Environmentally induced epigenetic transgenerational inheritance of altered SRY genomic binding during gonadal sex determination

Michael K. Skinner,* Ramji K. Bhandari, M. Muksitul Haque and Eric E. Nilsson

Center for Reproductive Biology, School of Biological Sciences, Washington State University, Pullman, WA, 99164-4236, USA

*Correspondence address. Center for Reproductive Biology, School of Biological Sciences, Washington State University, Pullman, WA 99164-4236, USA. Tel: 509-335-1524; Fax: 509-335-2176; E-mail: skinner@wsu.edu

1Present address: Division of Biological Sciences, University of Missouri, Columbia, MO 65211, USA.

Abstract

A critical transcription factor required for mammalian male sex determination is sex determining region on the Y chromosome (SRY). The expression of SRY in precursor Sertoli cells is one of the initial events in testis development. This study was designed to determine the impact of environmentally induced epigenetic transgenerational inheritance on SRY binding during gonadal sex determination in the male. The agricultural fungicide vinclozolin and vehicle control (dimethyl sulfoxide)-exposed gestating females (F0 generation) during gonadal sex determination promoted the transgenerational inheritance of differential DNA methylation in sperm of the F3 generation (great grand-offspring). The fetal gonads in F3 generation males were used to identify potential alterations in SRY binding sites in the developing Sertoli cells. Chromatin immunoprecipitation with an SRY antibody followed by genome-wide promoter tiling array (ChIP-Chip) was used to identify alterations in SRY binding. A total of 81 adjacent oligonucleotide sites and 173 single oligo SRY binding sites were identified to be altered transgenerationally in the Sertoli cell vinclozolin lineage F3 generation males. Observations demonstrate the majority of the previously identified normal SRY binding sites were not altered and the altered SRY binding sites were novel and new additional sites. The chromosomal locations, gene associations and potentially modified cellular pathways were investigated. In summary, environmentally induced epigenetic transgenerational inheritance of germline epimutations appears to alter the cellular differentiation and development of the precursor Sertoli cell SRY binding during gonadal sex determination that may influence the developmental origins of adult onset testis disease observed.

Key words: transgenerational; inheritance; vinclozolin; testis; infertility; developmental origins

Introduction

A number of environmental factors from toxicants to nutrition have been shown to promote the epigenetic transgenerational inheritance of disease and phenotypic variation in a growing number of different species, from plants to humans [1]. One of the first environmental toxicants found to promote the phenomenon was the agricultural fungicide vinclozolin and one of the main transgenerational diseases observed was associated...
with the testis and male infertility [2, 3]. Following the exposure of an F0 generation gestating female, the F3 and F4 generation males had spermatogenic cell apoptosis, reduced sperm number and motility, and increased male infertility [2–4]. Recently, the F3 generation vinclozolin lineage Sertoli cells were found to have an altered transgenerational transcriptome that correlated to the spermatogenic cell defect and testis disease [5].

The critical window for environmental exposure of the F0 generation gestating female was at the end of primordial germ cell (PGC) migration and colonization of the gonad through the onset of gonadal sex determination (embryonic days 8–14 in the rat) [1, 2, 6]. During this period of development, the PGCs have an erasure of the majority of DNA methylation that then at the onset of sex determination is initiated to re-methylate in a male- or female-specific manner to eventually generate the sperm or egg cells [7]. Environmental factors during this period appear to alter this epigenetic programming to promote imprinted-like differential DNA methylation regions (DMR) in the germ cells, termed epimutations [1], that transmit the transgenerational inheritance of germ cell epimutations and disease to subsequent generations [5, 8].

The process of mammalian sex determination was originally outlined by Jost [9–11] with the events of fetal gonadal sex determination followed by phenotypic sex determination. The testis determining factor proposed by Jost [9, 12] was subsequently identified and termed the sex determining region on the Y chromosome (SRY) [13–15]. SRY is a high mobility group (HMG) box transcription factor that binds DNA and can bend DNA [16]. SRY is highly conserved in mammals [14] and other organisms [17]. SRY is generally transiently expressed in Sertoli cells during the initiation of male gonadal sex determination. Subsequent to SRY expression, SOX9 is expressed, another HMG box factor, that also promotes a cascade of transcriptional events required for Sertoli cell differentiation and testis development [18, 19].

The cascade of transcriptional events involved in male gonadal sex determination has previously been studied and reviewed [20–22]. Sry is the initial event to promote this cascade of transcriptional events during precursor Sertoli cell differentiation and male gonadal sex determination.

Investigation of the downstream individual gene targets of SRY has identified Sox9 [18, 19], Tcf21 [23], Nt3 [24], and Chlb4 [25]. Investigation of the downstream targets of Sox9 initially identified individual genes such as anti-Müllerian hormone [26, 27], fibroblast growth factor 9 [28–30], and others [22]. SOX9 has the capacity to replace SRY binding at many of these sites later in Sertoli cell differentiation [18, 31]. Following the actions of SRY on target transcription factors like SOX9 and TCF21, subsequent cascades of transcriptional events and targets are regulated [23, 32, 33]. Genome-wide analysis of SRY targets in the rat identified 71 binding sites using an SRY chromatin immunoprecipitation followed by a promoter tiling array (SRY ChIP-Chip) [32]. These analyses demonstrate the more global actions of SRY at the onset of Sertoli cell differentiation.

In considering the molecular mechanisms involved in the environmentally induced epigenetic transgenerational inheritance of testis disease, the impacts on Sertoli cell differentiation were investigated. Vinclozolin-induced transgenerational adult onset spermatogenic cell apoptosis and testis abnormalities were found to involve alterations in the adult Sertoli cell transcriptome and epigenome [5]. The F3 generation vinclozolin lineage Sertoli cells had alterations in over 100 differential DMRs and over 400 genes had altered gene expression. Many of the previously identified genes involved in male infertility and testis disease were present in this altered transgenerational transcriptome [5]. Interestingly, the adult Sertoli cell altered gene set had eight genes associated with pyruvate production [5], which is the energy source/metabolite produced for use by the developing spermatogenic cells sequestered within the blood testis barrier [34, 35]. A decrease in pyruvate production would correlate to an increase in spermatogenic cell apoptosis observed [35–38]. Therefore, the epigenetic transgenerational inheritance mechanism appears to involve the germine (sperm) transmission of altered DMR-termed epimutations, such that the embryonic stem cell obtained after fertilization would have an altered epigenome, which would generate an altered epigenome and transcriptome in all cells derived from these stem cells [1, 5]. Later in development, the developing somatic cells will have susceptibility to develop disease due to the altered epigenomes and transcriptomes. This was shown for the Sertoli cells associated with testis disease and the granulosa cells associated with ovary disease in the F3 generation vinclozolin lineage animals compared with the control lineage animals [5, 8].

This study was designed to investigate the potential transgenerational alterations in SRY targets at the onset of Sertoli cell differentiation associated with the initiation of gonadal sex determination. The altered transgenerational Sertoli cell differentiation observed in the adult [2, 3, 5] was speculated to in part be due to induced alterations at the initial stages of Sertoli cell fate determination and differentiation. Previously, we demonstrated an altered testis [39] or PGC [40] transcriptome in vinclozolin F3 generation males, but this study is focused on Sertoli cells. Since SRY is only expressed in Sertoli cells in the fetal testis, potential alterations in SRY binding sites would suggest an altered transcriptional regulation at the onset of Sertoli cell differentiation.

Results

The experimental design involved the intraperitoneal exposure of gestating female rats to vinclozolin or a vehicle control (dimethyl sulfoxide) transiently from embryonic days E8 to E14 [2, 5]. Sister littersmates were divided into control and vinclozolin treatment groups and mated to similar males to minimize the genetic variation between the control and vinclozolin lineages. Sufficient females were used so no inbreeding (sibling or cousin) occurred in any generation. The F1 generation was bred within the lineage to generate the F2 generation, and these F2 generation bred to generate the F3 generation as previously described [1, 2, 5]. The only exposure was the F0 generation female. The F3 generation control and vinclozolin lineage embryonic day 13 (E13) embryos were collected and the gonads micro-dissected and then sexed with an SRY polymerase chain reaction (PCR) protocol previously described [41]. The male gonads were pooled from a minimum of three different litters and the pools used to collect DNA. Three different experiments were performed to collect three control and vinclozolin E13 F3 generation testis pools, each with different animals (n = 25 gonads/pool). The chromatin DNA (not denatured) from each pool was fragmented and used in an SRY chromatin immunoprecipitation (ChIP) procedure for each pool separately as previously described [32]. The control and vinclozolin SRY ChIP DNA were paired for a competitive hybridization on a genome-wide promoter tiling array (ChIP-Chip) assay. The hybridization data obtained were used to identify the SRY binding sites that were different in the F3 generation vinclozolin versus control lineage in E13 testis as previously described [32].

The number of single oligonucleotide tiling array probe differential SRY binding sites detected in the vinclozolin F3 generation lineage E13 testis was 173, and the number of ≥ 3 adjacent oligo tiling array probe sites was 81, Fig. 1. A minimum
statistical significance of each SRY binding site identification that was different between the control and vinclozolin lineage F3 generation E13 testis was \( P < 10^{-4} \), see Methods. The \( \geq 3 \) adjacent probe sites were selected for further investigation as this is a more stringent selection that avoids the potential false positives and negatives in the single probe sites identified. The complete list of the SRY binding sites is presented in Supplementary Table S1 for \( \geq 3 \) sites and Supplementary Table S2 for single probe sites. A comparison of the vinclozolin F3 generation E13 testis SRY binding sites with control animal SRY binding sites using the comparative hybridization protocol demonstrated that all the previously identified SRY binding sites [32] were the same in the control with no alterations in the vinclozolin lineage, Fig. 1B. Therefore, the previous sites in the control E13 testis identified [32] were essentially the same in the vinclozolin F3 generation E13 testis, as they had similar competitive hybridization. SRY binding sites identified in this study are altered in the vinclozolin lineage versus the control lineage F3 generation E13 testis precursor Sertoli cells. Therefore, the novel SRY binding sites identified are new additional sites in comparison to the control lineage.

Examples of novel SRY binding site profiles are presented in Fig. 2 for three representative sites. The list of all the 81 \( \geq 3 \) adjacent probe sites is presented in Supplementary Table S1. The adjacent oligo probe hybridization sites are presented with a \( P < 10^{-4} \) statistical difference for each individual probe in the adjacent region. The negative or reduced hybridization previously was identified as non-specific IgG binding sites that can interfere with the detection of positive SRY binding sites [32]. All the SRY binding sites are new positive binding at the regions identified. The chromosome locations of the altered SRY binding sites (\( \geq 3 \) adjacent probes) are presented in Fig. 3. SRY binding sites are present on most autosomes and the X-chromosome. The analysis used a competitive hybridization between the control and vinclozolin F3 generation lineages, and the 71 previously identified SRY binding sites in control animals (\( \geq 3 \) adjacent probe) [32] were not found to be altered in this analysis. The absence of these sites in this study indicates they were not altered in the vinclozolin lineage, Fig. 1B.

The genes associated with the 81 differential SRY binding sites are listed in Table 1 with the gene categories and function indicated. The complete lists of the adjacent and single oligo probe SRY binding sites are presented in Supplementary Tables S1 and S2. The primary gene categories associated with the SRY binding sites are presented in Fig. 4. The main gene categories/functions associated were similar to the gene categories previously shown to be associated with control animal SRY binding sites [32]. A pathway analysis of the SRY binding site-associated genes identified the Immune Influenza A pathway with \( \geq 3 \) genes associated with the list presented in Table 2. The single oligo SRY-altered binding site pathways involved are presented in Table 3. The vinclozolin induced epigenetic transgenerational inheritance of testis disease appears to have involved an alteration in SRY binding sites during the initial differentiation of Sertoli cells and onset of testis determination.

**Discussion**

Environmentally induced epigenetic transgenerational inheritance of disease and phenotypic variation involves the germline transmission of epigenetic information, in the absence of direct exposure [1, 42–55]. Since the mature germ cell (sperm or egg) transmits the altered epigenome to the embryonic stem cells, all cells derived from the embryonic stem cells will have an altered epigenome and transcriptome [1, 5, 8]. Each cell type will have a unique cascade of epigenetic and genetic events that leads to cell specific differentiation involving a unique epigenome and transcriptome [56]. Therefore, epigenetic transgenerational inheritance impacts all somatic cells which will alter the organism’s phenotype and in those tissues sensitive to the alteration potential disease susceptibility [1]. Because of the dependence of this phenomenon on the germline cell, the hypothesis was tested that a cell at the onset of its differentiation and cell fate determination will have a shift in its normal transcriptional events leading to the cell’s differentiation.

The Sertoli cell is one of the key somatic cells in the testis that supports the development and function of the developing germ cells undergoing spermatogenesis to develop into sperm in the epididymis [57–59]. In the adult, Sertoli cells from the seminiferous tubule provide the complex structural support for the developing spermatogenic cells from the spermatogonia stem cell state through the release of spermatozoa. During this process, the Sertoli cells provide the microenvironment and nutrient support required [58, 59], such that abnormalities in Sertoli cell function can lead to testis abnormalities and male infertility [60, 61]. In addition to the role of Sertoli cells in the adult male, Sertoli cells are also essential for the initial stages of germ cell development and gonadal sex determination [62–64]. When the PGCs migrate down the genital ridge and initially colonize the indifferent gonad, the PGC and somatic cells are in an undifferentiated state prior to the onset of gonadal sex determination. The somatic cells associated with the PGC in the gonad at the onset of sex determination initiate their cell fate determination to promote the PGC development into a male or female germline lineage associated with the initial testis or ovary development, termed gonadal sex determination and initially discussed by Jost [9–11]. Therefore, as observed in the adult, the precursor Sertoli cell in the initiation of testis development and conversion of the PGC to a male germ line lineage is critical for germ cell development and differentiation.

The initial event in cell fate determination and cellular differentiation of Sertoli cells involves the testis determining factor [13–15] identified as SRY. The SRY is an HMG-box protein that binds and can bend DNA to regulate gene expression. One of the initial downstream targets of SRY is the Sox9 gene that can initiate a cascade of molecular events to promote the differentiation of Sertoli cells [18, 19]. The onset of Sertoli cell differentiation by SRY marks the induction of gonadal sex determination of the testis and male germ cell differentiation. A number of downstream targets for SRY have been identified, with Sox9 induction being thought to be one of the primary regulatory events [18–22]. Previous studies have shown SRY can target the genes encoding an array of proteins such as SOX9 [18, 19] and TCF21 [23] that in turn can promote a subsequent cascade of transcriptional events [33]. For example, using a ChIP-Chip analysis for the rat, SOX9 was found to target 109 genes [32] and TCF21 targeted 121 genes [33]. Therefore, if an environmental factor could alter SRY genome targets, the abnormal epigenetic and developmental cascade would be expected to influence Sertoli cell differentiation. This would then persist to the adult Sertoli cell state and potentially associate with testis abnormalities and disease [5].

This study used the agricultural fungicide vinclozolin to promote the epigenetic transgenerational inheritance of testis disease into the F3 generation following a transient exposure of a pregnant F0 generation female during gonadal sex determination [2]. Previously, the adult F3 generation males have been shown to have spermatogenic cell apoptosis and male infertility [2–4]. The
embryonic day 13 (E13) testis from control and vinclozolin lineage F3 generation male rats was used for this study. The E13 stage in the rat correlates with the early stage of Sertoli cell differentiation and testis development when SRY has induced the expression of SOX9 and other target genes [32, 40]. The chromatin obtained from the E13 testis was used in a ChIP-Chip analysis with the SRY antibody to identify target binding sites for SRY. This was analyzed on a genome-wide promoter tiling array with a competitive hybridization of the control versus vinclozolin F3 generation E13 testis DNA using two different fluorescent labels. Observations indicated 81 altered SRY targets using a high stringency selection of three adjacent oligo probes with each having a statistically significant change in hybridization. With a less stringent single oligo probe analysis, 173 SRY binding sites were detected. To reduce the issue of false positives and negatives, the 81 SRY targets found to be different between the control versus vinclozolin lineage samples were used for further analysis. The 71 SRY binding target genes previously identified in control normal E13 testis [32] were not different between the control versus vinclozolin lineage samples. Therefore, the majority of the normal Sertoli cell developmental pathways and SRY targets were not affected, but 81 new and novel SRY targets were identified. The environmentally induced epigenetic transgenerational inheritance of the altered cellular transcriptomes and epigenomes in Sertoli cells did not alter the normal developmental molecular events but added additional molecular targets. Epigenetics has the ability to directly alter transcription factor binding and gene expression.

The gene associations of these altered SRY binding targets demonstrated a variety of affected gene functional categories. Pathway analysis identified the influenza A pathway affected with greater than four genes being the most significant pathways influenced by the altered SRY targets. These potential effects on the early Sertoli cell differentiation may indirectly influence the developing germ cells. Therefore, the gene targets associated with the altered SRY binding may have a significant impact on gonadal sex determination and eventually the differentiated state of adult Sertoli cells and spermatogenic cells.

A previous study demonstrated the adult Sertoli cell in the vinclozolin lineage F3 generation male had an altered epigenome and transcriptome compared with the control lineage males [5]. A gene pathway that was affected involved pyruvate production that is required for the developing germ cells within the blood tests barrier [34–38]. The reduction in pyruvate production may be directly correlated with the dramatic increase in spermatogenic cell apoptosis observed in the adult testis of vinclozolin lineage males [2, 3]. This study suggests the alterations observed in the adult Sertoli cell of vinclozolin lineage originated in part at the initiation of Sertoli cell fate determination during gonadal sex determination. Therefore, the developmental origins of the adult onset testis disease were in part identified. Although the disease does not develop until later in life, the developmental origins occurred at the initial cell fate determination.

In regards to the initial hypothesis tested, observations support the role of the germline in transmitting an altered epigenome to subsequently affect the developing embryonic stem cell epigenome that then impacts all derived somatic cell epigenomes and transcriptomes. The impact on all cells and tissues has the ability to alter a large number of disease states. Indeed, the vinclozolin lineage transgenerational animals have a large number of disease conditions observed [1–3] and phenotypic variation in areas such as behavior [65, 66]. The current observations indicate a direct impact on sex determination, key transcriptional events of SRY and its function, and somatic cell function involving Sertoli cell—germ cell interactions. Since the environment induced these alterations at a critical stage of development, environmentally induced epigenetic transgenerational inheritance of diseases and phenotypic variation is anticipated to have a critical role in biology.

## Methods

### Tissue Preparation

Harlan Sprague-Dawley rats were used for the study. All the rats were kept in a temperature controlled environment. Estrous cycles of female rats were monitored by cellular morphology from vaginal smears. Rats in early estrus were paired
with males overnight and mating confirmed by sperm positive smears, denoting day 0 of pregnancy. The breeding of control and vinclozolin lineage F0, F1, and F2 generations were as previously described [2–6]. The F0 generation females were exposed to vinclozolin or dimethyl sulfoxide through i.p. injection daily from E8 to E14 [2–6]. The F2 generation pregnant rats were euthanized at gestational embryonic day 13 (E13) of pregnancy, and F3 generation embryonic gonads were collected for

Figure 2. Representative SRY binding site profiles on (A) Chromosome 1, (B) Chromosome 5, and (C) Chromosome 10. Each probe hybridization is presented (bar) with the positive peak indicating the SRY binding site. The location of the SRY binding site is indicated with the positive peak. The gene and transcriptional start site (TSS) is listed for each.
chromatin immunoprecipitation. A somite count of 12–18 tail was also used to help stage the fetus for this developmental period as previously discussed [32, 33]. Sex was determined by PCR using primers specific for Sry on genomic DNA isolated from embryo tails as previously described [41]. All procedures were approved by the Washington State University Animal Care and Use Committee (IACUC approval no. 02568-034).

**In Vivo ChIP Assay**

A modified ChIP (cChIP) assay was adopted from O’Neill et al. [67] and performed according to Bhandari et al. [23]. The conditions for the native-ChIP (not including cross linking) were optimized for immunoprecipitating with SRY antibodies. The native-ChIP was used to identify high affinity binding sites and reduce low affinity sites. To run a replicate of the ChIP assay, at least 25 male gonads from thirty 13 dpc (12–18 tail somite stage) rat embryos were used per array. All three ChIP experiments were with different biological samples. *Drosophila* SL2 cells (American Type Culture Collection (ATCC) Catalog no. CRL-1963) were used as a carrier. Densely grown cells (~5 × 10^7 cells) were pelleted and washed three times in ice-cold phosphate-buffered saline, 5 mM sodium butyrate, and resuspended in 0.5 ml NB buffer (15 mM Tris-HCl, pH 7.4, 60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 0.5 mM 2-mercaptoethanol, 0.1 mM phenylmethanesulfonylfluoride (PMSF)). Testis samples were mixed with SL2 cells and homogenized to make single cell suspension. Nuclei were pelleted, resuspended in 10 ml NB buffer, 5% (vol/vol) sucrose, pelleted, and resuspended again in 1.5 ml digestion buffer (50 mM Tris-HCl pH 7.4, 0.32 M sucrose, 4 mM MgCl₂, 1 mM CaCl₂, 0.1 mM PMSF). Following micrococcal nuclease digestion (NEB, USA) for 5 minutes at 28°C, the digested samples were gently spun (800 x g) for 15 minutes and supernatant set aside on ice. The pellet was resuspended in 250 μl digestion buffer and again centrifuged gently at 800 x g for 15 minutes at 4 degrees. Both the supernatants were pooled, and a fraction (50 μl) out of it was kept aside as input. The remaining supernatant was incubated with either non-immune IgG or anti-SRY (Santa Cruz, CA) antibody at 4°C overnight. The specificity of these antibodies on western blots have been previously described and validated [23].

After incubation with 100 μl of pre-swollen protein A-Sepharose beads (SL2 DNA blocked) for 2 h at 4°C, the bead-bound immunoprecipitates were centrifuged gently and washed five times with wash buffer (50 mM TrisHCl pH 7.5, 10 mM EDTA, 5 mM Na butyrate, and 50–150 mM NaCl). The protein–DNA complexes were incubated at room temperature with elution buffer (1% SDS in TE) and centrifuged at 11500 x g for 10 minutes. Elution was repeated two times, and eluted DNA was pooled. Co-immunoprecipitated DNA was purified by phenol/chloroform extraction and ethanol precipitation. Final
concentration of immunoprecipitated DNA varied from 200 to 500 ng per assay. Three different experiments and ChIP assays were performed. Exactly 30 ng of immunoprecipitated DNA from each assay was amplified by whole-genome amplification kit developed by Sigma (Sigma no. WGA2 50 RXN). At least five separate whole-genome amplifications were performed, and DNA was pooled. Pooled whole-genome amplified DNA was purified by using Promega’s Wizard SV40 PCR cleanup kit

Table 1. SRY binding associated genes and gene function

| Gene name | NCBI_gene ID | Gene function | Chr | Cluster ID | Probes (no.) | Length bp |
|-----------|--------------|---------------|-----|------------|--------------|-----------|
| Il18bp    | 84388        | Signaling     | chr1| chr1:159475918–159478113 | 3 | 2196 |
| Tigd3     | 309174       | Development   | chr1| chr1:208529885–208532090 | 3 | 2206 |
| Rab31l1   | 171452       | Signaling     | chr1| chr1:212473968–212476572 | 6 | 2605 |
| Hps2      | 368128       | Metabolism    | chr1| chr1:247461058–247463393 | 4 | 2336 |
| Vom1r19   | 494298       | Receptor      | chr1| chr1:58783093–58785503  | 3 | 2211 |
| Fgfl6     | 308394       | Development   | chr1| chr1:77466423–77469105  | 8 | 2683 |
| Lce3l     | 686125       | Development   | chr2| chr2:185871514–185879666 | 3 | 2183 |
| Slc25a24  | 310791       | Transport     | chr2| chr2:204716693–204718991 | 3 | 2299 |
| RGD1309170| 362047       | Unknown       | chr2| chr2:235717341–235719802 | 5 | 2462 |
| Tor1b     | 311854       | Metabolism    | chr3| chr3:10018125–10020621  | 3 | 2497 |
| RGD1561852| 499893       | Unknown       | chr3| chr3:118912719–118914915 | 3 | 2197 |
| Slc43a1   | 311168       | Transport     | chr3| chr3:68044469–68046640   | 3 | 2172 |
| Olr694    | 295896       | Receptor      | chr3| chr3:73637127–73639417   | 4 | 2291 |
| Cat       | 24248        | Metabolism    | chr3| chr3:88649222–88651529   | 4 | 2308 |
| Olr766    | 405356       | Receptor      | chr3| chr3:97122942–97125269   | 3 | 2328 |
| Ef3e6ip   | 300069       | Translation   | chr7| chr7:117061657–117064644 | 10| 2988 |
| Uqcr1     | 301011       | Metabolism    | chr8| chr8:113951092–113953366 | 3 | 2275 |
| Actg1     | 287876       | Cytoskeleton  | chr8| chr10:109774391–109776911| 5 | 2521 |
| Epn2      | 60443        | Transport     | chr8| chr10:47741753–47743932  | 3 | 2180 |
| Ppp1r9b   | 84686        | Cytoskeleton  | chr10| chr10:83669966–83672713 | 5 | 2748 |
| Uchl1     | 29545        | Metabolism    | chr14| chr14:44123206–44125426 | 3 | 2221 |
| Dnaic3    | 63880        | Metabolism    | chr15| chr15:103881239–103883433| 3 | 2195 |
| Pzk       | 306203       | Signaling     | chr15| chr15:18817572–18819962 | 5 | 2391 |
| Pdch17    | 306055       | Cell adhesion | chr15| chr15:86380753–66389494 | 5 | 2442 |
| Chrna6    | 81721        | Receptor      | chr16| chr16:69007355–69009564 | 3 | 2210 |
| Pkd212    | 291689       | Transport     | chr18| chr18:26871615–26873827 | 3 | 2213 |
| Pparc1b   | 291567       | Transcription | chr18| chr18:53782172–53784447 | 3 | 2276 |
| Gd10      | 361434       | Cell cycle    | chr19| chr19:53540561–53542901 | 4 | 2341 |
| RT1-DMb   | 294273       | Immunity      | chr20| chr20:4838459–4841177  | 6 | 2719 |
| Zfp275    | 293849       | Transcription | chrX| chrX:159237289–159239865| 7 | 2577 |

Figure 4. Gene categories and function for SRY binding sites. The number of genes associated with various gene categories is presented.

Number of genes with listed function

Gene Category / Function

Environmentally induced epigenetic transgenerational inheritance
Within each array, probes were separated into groups by GC content, and each group was separately normalized using the LOESS normalization procedure [69]. This allowed for groups with optimal GC content, which exhibited a reduced quality issue, to receive a normalization curve specific to that group. After each array was normalized within array, the arrays were then normalized across arrays using the A-quantile normalization procedure [70]. Following normalization, the probe’s normalized M values (and then A) were replaced with the median value of all probe M values (and then A) within a sliding window of 1000 bp [71–73], due to the size of DNA fragments used. Following normalization, each probe’s M value represents the median intensity difference between Cy5 and Cy3 of a 1000 bp window. Significance was assigned to probe differences between experimental (SRY) and IgG control by calculating the median value of the intensity differences when compared with a normal distribution scaled to the experimental mean and standard deviation of M. Regions of interest were then determined by combining consecutive probes with significance P values < 10^{-3}. Significance was assigned to probe differences between experimental and control by calculating the median value of the increasing differences when compared with a normal distribution scaled to the experimental mean and standard deviation of the mean. A Z score and P value were computed from that distribution with the use of R code analysis. The statistical package was similar to the edgeR bioconductor package and used empirical Bayes methods [74]. The statistically significant peaks of hybridization were identified and P value associated with each peak presented. Each peak of interest was then annotated for the gene. Every promoter exceeding the intensity threshold was considered positive for SRY binding. The final list of SRY targets includes the promoter-proximal regions that were within the cutoff line (P ≤ 1 × 10^{-5}) were plotted (average of the three replicates). The genes that were not in the list but seemed to be masked by IgG negative signals were designated as questionable positives.

**Gene Network and Pathway Analysis**

In this study, gene networks for SRY downstream binding target genes were constructed separately using previously published criteria for developmental network analysis [75]. Global literature analysis of various gene lists was performed with Pathway Studio software using BiblioSphere Pathway Edition (Genomatix Software GmbH, Munchen, Federal Republic of Germany), which performs direct gene interaction (connection) analysis and relationship with cellular processes (indirect interactions).

The cellular signaling pathway analysis of direct downstream target genes was performed according to the protocol previously described [75]. The downstream binding targets of SRY and their associations with pathways were analyzed for KEGG (Kyoto Encyclopedia for Genes and Genome, Kyoto University, Japan) pathway enrichment using Pathway-Express, a web-based tool freely available as part of the Onto-Tools (http://vortex.cs.wayne.edu). A program based on literature analysis Pathway Studio (Ariadne, Genomics Inc., Rockville, MD) was used to evaluate cellular processes connected to binding targets associated genes. The analysis of statistical over-representation of genes within a pathway used a Fisher’s Exact

**Table 2: Cellular pathways associated with altered SRY binding sites: the 81 ≥ 3 adjacent oligo SRY binding sites**

| Gene   | Description                                      |
|--------|--------------------------------------------------|
| Actg1  | Actin, gamma 1                                   |
| RT1-DMb| RT1 class II, locus DMb                          |
| Dnajc3 | DnaJ (Hsp40) homolog, subfamily C, member 3      |

**Table 3: Cellular pathways associated with altered SRY binding sites: the 173 single oligo SRY binding sites**

| Pathway                                      | Description                                      |
|----------------------------------------------|--------------------------------------------------|
| Metabolic pathways                          |                                                  |
| Pnp                                          | Purine nucleoside phosphorylase (EC:2.4.2.1)     |
| Ppcs                                         | Phosphatidylcholine synthetase (EC:6.3.2.5)      |
| Uqrc1                                        | Ubiquinol-cytochrome c reductase core protein 1  |
| Cers2                                        | Ceramide synthase 2                              |
| Hpsae2                                       | Heparanase 2                                     |
| Fut4                                         | Fucosyltransferase 4 (alpha (1,3), myeloid-specific) (EC:2.4.1.152) |
| Mthfd2                                       | Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, Olfactory transduction pathways (7 altered) |
| Olr1443                                      | Olfactory receptor 1443                          |
| Olr694                                       | Olfactory receptor 694                           |
| Olr848                                       | Olfactory receptor 848                           |
| Olr1285                                      | Olfactory receptor 1285                          |
| Olr766                                       | Olfactory receptor 766                           |
| Olr51                                        | Olfactory receptor 51                            |
| Olr1654                                      | Olfactory receptor 1654                          |
| Ddit4                                        | DNA-damage-inducible transcript 4                |
| Notch1                                       | Notch 1                                          |
| Brca1                                        | Breast cancer 1, early onset                      |
| Cdc5                                         | Cell division cycle associated 5                 |

(Promega). Purified DNA was checked on the gel and sent to Nimblegen for ChIP-Chip hybridization (Nimblegen, Iceland). A threeplex array (3x720 RefSeq Promoter Array) was used for hybridization comparisons. The ChIP-Chip advanced technology used is more accurate and statistically stringent than the more classic PCR technology, such that we do not feel PCR of selected targets helps validate the data.

**Bioinformatics Analysis of ChIP-Chip Data**

The ChIP-Chip hybridization used a Roche Nimblegen’s Rat ChIP-Chip 3x720K RefSeq Promoter Array. The enrichment for each probe on the array was calculated as the log ratio of the intensities of hybridization for SRY ChIP DNA (Cy3) to control DNA from IgG control (Cy5). Array contained on average 4000 bp of promoter for each of 15 287 promoters in the rat genome corresponding to 15 600 RefSeq transcripts (~3880 bp upstream and 970 bp downstream from transcription start site). The analysis of ChIP-Chip data was performed as previously described [68]. For each hybridization experiment, raw data from both the Cy3 can Cy5 channels were imported into R [44] and data checked for quality and converted to MA values (M = Cy5-Cy3; A = (Cy5 + Cy3)/2). The R codes that were used for analysis and annotation are available in the following website: http://www.skinner.wsu.edu. All the tiling array Chip data were deposited in the NCBI GEO site (GEO # GSE72469).
test using $2 \times 2$ contingency table. The pathway analysis is distinct from the gene network analysis described above.

Acknowledgements

The authors acknowledge the advice and technical assistance of Dr Ingrid Sadler-Riggleman, Dr Daniel Beck, Ms Tiffany Hylkema, and Ms Jayleena Barton (Washington State University) and assistance of Ms Heather Johnson in the preparation of the manuscript.

Funding

This research was supported by an NIH Grant #: R01 ES012974-10 to M.K.S.

Supplementary data

Supplementary data is available at EnvEpig online.

Conflict of interest: None declared.

References

1. Skinner MK. Endocrine disruptor induction of epigenetic transgenerational inheritance of disease. Mol Cell Endocrinol 2014;398:4–12.
2. Anway MD, Cupp AS, Uzumcu M et al. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 2005;308:1466–9.
3. Anway MD, Memon MA, Uzumcu M et al. Transgenerational effect of the endocrine disruptor vinclozolin on male spermatogenesis. J Androl 2006;27:868–79.
4. Anway MD, Leathers C, Skinner MK. Endocrine disruptor vinclozolin induced epigenetic transgenerational adult-onset disease. Endocrinology 2006;147:5515–23.
5. Guererro-Bosagna C, Savenkova M, Haque MM et al. Environmentally induced epigenetic transgenerational inheritance of altered sertoli cell transcriptome and epigenome: molecular etiology of male infertility. PLoS One 2013;8:e59922.
6. Manikkam M, Guerrero-Bosagna C, Tracey R et al. Transgenerational actions of environmental compounds on reproductive disease and identification of epigenetic biomarkers of ancestral exposures. PLoS One 2012;7:e31901.
7. Seisenberger S, Peat JR, Reik W. Conceptual links between DNA methylation reprogramming in the early embryo and primordial germ cells. Curr Opin Cell Biol 2013;25:281–8.
8. Nilsson E, Larsen G, Manikkam M et al. Environmentally induced epigenetic transgenerational inheritance of ovarian disease. PLoS One 2012;7:e36129.
9. Jost A. Hormonal factors in the sex differentiation of the mammalian foetus. Philos Trans R Soc Lond B Biol Sci 1970;259:119–30.
10. Jost A. Recherches sur la différenciation sexuelle de l’embryon de lapin. Arch Anat Microsc Morph Exp 1947;36:271–315.
11. Jost A. Problems of fetal endocrinology: the gonadal and hypophyseal hormones. Recent Prog Horm Res 1953;8:379–418.
12. Sinclair AH. Human sex determination. J Exp Zool 1998;281:501–5.
13. Gubbay J, Collignon J, Koopman P et al. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature 1990;346:245–50.
14. Sinclair AH, Berta P, Palmer MS et al. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 1990;346:240–4.
15. Koopman P, Gubbay J, Vivian N et al. Male development of chromosomally female mice transgenic for Sry. Nature 1991;351:117–21.
16. Ferrari S, Harley VR, Pontiggia A et al. SRY, like HMG1, recognizes sharp angles in DNA. EMBO J 1992;11:4497–506.
17. Czaja W, Miller KY, Skinner MK et al. Structural and functional conservation of fetal MatA and human SRY sex-determining proteins. Nutr Commun 2014;5:5434.
18. Sekido R, Bar I, Narvaez V et al. SOX9 is up-regulated by the transient expression of SRY specifically in Sertoli cell precursors. Dev Biol 2004;274:271–9.
19. Morais da Silva S, Hacker A, Harley V et al. Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nat Genet 1996;14:62–8.
20. Kashimada K, Koopman P. Sry: the master switch in mammalian sex determination. Development 2010;137:3921–30.
21. Sekido R, Lovell-Badge R. Sex determination and SRY: down to a wink and a nudge? Trends Genet 2009;25:19–29.
22. Wilhelm D, Palmer S, Koopman P. Sex determination and gonadal development in mammals. Physiol Rev 2007;87:1–28.
23. Bhandari R, Saddler-Riggleman L, Clement TM et al. Basic helix-loop-helix transcription factor tcf21 is a downstream target of the male sex determining gene SRY. PLoS One 2011;6:e19935.
24. Clement TM, Bhandari RK, Saddler-Riggleman L et al. SRY directly regulates the neurotrophin-3 promoter during male sex determination and testis development in rats. Biol Reprod 2011;85:227–84.
25. Bradford ST, Hiramatsu R, Maddugoda MP et al. The cerebellin 4 precursor gene is a direct target of SRY and SOX9 in mice. Biol Reprod 2009;80:1178–88.
26. Arango NA, Lovell-Badge R, Behringer RR. Targeted mutagenesis of the endogenous mouse Mis gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. Cell 1999;99:409–19.
27. De Santa Barbara P, Bonnaud N, Boizet B et al. Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Mullerian hormone gene. Mol Cell Biol 1998;18:6653–65.
28. Kim Y, Bingham N, Sekido R et al. Fibroblast growth factor receptor 2 regulates proliferation and Sertoli differentiation during male sex determination. Proc Natl Acad Sci U S A 2007;104:16558–63.
29. Kim Y, Capel B. Balancing the bipotential gonad between alternative organ fates: a new perspective on an old problem. Dev Dyn 2006;235:2292–300.
30. Wilhelm D, Hiramatsu R, Mizusaki H et al. SOX9 regulates prostaglandin D synthase gene transcription in vivo to ensure testis development. J Biol Chem 2007;282:10553–60.
31. Schmahl J, Kim Y, Calvin JS et al. Fgf9 induces proliferation and nuclear localization of FGFR2 in Sertoli precursors during male sex determination. Development 2004;131:3627–36.
32. Bhandari R, Haque MM, Skinner M. Global genome analysis of the downstream binding targets of testis determining factor SRY and SOX9. PLoS One 2012;7:e43380.
33. Bhandari RK, Schinke EN, Haque MM et al. SRY induced TCF21 genome-wide targets and cascade of bHLH factors during sertoli cell differentiation and male sex determination in rats. Biol Reprod 2012;87:131.
34. Grootegoed JA, Jansen R, Van der Molen HJ. The role of glucose, pyruvate and lactate in ATP production by rat spermatocytes and spermatids. Biochim Biophys Acta 1984;767:248–56.

35. Rato L, Alves MG, Socorro S et al. Metabolic regulation is important for spermatogenesis. Nat Rev Urol 2012;9:330–8.

36. Erkkila K, Aito H, Aalto K et al. Lactate inhibits germ cell apoptosis in the human testis. Mol Hum Reprod 2002;8:109–17.

37. Hirai K, Sasaki H, Yamamoto H et al. HST-1/FGF-4 protects testicular germ cells from apoptosis under heat-stress condition. Exp Cell Res 2004;294:77–85.

38. Raychoudhury SS, Kubinski D. Polycyclic aromatic hydrocarbon-induced cytotoxicity in cultured rat Sertoli cells: important for spermatogenesis. Exp Cell Res 2004;294:77–85.

39. Clement TM, Savenkova MI, Settles M et al. Alterations in the developing testis transcriptome following embryonic vinclozolin exposure. Reprod Toxicol 2010;30:353–64.

40. Skinner M, Guerrero-Bosagna C, Haque MM et al. Environmentally induced transgenerational epigenetic reprogramming of primordial germ cells and subsequent germine. PLoS One 2013;8:e66318.

41. Levine E, Cupp AS, Miyashiro L et al. Role of transforming growth factor-alpha and the epidermal growth factor receptor in embryonic rat testis development. Biol Reprod 2000;62:477–90.

42. Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. Nat Rev Genet 2007;8:253–62.

43. Martos SN, Tang WY, Wang Z. Elusive inheritance: transgenerational effects and epigenetic inheritance in human environmental disease. Prog Biophys Mol Biol 2015;118:44–54.

44. R Development Core Team. R: A Language for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. http://www.R-project.org, 2010.

45. Metz GA, Ng JW, Kovalchuk I et al. Ancestral experience as a game changer in stress vulnerability and disease outcomes. Bioessays 2015;37:602–11.

46. Szfy M. Nongenetic inheritance and transgenerational epigenetics. Trends Mol Med 2015;21:134–44.

47. Dias BG, Maddox SA, Kengel T et al. Epigenetic mechanisms underlying learning and the inheritance of learned behaviors. Trends Neurosci 2014;38:96–107.

48. McCrery JR. Distinctions between transgenerational and non-transgenerational epimutations. Mol Cell Endocrinol 2014;398:13–23.

49. Pembrey M, Saffery R, Bygren LO. Human transgenerational responses to early-life experience: potential impact on development, health and biomedical research. J Med Genet 2014;52:563–72.

50. Kawashima T, Berger F. Epigenetic reprogramming in plant sexual reproduction. Nat Rev Genet 2014;15:613–24.

51. Rissman EF, Adli M. Minireview: transgenerational epigenetic inheritance: focus on endocrine disrupting compounds. Endocrinology 2014;155:2770–80.

52. Padilla PA, Garcia AM, Ladage ML et al. Caenorhabditis elegans: an old genetic model can learn new epigenetic tricks. Integr Comp Biol 2014;54:52–60.

53. Jensen P. Transgenerational epigenetic effects on animal behaviour. Prog Biophys Mol Biol 2013;113:447–54.

54. Bohacek J, Gapp K, Saab B et al. Transgenerational epigenetic effects on brain functions. Biol Psychiatry 2013;73:313–20.