |
| | Chromogranin B (Chgb) | 0.21 | | 1368034_at |
| Proteolysis | Calpain 8 (Capn8) | 1.89 | A | 1387292_s_at |
| | ClpB caseinolytic peptidase B homolog (E. coli, Clpb) | 1.65 | | 1367997_at |
| | Elastase 3B, pancreatic (Ela3b) | 1.55 | | 1382532_at |
| | Zinc finger protein 68 (Zfp68) | 0.65 | | 1392835_at |
| | Protease, serine, 35 (Prss35) | 0.33 | A | 1379747_at |
| Receptors & Binding Proteins | Glutamate receptor, ionotropic, N-methyl D-aspartate-like 1A | 3.77 | | 1378849_at |
| | Platelet derived growth factor receptor, beta polypeptide (Pdgfrb) | 1.91 | E16, F | 1379211_at |
| | Natriuretic peptide receptor 1 (Npr1) | 1.51 | | 1368201_at |
| | Angiotensin II receptor, type 2 (Agtr2) | 0.35 | | 1398288_at |
| Growth Factors, Cyto&Chemokines | Nerve growth factor receptor(TNFR superfamily, member16, Ngfr) | 1.90 | | 1368148_at |
| | Wingless-related MMTV integration site 6 (Wnt6) | 1.57 | | 1376063_at |
| | Growth hormone receptor (Ghr) | 0.55 | | 1368924_at |
| | Osteoglycin (Ogn) | 0.32 | A, C | 1383263_at |
| Cell Cycle | Centromere protein T (Cenpt) | 1.87 | E16 | 1375937_a_at |
| | Telomeric repeat binding factor 2 (Terf2) | 1.65 | | 1398208_s_at |
| | Stromal antigen 2 (Stag2) | 0.61 | A | 1382332_at |
| Immune Response | RT1 class II, locus Bb (RT1-Bp) | 24.64 | | 1371033_at |

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|---|---|---|---|---|
| | Thymus cell antigen 1, theta (Thy1) | 0.15 | | 1369652_at |
| Apoptosis | NCK interacting protein with SH3 domain (Nckipsd) | 1.63 | | 1391425_at |
| Electron Transport | Cytochrome P450, family26, subfamily b, polypeptide 1 (Cyp26b1) | 2.16 | A | 1376667_at |
| DNA Repair | Family with sequence similarity 84, member A (Fam84a) | 1.55 | | 1374699_at |
| Golgi Apparatus | Similar to Vps41 protein | 0.59 | A | 1397512_at |
| Miscellaneous& Unknown | Hypothetical gene supported by BC082068 | 2.51 | | 1373177_x_at |
| | Similar to hypothetical protein FLJ20154 | 1.84 | | 1375369_at |
| | Similar to FLJ46082 protein | 1.65 | | 1375992_at |
| | Transmembrane protein 178 (Tmem178) | 1.66 | | 1376106_at |
| | Hypothetical protein LOC691153 | 2.19 | | 1376188_at |
| | Reprimo-like (Rprml) | 1.93 | | 1377492_at |
| | Hypothetical protein LOC678970 | 1.88 | | 1378540_at |
| | Hypothetical protein LOC680656 | 1.92 | | 1378755_at |
| | Kelch domain containing 5 (Klhdc5) | 0.48 | | 1379615_at |
| | LOC360807 | 0.64 | | 1382415_at |
| | Similar to hypothetical protein MGC52110 | 0.56 | A | 1385889_at |
| | Similar to CG3570-PA | 0.63 | | 1390394_at |
| | BTB (POZ) domain containing 11 (Btbd11) | 1.61 | | 1390873_at |
| | Similar to chromosome 1 open reading frame 63 | 0.57 | A | 1390943_at |
| | C1q domain containing 2 (C1qdc2) | 1.77 | | 1392200_a_at |
| | Hypothetical LOC302495 | 0.67 | | 1393050_at |
| | SAP30-like (Sap30l) | 1.72 | A | 1393651_at |
| | Lin-28 homolog (C. elegans, Lin28) | 1.88 | | 1393904_at |
| | Hypothetical LOC100125371 | 0.53 | A | 1394388_at |
| | Hypothetical protein LOC688832 | 1.74 | | 1395044_at |
| | Similar to hypothetical protein FLJ10652 | 0.38 | A | 1395297_at |
| | Malignant fibrous histiocytoma amplified sequence 1 (Mfhas1) | 0.56 | E16 | 1398648_at |
| EST's | --- | 0.26 | E16, V | 1371776_at |
| | --- | 1.94 | | 1372226_at |
| | --- | 1.63 | | 1372937_at |
| | --- | 0.60 | | 1373053_at |
| | --- | 0.63 | | 1374259_at |
| | --- | 1.53 | | 1374305_at |
| | --- | 2.98 | | 1374337_at |
| | --- | 1.58 | | 1374531_at |
| | --- | 1.58 | | 1374731_at |
| | --- | 0.61 | | 1374780_at |
| | --- | 1.79 | | 1374822_at |
| | --- | 1.66 | A | 1374992_at |
| | --- | 0.59 | A | 1375343_at |
| | --- | 0.64 | | 1375676_at |
| | --- | 0.27 | E16 | 1375680_at |
| | --- | 0.22 | E16 | 1375751_at |
| | --- | 1.63 | E16 | 1376226_at |
| | --- | 1.63 | | 1376808_at |
| | --- | 2.01 | | 1377495_at |
| | --- | 0.54 | A | 1377686_at |
| | --- | 1.52 | | 1377790_at |
| | --- | 1.65 | | 1377865_at |
| | --- | 1.72 | | 1378006_at |
| | --- | 1.66 | | 1378093_at |
| | --- | 1.84 | | 1378876_at |
| | --- | 0.65 | | 1379094_at |
| | --- | 1.78 | | 1379324_at |
| | --- | 0.65 | A | 1379429_at |
| | --- | 0.56 | A | 1379830_at |
| | --- | 1.88 | | 1380209_at |
| | --- | 1.73 | | 1380345_at |
| | --- | 0.26 | A | 1380552_at |
| | --- | 1.58 | | 1380596_at |
| | --- | 1.82 | A | 1380734_at |
| | --- | 2.00 | E16 | 1381063_at |
| | --- | 2.27 | | 1381269_at |
| | --- | 0.64 | | 1381481_at |
| | --- | 0.63 | A | 1382013_at |
| | --- | 2.00 | | 1382291_at |
| | --- | 0.59 | | 1382294_at |
| | --- | 0.64 | | 1382416_at |
| | --- | 1.72 | | 1382794_at |

| | | | |
|---|---|---|---|
| --- | 0.60 | | 1383338_at |
| --- | 0.60 | | 1383510_at |
| --- | 0.63 | | 1383705_at |
| --- | 1.72 | | 1384138_at |
| --- | 0.61 | | 1384562_at |
| --- | 0.58 | | 1384668_at |
| --- | 0.63 | A | 1384854_at |
| --- | 0.65 | | 1384889_at |
| --- | 1.76 | | 1385868_at |
| --- | 0.41 | | 1385892_at |
| --- | 0.51 | A | 1386064_at |
| --- | 2.23 | | 1386186_s_at |
| --- | 1.78 | | 1386557_at |
| --- | 1.63 | | 1390523_at |
| --- | 0.65 | | 1390545_at |
| --- | 0.53 | | 1390866_at |
| --- | 0.54 | E16 | 1391256_at |
| --- | 1.65 | | 1391439_at |
| --- | 0.63 | | 1391581_at |
| Similar to RIKEN cDNA 6330416G13 gene | 1.56 | E16 | 1391817_at |
| --- | 0.42 | | 1392166_at |
| --- | 0.52 | | 1392968_at |
| --- | 1.62 | | 1393311_at |
| --- | 2.69 | A | 1394807_at |
| --- | 0.53 | A | 1395010_at |
| --- | 1.53 | | 1395014_at |
| --- | 1.68 | A | 1396288_s_at |
| --- | 0.47 | | 1396420_at |
| --- | 1.54 | | 1397168_at |
| --- | 1.74 | | 1397924_at |
| --- | 1.61 | E16 | 1398394_at |
| --- | 0.29 | A | 1398716_at |

*For any gene identified in multiple lists, the other lists in which it appears are indicated in the Cross Listed column. E13= altered by vinclozolin at E13, E16= altered by vinclozolin at E16, V= altered by vinclozolin in organ cultures, F= altered by flutamide in organ cultures, A= appeared in a list of F1 E16 vinclozolin altered transcripts published by Anway et al 2008, C=appeared in a list of testis differentiation candidates published by Clement et al 2007

Table 3-S3. E16 vinclozolin altered transcripts

| Category | Gene | V/C | Cross Listed* | Affy ID |
|---|---|---|---|---|
| Transcription | Similar to Transcription factor 7-like2 (HMG box transc. factor4,TCF-4) | 2.26 | | 1379815_at |
| | Zinc finger protein 292 (Zfp292) | 2.11 | A | 1376917_at |
| | Nuclear receptor co-repressor 1 (Ncor1) | 2.05 | A, F | 1384293_at |
| | THO complex 2 (Thoc2) | 2.01 | | 1396803_at |
| | Zinc finger protein 346 (Zfp346) | 2.00 | | 1390641_at |
| | DEAD (Asp-Glu-Ala-Asp) box polypeptide 42 (Ddx42) | 2.00 | | 1379896_at |
| | RNA binding motif protein 5 (Rbm5) | 1.94 | | 1398595_at |
| | Transformed mouse 3T3 cell double minute 4 (Mdm4) | 1.93 | | 1382417_at |
| | Tankyrase,TRF1-interac. ADPribose polymerase2 (Tnks2) | 1.93 | A | 1395047_at |
| | General transcription factor II H, polypeptide 2 (Gtf2h2) | 1.92 | | 1382030_at |
| | Similar to modulator of estrogen induced transcription | 1.85 | | 1381175_at |
| | Serologically defined colon cancer antigen 1 (Sdccag1) | 1.84 | E14 | 1396116_at |
| | Cdc42 guanine nucleotide exchange factor (GEF) 9 (Arhgef9) | 1.80 | | 1387199_a_at |
| | Mitochondrial tumor suppressor 1 (Mtus1) | 1.79 | A | 1372457_at |
| | Signal sequence receptor, alpha (Ssr1) | 1.64 | A | 1390767_at |
| | Protein kinase C binding protein 1 (Prkcbp1) | 1.60 | | 1390305_at |
| | Zinc finger and BTB domain containing 43 (Zbtb43) | 1.59 | | 1373763_at |
| | Myeloid/lymphoid leukemia translocated to, 4 (Mllt4) | 1.59 | A | 1387392_at |
| | Regulator of telomere elongation helicase 1 (Rtel1) | 1.57 | | 1391964_at |
| | Pirin (Pir) | 0.65 | | 1377662_at |
| | N-myc (and STAT) interactor (Nmi) | 0.62 | | 1393144_at |
| | Coiled-coil domain containing 95 (Ccdc95) | 0.62 | | 1377614_at |
| | RAS-like family 11 member B (Rasl11b) | 0.56 | | 1383322_at |
| Metabolism & Transport | NECAP endocytosis associated 1 (Necap1) | 2.30 | A | 1394904_at |
| | Carbohydrate sulfotransferase 2 (Chst2) | 1.94 | E14, A | 1379300_at |
| | ATPase, Ca++ transporting, plasma membrane 1 (Atp2b1) | 1.93 | | 1394714_at |
| | ATPase, Cu++ transporting, beta polypeptide (Atp7b) | 1.84 | | 1370324_at |
| | Threonine synthase-like 1 (bacterial, Thnsl1) | 1.81 | | 1383818_at |
| | Nucleoporin like 1 (Nupl1) | 1.75 | | 1388198_at |
| | N-acetylglucosamine-1-phosphate transferase, a&B sub. (Gnptab) | 1.71 | | 1397758_at |
| | Abhydrolase domain containing 14A (Abhd14a) | 1.69 | | 1372976_at |
| | Aldehyde dehydrogenase 1 family, member B1 (Alhd1b1) | 1.67 | | 1383472_at |
| | Mannosidase, beta A, lysosomal (Manba) | 1.60 | | 1371875_at |
| | ATPase, Na+/K+ transporting, beta 2 polypeptide (Atp1b2) | 1.59 | | 1379906_at |
| | D-dopachrome tautomerase (Ddt) | 1.57 | | 1367793_at |
| | Sterol O-acyltransferase 1 (Soat1) | 1.50 | | 1373869_at |
| | Protein disulfide isomerase-associated 5 (Pdia5) | 0.65 | | 1374828_at |
| | Sarcosine dehydrogenase (Sardh) | 0.61 | E14 | 1370573_at |
| | Molybdenum cofactor synthesis 2 (Mocs2) | 0.54 | | 1372177_at |
| | Epoxide hydrolase 2, cytoplasmic (Ephx2) | 0.53 | E14 | 1369663_at |
| Cytoskeleton-ECM | Myosin binding protein H-like (Mybphl) | 2.57 | | 1378970_at |
| | Cadherin 1 (Cdh1) | 2.47 | | 1386947_at |
| | Grancalcin (Gca) | 2.11 | | 1390557_at |
| | Troponin T2, cardiac (Tnnt2) | 1.87 | | 1367592_at |
| | Claudin 11 (Cldn11) | 1.82 | A | 1369609_at |
| | Laminin, alpha 1 (Lama1) | 1.79 | | 1392926_at |
| | Nidogen 1 (Nid1) | 1.76 | A | 1371032_at |
| | Dynamin 2 (Dnm2) | 1.66 | | 1369661_at |
| | Structural maintenance of chromosomes 4 (Smc4) | 1.65 | | 1383008_at |
| | Sparc/osteonectin, cwcv&kazal-like domains proteoglycan 2 (Spock2) | 1.58 | E14, C | 1388452_at |
| | ARP3 actin-related protein 3 homolog (yeast, Actr3) | 1.57 | A | 1395886_at |
| | Elastin microfibril interfacer 1 (Emilin1) | 0.64 | | 1372325_at |
| | Smoothelin-like 2 (Smtnl2) | 0.58 | | 1385171_s_at |
| | Tubulin, beta 6 (Tubb6) | 0.56 | | 1376100_at |
| | Troponin I type 2 (skeletal, fast) (Tnni2) | 0.47 | | 1367964_at |
| Signaling | Similar to protein tyrosine phosphatase, receptor type, D | 2.59 | | 1395148_at |
| | Sushi domain containing 3 (Susd3) | 2.31 | | 1379960_at |
| | Mitogen-activated protein kinase kinase kinase kinase 4 (Map4k4) | 2.27 | | 1381790_at |
| | Mitogen-activated protein kinase 1 interacting protein 1-like (Mapk1ip1l) | 2.03 | | 1392133_at |
| | Rho GTPase-activating protein (Grit) | 1.85 | | 1377061_at |
| | TAO kinase 2 (Taok2) | 1.72 | | 1387140_at |
| | Steroidogenic acute regulatory protein (Star) | 1.62 | | 1368406_at |
| | Dual specificity phosphatase-like 15 (Dusp15) | 1.57 | | 1385912_at |

| | | | | |
|---|---|---|---|---|
| | Similar to NMDA receptor regulated 1-like | 1.56 | | 1383728_at |
| | Pleckstrin homology-like domain, family A, member 3 (Phlda3) | 0.59 | | 1375224_at |
| | Rho GTPase activating protein 22 (Arhgap22) | 0.53 | | 1391083_at |
| | Protein kinase C, zeta (Prkcz) | 0.53 | | 1370197_a_at |
| | Heat shock protein 12B (Hspa12b) | 0.50 | | 1389604_at |
| | Dual specificity phosphatase 6 (Dusp6) | 0.36 | | 1382778_at |
| Receptors & Binding Proteins | Platelet derived growth factor receptor, beta polypeptide (Pdgfrb) | 3.87 | E14, F | 1379211_at |
| | Thyroid hormone receptor alpha (Thra) | 1.83 | | 1370691_a_at |
| | Endothelin receptor type A (Ednra) | 1.81 | A | 1369511_at |
| | Scavenger receptor class B, member 1 (Scarb1) | 1.75 | A | 1367855_at |
| | EF-hand calcium binding domain 2 (Efcab2) | 1.64 | | 1375646_at |
| | Taste receptor, type 1, member 2 (Tas1r2) | 1.61 | | 1382743_at |
| | Synaptojanin 2 binding protein (Synj2bp) | 1.58 | | 1368040_at |
| | ATP-binding cassette, sub-family C (CFTR/MRP), member 1 (Abcc1) | 1.57 | | 1371005_at |
| | Receptor accessory protein 6 (Reep6) | 1.57 | A | 1372841_at |
| | Fibroblast growth factor receptor-like 1 (Fgfrl1) | 0.52 | | 1390374_at |
| | Amyloid beta (A4) precursor protein-binding, familyA,member3 (Apba3) | 0.50 | | 1368389_at |
| Proteolysis | Similar to a disintegrin and metalloprotease domain 4 | 1.93 | | 1394612_at |
| | Similar to ubiquitin protein ligase E3 component n-recognin 2 | 1.91 | | 1391304_at |
| | Cullin 5 (Cul5) | 1.86 | | 1369068_at |
| | Carboxypeptidase A1 (Cpa1) | 1.75 | | 1369657_at |
| | Ubiquitin-conjugating enzyme E2T (putative, Ube2t) | 1.62 | | 1390481_a_at |
| | Archaelysin family metallopeptidase 2 (Amz2) | 0.66 | | 1388941_at |
| Growth Factors, Cyto+Chemokines | Prolactin receptor (Prlr) | 1.99 | | 1370384_a_at |
| | Insulin-like 3 (Insl3) | 1.93 | | 1388241_at |
| | Spondin 1, (f-spondin) extracellular matrix protein (Spon1) | 1.79 | C | 1370312_at |
| | Chemokine (C-X-C motif) receptor 4 (Cxcr4) | 1.71 | | 1370097_a_at |
| | Stanniocalcin 1 (Stc1) | 1.59 | | 1396101_at |
| Development | Synaptic vesicle glycoprotein 2b (Sv2b) | 2.36 | | 1369628_at |
| | Deleted in liver cancer 1 (Dlc1) | 1.94 | | 1389894_at |
| | Neural precursor cell expressed, develop. down-regulated 9 (Nedd9) | 1.65 | | 1396053_at |
| | Rotatin (Rttn) | 1.64 | | 1382747_at |
| | Similar to spermatogenesis associated glutamate (E)-rich protein 4f | 0.26 | | 1397562_at |
| Translation & Protein Modification | tRNA methyltransferase 6 homolog (S. Cerevisiae, Trmt6) | 1.93 | | 1377625_at |
| | ESF1, nucleolar pre-rRNA processing protein(S. cerevisiae, Esf1) | 1.90 | | 1394784_at |
| | Eukaryotic translation initiation factor 4 gamma, 1 (Eif4g1) | 1.83 | | 1395702_at |
| | Serine/arginine repetitive matrix 2 (Srrm2) | 1.72 | | 1390048_at |
| | Splicing factor, arginine/serine-rich 18 (Sfrs18) | 1.64 | | 1373534_at |
| Immune Response | HLA-B associated transcript 2 (Bat2) | 2.19 | | 1388536_at |
| | Coagulation factor VIII (F8) | 1.71 | A | 1380190_at |
| | Interferon (alpha and beta) receptor 1 (Ifnar1) | 1.62 | | 1398055_at |
| | RT1 class Ib, locus Aw2 (RT-1Aw2) | 0.44 | A | 1369110_x_at |
| Cell Cycle | Retinoblastoma binding protein 6 (Rbbp6) | 1.70 | A | 1378544_at |
| | Anaphase promoting complex subunit 4 (Anapc4) | 1.67 | | 1381153_at |
| | Centromere protein T (Cenpt) | 1.58 | E14 | 1375937_a_at |
| | Spindlin family, member 2 (Spin2) | 0.64 | | 1384023_at |
| Epigenetics | Chromodomain helicase DNA binding protein 6 (Chd6) | 2.07 | | 1398225_at |
| | Cytotoxic granule-associated RNA binding protein 1 (Tia1) | 1.74 | | 1397692_at |
| | Protein arginine N-methyltransferase 2 (Prmt2) | 0.66 | | 1376025_at |
| Electron Transport | Similar to MICAL CG33208-PB, isoform B | 2.16 | | 1379999_at |
| Miscel & Unknown | RGD1563912 | 3.17 | | 1375305_at |
| | Trinucleotide repeat containing 6a (Tnrc6a) | 3.14 | | 1375664_at |
| | Similar to KIAA1731 protein | 2.85 | | 1394697_at |
| | Similar to F33H2.2 | 2.73 | | 1381859_at |
| | Hypothetical LOC363737 /// similar to hypothetical protein FLJ20436 | 2.47 | | 1381108_at |
| | Kelch domain containing 3 (Klhdc3) | 2.36 | | 1392378_at |
| | Hypothetical LOC290577 | 2.29 | | 1378938_at |
| | SPECC1-like (Specc1l) | 1.88 | | 1380790_at |
| | C-Maf-inducing protein (Cmip) | 1.88 | A | 1382064_at |
| | Family with sequence similarity 103, member A1 (Fam103a1) | 1.81 | | 1376780_at |
| | R3H domain containing 2 (R3hdm2) | 1.79 | A | 1382066_at |
| | Proline rich 12 (Prr12) | 1.70 | | 1375023_at |
| | Midasin homolog (yeast, Mdn1) | 1.59 | | 1379417_at |
| | EP300 interacting inhibitor of differentiation 2 (Eid2) | 0.65 | | 1393058_at |
| | Similar to F28C1.3a | 0.59 | | 1372405_at |
| | Malignant fibrous histiocytoma amplified sequence 1 (Mfhas1) | 0.43 | E14 | 1398648_at |
| EST's | --- | 4.83 | A, V | 1380644_at |

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|---|---|---|---|
| --- | 4.10 | | 1381733_at |
| --- | 3.93 | V | 1391788_at |
| --- | 3.89 | F | 1378313_at |
| --- | 3.68 | E14, A | 1375751_at |
| --- | 3.44 | A | 1380758_at |
| --- | 3.31 | | 1395728_at |
| --- | 3.30 | E13 | 1396596_at |
| --- | 3.14 | A | 1381036_at |
| --- | 3.01 | | 1392730_at |
| --- | 2.99 | | 1390644_at |
| --- | 2.97 | A | 1393548_at |
| --- | 2.94 | | 1377329_at |
| --- | 2.92 | | 1397138_at |
| --- | 2.91 | F | 1381425_at |
| --- | 2.87 | | 1375513_at |
| --- | 2.86 | | 1392192_at |
| --- | 2.84 | | 1390840_at |
| --- | 2.84 | | 1375233_at |
| --- | 2.76 | A | 1380332_at |
| --- | 2.76 | A | 1397157_at |
| --- | 2.74 | A | 1391060_at |
| --- | 2.74 | | 1393662_at |
| --- | 2.73 | A | 1392704_at |
| --- | 2.71 | | 1381635_at |
| --- | 2.67 | | 1385399_at |
| --- | 2.66 | | 1395319_at |
| --- | 2.65 | | 1382998_at |
| --- | 2.62 | | 1396663_at |
| --- | 2.60 | | 1384058_at |
| --- | 2.60 | | 1396539_at |
| --- | 2.60 | A | 1376531_at |
| --- | 2.60 | A | 1397624_at |
| --- | 2.59 | | 1378814_at |
| --- | 2.57 | | 1380145_at |
| --- | 2.56 | | 1384587_at |
| --- | 2.56 | A | 1382966_at |
| --- | 2.53 | | 1381140_at |
| --- | 2.53 | | 1381731_at |
| --- | 2.53 | | 1397316_at |
| --- | 2.52 | | 1377973_at |
| --- | 2.45 | | 1397240_at |
| --- | 2.44 | | 1396102_at |
| --- | 2.43 | | 1392056_at |
| --- | 2.43 | | 1397101_at |
| --- | 2.42 | | 1395101_at |
| --- | 2.42 | E14, A | 1375680_at |
| --- | 2.41 | | 1398736_at |
| --- | 2.39 | | 1394569_at |
| --- | 2.37 | | 1380940_at |
| --- | 2.36 | | 1380097_at |
| --- | 2.34 | A | 1381207_at |
| --- | 2.33 | | 1395345_at |
| --- | 2.30 | | 1392389_at |
| --- | 2.30 | | 1379106_at |
| --- | 2.29 | | 1381520_at |
| --- | 2.29 | | 1380197_at |
| --- | 2.29 | | 1378223_at |
| --- | 2.29 | | 1397028_at |
| --- | 2.28 | | 1379259_at |
| --- | 2.27 | | 1378167_at |
| --- | 2.27 | | 1392024_at |
| --- | 2.27 | | 1397489_at |
| --- | 2.26 | | 1392368_at |
| --- | 2.26 | | 1397348_at |
| --- | 2.23 | | 1395048_at |
| --- | 2.23 | | 1381319_at |
| --- | 2.22 | | 1385350_at |
| --- | 2.22 | | 1397481_at |
| --- | 2.21 | | 1380111_at |
| --- | 2.21 | | 1376938_at |

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|---|---|---|---|
| --- | 2.20 | | 1392182_at |
| --- | 2.20 | | 1390694_at |
| --- | 2.20 | | 1380858_at |
| Similar to RIKEN cDNA 4833418A01 | 2.19 | | 1385204_at |
| --- | 2.18 | | 1397944_at |
| --- | 2.18 | | 1375404_at |
| --- | 2.18 | | 1386224_at |
| --- | 2.17 | | 1394574_at |
| --- | 2.16 | E14 | 1398394_at |
| Similar to RIKEN cDNA 3110040N11 | 2.16 | | 1392745_at |
| --- | 2.16 | | 1396373_at |
| --- | 2.15 | | 1378614_at |
| Similar to RIKEN cDNA 2610200G18 | 2.14 | | 1393191_at |
| --- | 2.13 | | 1378510_at |
| --- | 2.13 | | 1397749_at |
| --- | 2.12 | | 1385380_at |
| --- | 2.11 | A | 1380027_at |
| --- | 2.10 | A | 1394779_at |
| --- | 2.10 | A | 1392219_at |
| --- | 2.10 | | 1378682_at |
| --- | 2.09 | A | 1378062_at |
| --- | 2.09 | | AFFX_ratb1/X 12957_at |
| --- | 2.06 | | 1381483_at |
| --- | 2.06 | | 1386039_x_at |
| --- | 2.06 | A | 1382565_at |
| --- | 2.06 | A | 1390743_at |
| --- | 2.05 | | 1375814_at |
| --- | 2.05 | | 1391959_at |
| --- | 2.04 | | 1378038_at |
| --- | 2.04 | | 1390759_at |
| --- | 2.01 | | 1397120_at |
| --- | 2.00 | | 1395953_at |
| --- | 2.00 | | 1385385_at |
| --- | 2.00 | A | 1379575_at |
| --- | 1.99 | | 1381253_at |
| --- | 1.99 | A | 1383332_at |
| --- | 1.98 | | 1397177_at |
| --- | 1.98 | | 1397030_at |
| --- | 1.96 | | 1374022_at |
| --- | 1.96 | | 1380195_at |
| --- | 1.96 | | 1394698_at |
| --- | 1.94 | | 1381529_at |
| --- | 1.94 | A | 1378654_at |
| --- | 1.94 | A | 1373343_at |
| --- | 1.93 | | 1393512_at |
| --- | 1.92 | | 1392146_at |
| --- | 1.92 | | 1380745_at |
| --- | 1.91 | | 1395703_at |
| --- | 1.91 | | 1379164_at |
| --- | 1.91 | E13, A | 1375601_at |
| --- | 1.90 | | 1397112_at |
| --- | 1.88 | | 1380942_at |
| --- | 1.87 | | 1392186_at |
| --- | 1.87 | | 1395241_at |
| --- | 1.87 | E13 | 1379866_at |
| --- | 1.86 | | 1378671_at |
| --- | 1.86 | | 1377207_at |
| --- | 1.84 | A | 1391006_at |
| --- | 1.83 | E14 | 1381063_at |
| --- | 1.82 | | 1375217_at |
| --- | 1.82 | | 1393600_at |
| --- | 1.82 | | 1385164_at |
| --- | 1.81 | | 1375374_at |
| --- | 1.81 | | 1381420_at |
| --- | 1.80 | | 1391863_at |
| --- | 1.79 | | 1377175_at |
| --- | 1.79 | A | 1375388_at |
| --- | 1.79 | | 1393582_at |
| --- | 1.79 | | 1372983_at |

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|---|---|---|---|
| --- | 1.78 | A | 1389953_at |
| --- | 1.78 | | 1390202_at |
| --- | 1.76 | | 1377706_x_at |
| --- | 1.76 | | 1380361_at |
| --- | 1.76 | A | 1379098_at |
| --- | 1.76 | | 1373908_at |
| --- | 1.74 | | 1380155_at |
| --- | 1.73 | | 1396759_at |
| --- | 1.72 | | 1379555_at |
| --- | 1.72 | | 1391710_at |
| --- | 1.72 | | 1379089_at |
| --- | 1.70 | A | 1386540_at |
| --- | 1.70 | | 1395092_at |
| --- | 1.70 | | 1396190_x_at |
| --- | 1.69 | | 1386565_at |
| --- | 1.66 | | 1392774_at |
| --- | 1.66 | | 1376969_at |
| --- | 1.66 | | 1389046_at |
| --- | 1.65 | | 1380821_a_at |
| --- | 1.64 | | 1390093_at |
| --- | 1.63 | | 1393156_at |
| --- | 1.62 | | 1389565_at |
| Similar to RIKEN cDNA 6330416G13 gene | 1.61 | E14 | 1391817_at |
| --- | 1.60 | A | 1390351_at |
| --- | 1.59 | A | 1382952_at |
| --- | 1.59 | | 1396264_at |
| --- | 1.59 | | 1381035_at |
| --- | 1.56 | A | 1392140_at |
| --- | 1.55 | E14 | 1376226_at |
| --- | 1.54 | | 1377161_at |
| --- | 0.66 | | 1379356_at |
| --- | 0.62 | | 1378154_at |
| --- | 0.61 | | 1393506_at |
| --- | 0.61 | | 1388451_at |
| --- | 0.60 | | 1394829_at |
| --- | 0.59 | | 1382255_at |
| --- | 0.58 | E14, V | 1371776_at |
| --- | 0.58 | E14, A | 1391256_at |
| --- | 0.40 | E13 | 1391886_at |

*For any gene identified in multiple lists, the other lists in which it appears are indicated in the Cross Listed column. E13= altered by vinclozolin at E13, E14= altered by vinclozolin at E14, V= altered by vinclozolin in organ cultures, F= altered by flutamide in organ cultures, A= appeared in a list of F1 E16 vinclozolin altered transcripts published by Anway et al 2008, C=appeared in a list of testis differentiation candidates published by Clement et al 2007

Table 3-S4. Vinclozolin altered E13 cultured testis transcripts

| Category | Gene | V/C | Cross Listed* | Systematic |
|---|---|---|---|---|
| Signaling | Heat shock protein 4 (Hspa4) | 0.36 | | 1370344_at |
| | Similar to RCK | 0.44 | A | 1389868_at |
| | Bone marrow stromal cell antigen 2 (Bst2) | 0.55 | A | 1390738_at |
| Transcription | TCDD-inducible poly(ADP-ribose) polymerase (Tiparp) | 0.37 | | 1385407_at |
| Translation | | | | |
| Prot.Modification | Chaperonin subunit 6a (zeta) (Cct6a) | 0.38 | A | 1377006_at |
| Metabolism | Reticulon 4 (Rtn4) | 0.44 | | 1388027_a_at |
| Miscel & Unknown | Family with sequence similarity 134, member C (Fam134c) | 0.45 | E16, A | 1376087_at |
| Development | Anterior pharynx defective 1a homolog (C. elegans, Aph1a) | 0.46 | | 1388885_at |
| Receptor & Binding Proteins | Fc receptor-like A (Fcrla) | 2.09 | | 1392886_a_at |
| EST's | --- | 0.40 | | 1393782_at |
| | --- | 0.41 | E16, A | 1380644_at |
| | --- | 0.43 | A | 1379511_at |
| | --- | 0.43 | | 1376438_at |
| | --- | 0.44 | | 1393705_at |
| | --- | 0.46 | E14 | 1371776_at |
| | --- | 0.46 | A | 1394283_at |
| | --- | 0.46 | | 1391788_at |
| | --- | 0.47 | | 1377321_at |
| | --- | 0.52 | | 1392702_at |

*For any gene identified in multiple lists, the other lists in which it appears are indicated in the Cross Listed column. E13= altered by vinclozolin at E13, E14= altered by vinclozolin at E14, E16= altered by vinclozolin at E16, A= appeared in a list of F1 E16 vinclozolin altered transcripts published by Anway et al 2008.

Table 3-S5. Flutamide altered E13 cultured testis transcripts

| Category | Gene | V/C | Cross Listed* | Systematic |
|---|---|---|---|---|
| Transcription | TSC22 domain family, member 4 (Tsc22d4) | 1.59 | | 1375958_at |
| | Nuclear receptor co-repressor 1 (Ncor1) | 1.56 | E16 | 1384293_at |
| | Wiskott-Aldrich syndrome-like (human, Wasl) | 1.54 | | 1391625_at |
| | SWI/SNF matrix asso. actin dep. reg. of chromatin (Smarca4) | 1.51 | | 1375469_at |
| | DnaJ (Hsp40) homolog, subfamily A, member 3 (Dnaja3) | 1.48 | | 1378392_at |
| Translation Prot.Modification | Similar to 60S ribosomal protein L3 (L4) | 2.03 | | 1396049_x_at |
| | Pumilio 1 (Drosophila, Pum1) | 1.53 | | 1375396_at |
| | Pinin (Pnn) | 1.49 | | 1396463_at |
| Metabolism & Transport | Beta globin minor gene | 1.69 | | 1371102_x_at |
| | Glutaminyl-peptide cyclotransferase-like (Qpctl) | 0.57 | | 1395623_at |
| | Similar to vesicle transport w/interaction w/ t-SNAREs 1B homolog | 1.90 | | 1393304_at |
| Signaling | Similar to Myeloid/lymphoid or mixed-lineage leukemia protein 3 Homolog Histone-lysine N-methyltransferase | 1.62 | | 1382658_at |
| | Heat shock protein 90, alpha, class A member 1 (Hsp90aa1) | 1.56 | | 1388850_at |
| Cytosceleton-ECM | Tubulin, gamma complex associated protein 3 (Tubgcp3) | 1.82 | | 1372660_at |
| | Tubulin, gamma complex associated protein 6 (Tubgcp6) | 0.68 | | 1374887_at |
| Epigenetics | Methyltransferase 11 domain containing 1 (Mett11d1) | 1.51 | | 1376497_a_at |
| Growth Factors | | | | |
| Cyto+Chemokines | Interleukin 6 signal transducer (Il6st) | 1.63 | | 1370957_at |
| Apoptosis | Apoptotic chromatin condensation inducer 1 (Acin1) | 0.62 | | 1396098_at |
| Proteolysis | Zinc finger protein 451 (Zfp451) | 1.52 | | 1394654_at |
| Receptors & Binding Proteins | Platelet derived growth factor receptor, beta polypeptide (Pdgfrb) | 1.56 | E16 | 1379211_at |
| Misc & Unknown | Late cornified envelope 1C (Lce1c) | 1.64 | | 1374986_x_at |
| | Sterile alpha motif domain containing 4B (Samd4b) | 1.63 | | 1386854_at |
| | Similar to hypothetical protein FLJ31528 | 1.55 | | 1399087_at |
| | GRB10 interacting GYF protein 1 (Gigyf1) | 1.53 | | 1381469_a_at |
| | Similar to Protein C7orf26 homolog | 0.65 | | 1389771_at |
| | Bromodomain containing 4 (Brd4) | 1.66 | | 1375650_at |
| EST's | --- | 2.37 | | 1375707_at |
| | --- | 1.84 | | 1397257_at |
| | --- | 1.68 | | 1395381_at |
| | --- | 1.67 | | 1384802_at |
| | --- | 1.64 | E16 | 1381425_at |
| | --- | 1.61 | | 1389488_at |
| | --- | 1.61 | E16 | 1378313_at |
| | --- | 1.60 | | 1380397_at |
| | --- | 1.59 | | 1394951_at |
| | --- | 1.59 | | 1378249_x_at |
| | --- | 1.57 | | 1391464_at |
| | --- | 1.57 | | 1389841_at |
| | --- | 1.56 | | 1376696_at |
| | --- | 1.56 | | 1389918_at |
| | --- | 1.55 | | 1393739_at |
| | --- | 1.53 | | 1375079_at |
| | --- | 0.68 | | 1391098_x_at |

*For any gene identified in multiple lists, the other lists in which it appears are indicated in the Cross Listed column. E13= altered by vinclozolin at E13, E14= altered by vinclozolin at E14, E16= altered by vinclozolin at E16.