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The Sex-Determining Factors SRY and SOX9 Regulate Similar Target Genes and Promote Testis Cord Formation during Testicular Differentiation

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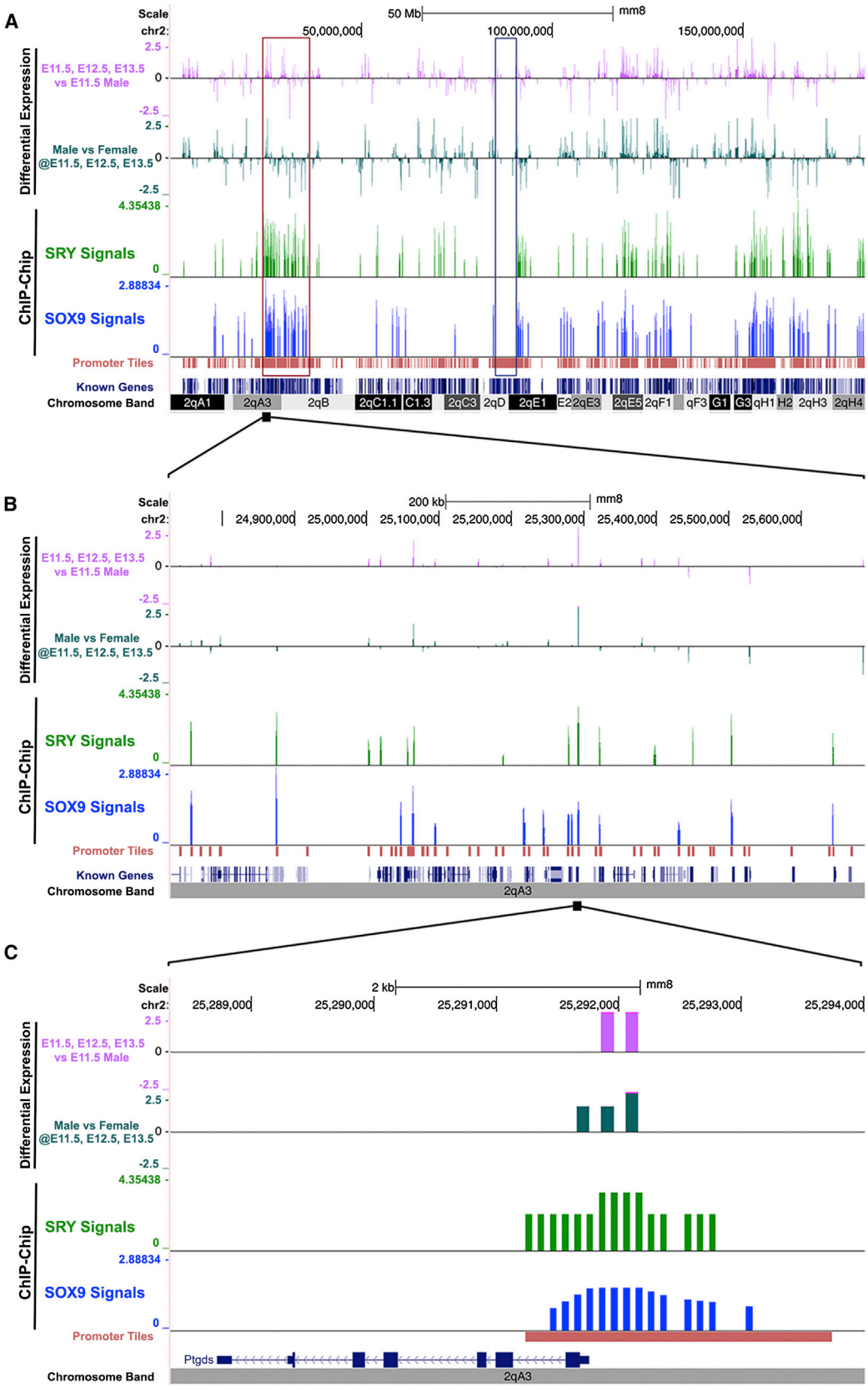
SUMMARY

Male sex determination is mediated sequentially by sex-determining region Y (SRY) and related SRY-box 9 (SOX9) transcription factors. To understand the gene regulatory hierarchy for SRY and SOX9, a series of chromatin immunoprecipitation and whole-genome promoter tiling microarray (ChIP-Chip) experiments were conducted with mouse gonadal cells at the time of sex determination. SRY and SOX9 bind to the promoters of many common targets involved in testis differentiation and regulate their expression in Sertoli cells. SRY binds to various ovarian differentiation genes and represses their activation through WNT/β-catenin signaling. Sertoli cell-Sertoli cell junction signaling, important for testis cord formation, is the top canonical pathway among the SRY and SOX9 targets. Hence, SRY determines Sertoli cell fate by repressing ovarian and activating testicular differentiation genes, promotes early Sertoli cells to form testis cord, and then passes on its functions to SOX9, which regulates common targets and activates its own gene regulatory program, beyond SRY actions, in sex determination.

INTRODUCTION

The Y chromosome plays a pivotal role in mammalian sex determination. The discovery of the sex-determining region Y (*Sry*) gene as the sex-determining gene on this chromosome marks the most significant development in the field (Gubbay et al., 1990; Sinclair et al., 1990). SRY is postulated to determine the fate of the supporting cells into the Sertoli cell lineage, the foremost event of the developmental cascade (McLaren, 1991). The Sertoli cells, in turn, bring about a variety of cellular and developmental events, including pre-Sertoli cell proliferation, recruitment of additional supporting cells into Sertoli cells, blockage of meiosis in germ cells, and the formation of testis cords, the first visible structure of the embryonic testis. Recent studies suggest that SRY synergizes with steroidogenic factor 1 (SF1, also known as nuclear receptor subfamily 5, group A, member 1 [NR5A1]) and binds to a 1.4 kb core domain of the testis enhancer of *Sox9* (*TESCO*), between −13 and −10 kb upstream of the transcription start site of the related SRY-box 9 (*Sox9*) gene (Sekido and Lovell-Badge, 2008), and greatly upregulates *Sox9* expression. SOX9, in turn, interacts with SF1 and binds to the same or similar binding sites within *TESCO*, propagates its own expression in a positive feedback loop, and propels testis differentiation beyond SRY actions. Because ectopic expression of *Sox9*, by either transgenic means or activating mutations (Bishop et al., 2000; Qin et al., 2004; Vidal et al., 2001), results in male (M) sex differentiation without a functional *Sry*, it has been argued that the primary role of *Sry* is to activate *Sox9* in this Y chromosome-initiated developmental process (Sekido and Lovell-Badge, 2009).

Various studies, however, suggest that SRY might serve diverse functions in not only activating *Sox9* but also regulating other important sex-determining genes, reprogramming the chromatin landscape, and epigenetically establishing the supporting cells to a Sertoli cell lineage (Oh and Lau, 2006). SRY lacks a transcriptional domain present in most SOX proteins and needs to recruit corepressors and coactivators to accomplish its transcriptional and/or epigenetic functions. Indeed, numerous SRY interactive proteins with a variety of transcriptional or chromatin modulating properties have been identified with several molecular approaches (Lau and Li, 2009; Li et al., 2006; Oh et al., 2005; Peng et al., 2009; Sekido and Lovell-Badge, 2008; Wu et al., 2009). SRY forms transcription complexes with either SF1 or SP1 and transactivates the respective target genes (Sekido and Lovell-Badge, 2008; Wu et al., 2009). SRY could interact with nuclear β-catenin and repress WNT signaling-dependent gene expression (Lau and Li, 2009). SRY also interacts with numerous transcription factors (Wissmüller et al., 2006) and modulates the respective transcriptional activities. SRY binds the Krüppel-associated box-only (KRAB-O) protein and recruits a gene-silencing complex, consisting of the KAP1-HP1 (KRAB-associated protein 1-heterochromatin protein 1), to the promoters and represses the target genes (Oh et al., 2005; Peng et al., 2009). SRY also interacts with poly(ADP-ribose) polymerase 1 (PARP1), which could



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poly(ADP-ribosyl)ate nuclear proteins and modulate their affinity to their respective target sequences (Li et al., 2006). Hence, SRY could reprogram epigenetically the chromatin landscape of its targets and permanently establish the Sertoli cell lineage in the supporting cells of the developing testis. At present, the full spectrum of SRY-containing transcription and chromatin remodeling complexes in the Sertoli precursors is still unknown. These observations, however, raise some important questions. Is *Sox9* the only target gene for SRY? If not, what are the other target genes for the SRY-containing transcription complexes? Are any of its targets involved in the cascade of cellular events in testis differentiation? Furthermore, because SOX9 is responsible for propagating testis differentiation beyond SRY actions and their DNA binding high-mobility group boxes are functionally interchangeable (Bergstrom et al., 2000), do SRY and SOX9 bind and regulate similar target genes in the same developmental pathway?

To address the above questions, we had conducted a series of experiments using the chromatin immunoprecipitation (ChIP) and whole-genome promoter tiling microarray (ChIP-Chip) strategy on fetal gonads from mouse embryos at embryonic day 11.5 (E11.5) and E12.5 developmental stages to identify the target genes for SRY and SOX9, respectively. Our results provided some answers to these questions, suggesting that SRY-containing transcription complexes bind to the promoters of a large number of target genes, including numerous known testis and ovarian-differentiating genes. SRY and SOX9 share and regulate a significant number of common targets, which serve important functions in early events in testis differentiation, including Sertoli cell fate determination, M germ cell definition, and testis cord formation and differentiation.

RESULTS

SRY and SOX9 Recognize a Large Number of Common Target Genes during Sex Determination

Using two specific antibodies against the mouse SRY and SOX9, respectively (Figure S1), gonadal cells from E11.5 embryos were processed for SRY ChIP, at which time SRY expression was at its peak, whereas M gonadal cells from E12.5 embryos were used for SOX9 ChIP, at which time SRY expression was at its minimum or had disappeared (Taketo et al., 2005). We surmise that using highly specific antibodies and gonadal cells at distinct embryonic stages had greatly enhanced the specificity of the respective SRY or SOX9 bound chromatin in our ChIP-Chip studies. The crude ChIP data were further analyzed with the Mpeak program (Zheng et al., 2013), which identified statistically significant binding peaks by scanning the hybridization signals along parallel promoter tiling regions between two biological replicates. Using an optimal threshold value of 1 for prefiltering and p value <0.01, we had identified a total of 3,083 target genes for SRY in E11.5 gonads and 1,903 target genes for SOX9 in E12.5 M gonads, respectively (Tables S1 and S2). These two transcriptional factors regulate a substantially large number (907) of common genes, about one-third and half of the SRY and SOX9 targets, respectively. These observations are significant because the respective targets were identified with independent and biologically replicated experiments with gonadal cells at two distinct developmental stages, i.e., E11.5 for SRY and E12.5 for SOX9, suggesting that SOX9 must have assumed a sizable portion of the SRY regulatory program in the process.

A total of 126 and 84 target genes respectively for SRY and SOX9 previously demonstrated to be associated with sex determination, to possess high Mpeak (binding) scores, and/or to be highly differentially expressed between M and female (F) supporting cells, were selected for confirmation analysis with gene-specific primer pairs (Table S3) and quantitative PCR with corresponding immunoprecipitated chromatin DNAs. Primers from the SRY and SOX9 binding sites at *TESCO* (Sekido and Lovell-Badge, 2008) and the mouse cerebellum 4 precursor (*Cbln4*) (Bradford et al., 2009) were used as positive controls. This initial analysis showed 89.7% and 88.1% confirmation for SRY and SOX9 binding to the respective target genes (Figure S2).

SRY and SOX9 Bind Preferentially to the Same Regions at the Promoters of Their Common Targets

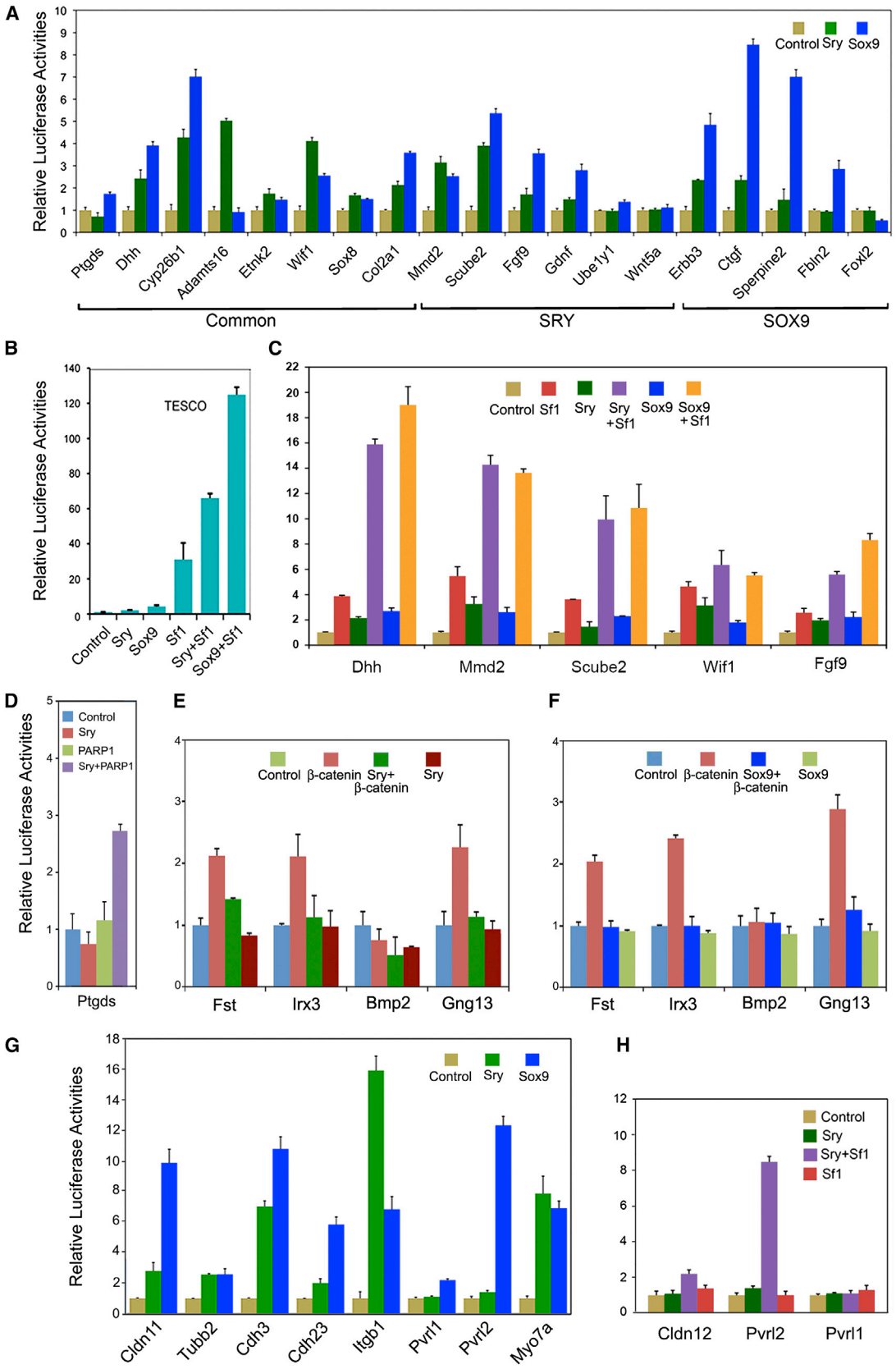
To determine the overall distributions of SRY and SOX9 binding signals in the entire mouse genome and to compare them to those of differentially expressed genes, the expression levels between M and F supporting cells at E11.5, E12.5, and E13.5 stages and between E12.5/E13.5 and E11.5 were calculated from microarray expression data (Jameson et al., 2012) and tabulated as log2 ratios at the transcription start site of each annotated gene and analyzed with the Genome Browser (Karolchik et al., 2011). At the chromosome level, SRY and SOX9 ChIP-Chip signals were preferentially localized at chromosomal regions containing differentially expressed genes (Figure 1A). At the chromosomal subband level, SRY and SOX9 bindings could be identified as differentially regulated genes in M supporting cells or among the three developmental stages (Figure 1B). At the specific gene level, the binding patterns of SRY and SOX9 were almost identical at the promoters of their common target genes, e.g., prostaglandin D2 synthase (*Ptgds*) (Figure 1C). These patterns of the SRY and SOX9 bindings were most prevalent among their common targets (Figure S3), suggesting that they could bind to the same, if not identical, regions and regulate their common target genes in similar manners.

Figure 1. SRY and SOX9 ChIP-Chip Signals and Gene Differential Expression Patterns on Mouse Chromosome 2, as Visualized by the Genome Browser

(A) Distributions of SRY (green) and SOX9 (blue) binding signals on mouse chromosome 2 as visualized with the Genome Browser, and compared to differentially expressed genes between M and F supporting cells at E11.5, E12.5, and E13.5 gonads (jade), and between supporting cells of M gonads at E12.5 versus E11.5 and E13.5 versus E11.5 stages (pink). Examples of significant and minimal SRY and SOX9 bindings and differential gene expression in supporting cells are boxed in dark red and dark blue, respectively.

(B) Enlargement at chromosome band 2qA3, showing SRY and SOX9 signals among common targets.

(C) Signals at individual gene level, showing almost identical patterns for SRY and SOX9 signals on the *Ptgds* promoter.



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SRY and SOX9 Bind and Regulate the Promoter Activities of Common and Unique Target Genes in a Mouse Sertoli Cell Line

Among the SRY and SOX9 targets, there are numerous notable ones, such as *Ptgds* (Wilhelm et al., 2007), desert hedgehog (*Dhh*) (Clark et al., 2000; Yao et al., 2002), cytochrome P450, family 26, subfamily B, polypeptide 1 (*Cyp26b1*) (Bowles et al., 2006), fibroblast growth factor 9 (*Fgf9*) (Colvin et al., 2001), glial cell line-derived neurotrophic factor (*Gdnf*) (Oatley and Brinster, 2008), and others previously demonstrated to serve vital functions in sex determination and differentiation (Table S4). To evaluate the functional effects of SRY and SOX9 on the target genes, promoters containing the specific SRY/SOX9 binding peaks and transcription start sites were inserted immediately upstream of a promoter-less luciferase reporter and analyzed with transfection assays in a mouse Sertoli cell line, MSC1 (McGuinness et al., 1994), with and without a mouse *Sry* or *Sox9* transgene. A luciferase reporter directed by the *TESCO* enhancer and a minimal promoter (Sekido and Lovell-Badge, 2008) was used as a positive control. Transfection of MSC1 cells with this *TESCO* reporter with either *Sry* or *Sox9* resulted in moderate increases in reporter activities, as compared to those with *TESCO* reporter alone (Figure 2B). Significantly, inclusion of *Sf1* (*Nr5a1*) expression vector in the transfections greatly stimulated the reporter activities, which were further amplified with either *Sry* or *Sox9* coexpression. These results corroborated with those from the initial study on *TESCO* (Sekido and Lovell-Badge, 2008), thereby supporting the validity of our promoter assay system.

Initially, 23 putative target genes (8 common, 10 SRY-unique, and 5 SOX9-unique targets) were selected for detailed promoter characterization in MSC1 cells (Tables S1 and S2 for remainders of SRY and SOX9 targets). Most promoters of the selected target genes were capable of responding to the transactivation effects of a cotransfected *Sry* or *Sox9* gene (Figure 2A). Interestingly, several specific targets for SRY (e.g., *Mmd2*, *Scube2*, *Fgf9*, and *Gdnf*) were capable of responding to SOX9 stimulation and vice versa (e.g., *Erbb3* and *Ctgf*) in this promoter assay system. We surmise that the respective transcription factors, i.e., SRY or SOX9, were expressed at high levels in the transiently transfected MSC1 cells, and their DNA binding domains are functionally interchangeable (Bergstrom et al., 2000); they could bind to the *cis* elements of each other's target promoters, thereby stimulating the luciferase reporter activities.

We further noted that the promoter from *Adamts16* lacked any response to a cotransfected *Sox9* gene, and those from *Ube1y1* and *Wnt5a* showed minimal stimulation by either the *Sry* or *Sox9* expression. The *Ptgds* promoter-directed reporter showed only moderate stimulation by *Sox9*, but not *Sry*, as reported by Wilhelm et al. (2007). Currently, the molecular mechanism(s) for such unresponsiveness of the respective promoters in this assay system is unknown. Conceivably, yet-to-be defined critical cofactors for SRY or SOX9 for their regulatory activities on the respective promoters were absent or insufficient in the MSC1 cells. Because SF1 has been demonstrated to work collaboratively with SRY in transcriptional regulation of their responsive genes, i.e., *Sox9* (Sekido and Lovell-Badge, 2008), we had selected a few target promoter-luciferase constructs for further analysis. Inclusion of *Sf1* in the *Sry* transfection greatly exacerbated the activities of the reporter in the promoter assays (Figure 2C). Because SOX9 could also interact with SF1 and regulate its own gene transcription in a positive feedback loop, we had performed parallel *Sox9* transfection assays similarly as those for *Sry* and *Sf1*. Our results demonstrated that indeed SOX9 could synergistically interact with SF1 and transactivate the same reporters directed by the respective SRY or common target promoters, similar to SRY (Figure 2C).

Interestingly, as a SRY interactive protein, cotransfection of *PARP1* with *Sry* could stimulate the *Ptgds* promoter-directed luciferase activities to about 3-fold in MSC1 cells, as compared to control, and those with *Sry* or *PARP1* alone (Figure 2D). Such SRY-PARP1 stimulation of *Ptgds* promoter was at a similar level as that by SOX9 (Figure 2A). These results support our postulation of a likely requirement(s) of cofactors for SRY and/or SOX9 transactivation of their respective target genes.

SRY Represses Ovarian-Determining Genes by Interfering with Their Activation by WNT/β-catenin Signaling

As the M sex determinant, SRY not only activates the testicular differentiation but also likely represses the ovarian-determining program(s). Several key ovarian-differentiating genes, i.e., follistatin (*Fst*), Iroquois-related homeobox 3 (*Irx3*), bone morphogenetic protein 2 (*Bmp2*), and guanine nucleotide binding protein (G protein), gamma 13 (*Gng13*), were SRY targets, but not SOX9. Because β-catenin is the major mediator of the RSPO1-WNT4 signaling responsible for activating the early events of ovarian determination and differentiation (Maatouk et al., 2008), we had evaluated the effects of SRY in β-catenin transactivation of reporter genes directed by the respective promoters of these ovarian-determining genes in a granulosa cell line, KK1. Our results showed that β-catenin could indeed stimulate the activities of three of four (i.e., *Fst*, *Irx3*, and *Gng13*) target gene promoter-directed reporters. Expression of *Sry* in transfected cells reduced such reporter activities to the same levels

Figure 2. Promoter Assays on Selected Target Genes of SRY and SOX9

(A) Individual promoter assays on SRY and SOX9 targets in the Sertoli cell line, MSC1.

(B) SRY and SOX9 exacerbation of SF1 stimulation of a *TESCO*-directed luciferase in MSC1 cells.

(C and D) Promoter assays showing SRY and SOX9 exacerbation of SF1 (C) and SRY and *PARP1* exacerbation (D) on respective targets.

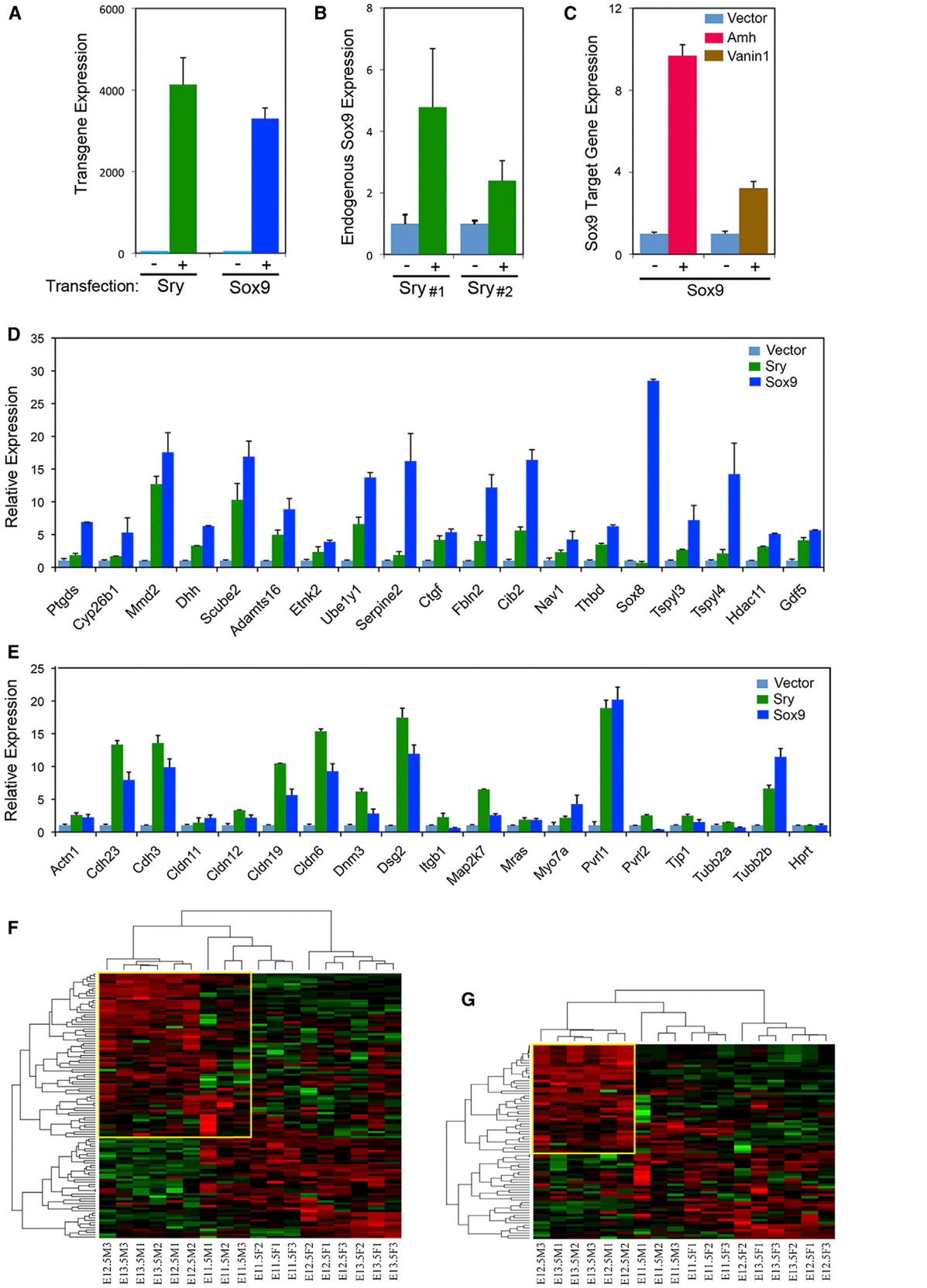
(E) SRY ovarian target promoter-directed reporter assay in KK1 granulosa cell line, showing their upregulation by WNT/β-catenin signaling and suppression by SRY coexpression.

(F) Similar transfection assays, showing SOX9 repression of WNT/β-catenin activation of SRY ovarian targets.

(G) Similar promoter-directed reporter assays, as in (A), among target genes in the Sertoli cell-Sertoli junction signaling.

(H) *Sf1* could exacerbate the transactivation of reporters directed by the promoter of *Pvrl2*, but only slightly by that of *Cldn12* and none at all by that of *Pvrl1*.

See Tables S1 and S2 for all SRY and SOX9 targets at E11.5 and E12.5 gonads, respectively. Error bars represent SDs derived from three independent experiments.



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as those without β-catenin transactivation (Figure 2E). Our experiments showed that SRY could antagonize the WNT/β-catenin transcriptional activities of ovarian-differentiating genes in a granulosa cell line.

Various studies indicated that ectopic expression of *Sox9*, in the absence of *Sry*, could induce M sex differentiation (Bishop et al., 2000; Qin et al., 2004; Vidal et al., 2001), suggesting that SOX9 could assume SRY functions, including repressing of WNT/β-catenin activation of ovarian-differentiating genes. To evaluate such a possibility, similar transfection assays were conducted with a mouse *Sox9* construct. Our results showed that indeed SOX9 suppressed the β-catenin-mediated transactivation of the same reporters, except that from *Bmp2* gene (Figure 2F), suggesting that SOX9 is capable of interfering the WNT/β-catenin signaling and repressing the ovarian-differentiating genes, when it is ectopically expressed during sex determination.

SRY and SOX9 Upregulate the Expression of Endogenous Testis-Differentiating Genes in Mouse Sertoli Cells

The effects of ectopically expressed SRY or SOX9 in the Sertoli cells were examined using a transient transfection strategy. A *Sry* or *Sox9* transgene was coexpressed with the GFP in MSC1 cells. Positive cells were purified by preparative flow cytometry using GFP as a biomarker and analyzed by quantitative RT-PCR (qRT-PCR). The results showed that the *Sry* and *Sox9* transgenes were expressed at high levels in the corresponding transfected MSC1 cells (Figure 3A). As a target gene for SRY, the endogenous *Sox9* gene was upregulated in Sertoli cells transiently expressing SRY (Figure 3B). Similarly, expression levels of known SOX9 targets, *Amh* and *Vanin1*, in SOX9-expressing MSC1 cells increased significantly over the vector-transfected control (Figure 3C). This initial analysis suggested that expression of SRY and SOX9 could have significant effects on the respective endogenous target genes. Using various gene-specific primers (Table S3), we had examined the expression of 30 target genes for SRY and SOX9 in the transient transfected Sertoli cells. Our results showed that approximately two-thirds of endogenous target genes were stimulated to various levels in *Sry*- or *Sox9*-transfected cells (Figure 3D).

SRY and SOX9 Targets Serve Important Roles in Testis Cord Formation

To decipher the probable functions and pathways regulated by SRY and SOX9 during testis development, we had analyzed the respective targets with a knowledge base Gene Ontology analysis system (Ingenuity Pathway Analysis [IPA]). We surmise that upregulated genes in M supporting cells could serve positive functions in testicular differentiation; hence, we had also analyzed SRY and SOX9 targets upregulated in M as compared to F supporting cells (i.e., M versus F at E11.5, E12.5, and E13.5 stages) and among the M supporting cells at three early stages of gonadal differentiation (Jameson et al., 2012).

Using the entire target sets for SRY and SOX9, IPA showed that they were significantly enriched in similar biological processes pertaining to cellular growth and proliferation and organ and embryo development. Notably, the Sertoli cell-Sertoli cell junction signaling ranked second among the 587 canonical pathways in the IPA database for the SRY target genes and, to a lesser extent, those of SOX9 (Table 1). Significantly, this signaling pathway was the top canonical pathway among the SRY targets upregulated in M supporting cells at all three gonadal developmental stages, and between M supporting cells of E12.5/E13.5 versus E11.5 stage, and again ranked similarly prominent among the upregulated SOX9 targets. Importantly, other epithelial adherens, tight junction, and actin cytoskeleton signalings were also among the top canonical pathways with the SRY and SOX9 targets (Table 1).

The Sertoli cell-Sertoli cell junction signaling pathway was initially established for Sertoli cell-Sertoli cell junction formation in the postnatal and adult testis and is important for the development of the blood-testis barrier (BTB) of the seminiferous tubules. The BTB consists of tight junctions and adherens junctions between Sertoli cells, which are dynamically remodeled allowing the spermatogenic cells to migrate across the base of the seminiferous epithelium to the lumen during their maturation (Cheng and Mruk, 2012). The identifications of Sertoli cell-Sertoli cell junction signaling, actin cytoskeleton signaling, and tight junction signaling as top canonical pathways among the SRY and SOX9 targets, especially those upregulated in early Sertoli cells, suggest that they are important in regulating cell adhesion, tight junction dynamics, and actin cytoskeletal anchoring in early testis cord formation and tubulogenesis in the M gonad (Cool et al., 2012).

Quantitative PCR analysis confirmed the SRY and SOX9 binding to ~90% of targets involved in the Sertoli cell-Sertoli cell junction signaling, similar to those previously studied (Figures S2D and S2G). Furthermore, promoters of selected target genes were capable of being activated by a cotransfected *Sry* or *Sox9* transgene in a reporter assay system (Figure 2G).

Figure 3. Effects of *Sry* and *Sox9* Transgene on Gene Expression of Selected Endogenous Targets in MSC1 Cells

(A) Detection of high-level expression of the transfected *Sry* and *Sox9* genes in MSC1 cells by qRT-PCR.

(B) Upregulation of endogenous *Sox9* gene in MSC1 cells by a *Sry* transgene.

(C) Upregulation of two known SOX9 targets, i.e., *Amh* and *Vanin1*, in SOX9-exressing MSC1 cells.

(D) Differential upregulation of selected endogenous SRY and SOX9 targets in respectively transfected MSC1 cells.

(E) Upregulation of endogenous genes belonging to the Sertoli cell-Sertoli cell junction signaling pathway in MSC1 cells by *Sry* and *Sox9* transgene, as compared to *Hprt* control.

(F and G) Expression levels (heatmap) of (F) SRY targets and (G) SOX9 targets in the Sertoli cell-Sertoli cell junction signaling in M and F supporting cells of mouse gonads at E11.5, E12.5, and E13.5 stages. A subset of genes was preferentially upregulated among the SRY and SOX9 targets (boxed) in M supporting cells at the E11.5–E13.5 stages (left columns). Both M and F supporting cells at the E11.5 stage showed similar, but distinct, expression patterns (middle). The F supporting cells showed minimal changes toward the E12.5 and E13.5 stages (right). E11.5M1 represents *E11.5* stage *m*ale supporting cells sample *1*.

See Table S4 for phenotypes associated with dysfunctions in sex determination of selected SRY/SOX9 targets. Error bars represent SDs derived from three independent experiments.

Table 1. Sertoli Cell-Sertoli Cell Junction Signaling and Other Epithelial Adherens, Tight Junction, and Actin Cytoskeleton Signaling Pathways among the SRY and SOX9 Targets

Rank	Ingenuity Canonical Pathways[a]	p Value	Ratio
SRY Targets (total targets in E11.5 supporting cells)			
2	Sertoli cell-Sertoli cell junction signaling	2.04×10^{-6}	2.63×10^{-1}
6	Actin cytoskeleton signaling	1.32×10^{-5}	2.32×10^{-1}
11	Tight junction signaling	8.13×10^{-5}	2.53×10^{-1}
SRY Targets Upregulated in M at E11.5			
1	Sertoli cell-Sertoli cell junction signaling	3.02×10^{-5}	3.76×10^{-2}
2	Remodeling of epithelial adherens junctions	3.63×10^{-4}	5.97×10^{-2}
5	Actin cytoskeleton signaling	7.41×10^{-4}	2.58×10^{-2}
6	Epithelial adherens junction signaling	8.51×10^{-4}	3.45×10^{-2}
8	Germ cell-Sertoli cell junction signaling	1.12×10^{-3}	3.16×10^{-2}
SRY Targets Upregulated in M at E12.5			
1	Sertoli cell-Sertoli cell junction signaling	2.19×10^{-3}	4.3×10^{-2}
3	Actin cytoskeleton signaling	6.92×10^{-3}	3.43×10^{-2}
6	Epithelial adherens junction signaling	1.17×10^{-2}	4.14×10^{-2}
SRY Targets Upregulated in M at E13.5			
1	Sertoli cell-Sertoli cell junction signaling	6.31×10^{-4}	4.30×10^{-2}
3	Epithelial adherens junction signaling	4.79×10^{-3}	4.14×10^{-2}
4	Remodeling of epithelial adherens junctions	5.75×10^{-3}	5.97×10^{-2}
5	Germ cell-Sertoli cell junction signaling	6.61×10^{-3}	3.80×10^{-2}
7	Actin cytoskeleton signaling	8.12×10^{-3}	3.00×10^{-2}
SRY Targets Upregulated in E12.5 versus E11.5 M			
1	Sertoli cell-Sertoli cell junction signaling	3.89×10^{-5}	4.84×10^{-2}
3	Germ cell-Sertoli cell junction signaling	6.17×10^{-4}	4.43×10^{-2}
SRY Targets Upregulated in E13.5 versus E11.5 M			
1	Sertoli cell-Sertoli cell junction signaling	4.37×10^{-5}	4.84×10^{-2}
3	Germ cell-Sertoli cell junction signaling	6.61×10^{-4}	4.43×10^{-2}
SOX9 Targets (total targets in E12.5 supporting cells)			
18	Tight junction signaling	3.02×10^{-3}	1.58×10^{-1}
26	Sertoli cell-Sertoli cell junction signaling	5.13×10^{-3}	1.45×10^{-1}
SOX9 Targets Upregulated in M at E11.5			
3	Integrin signaling	3.02×10^{-3}	3.37×10^{-2}
13	Paxillin signaling	1.66×10^{-2}	3.57×10^{-2}
14	Germ cell-Sertoli cell junction signaling	1.74×10^{-2}	3.14×10^{-2}
SOX9 Targets Upregulated in M at E12.5			
3	Tight junction signaling	1.70×10^{-3}	4.43×10^{-2}
4	Integrin signaling	1.74×10^{-3}	3.85×10^{-2}
9	Sertoli cell-Sertoli cell junction signaling	3.80×10^{-3}	3.74×10^{-2}
SOX9 Targets Upregulated in M E13.5			
2	Integrin signaling	1.32×10^{-3}	3.85×10^{-2}
3	Tight junction signaling	1.32×10^{-3}	4.43×10^{-2}
7	Sertoli cell-Sertoli cell junction signaling	3.02×10^{-3}	3.74×10^{-2}
SOX9 Targets Upregulated in E12.5 versus E11.5 M			
2	Sertoli cell-Sertoli cell junction signaling	5.37×10^{-3}	2.69×10^{-2}
6	Tight junction signaling	1.66×10^{-2}	2.53×10^{-2}
10	Integrin signaling	3.71×10^{-2}	1.93×10^{-2}
13	Actin cytoskeleton signaling	4.79×10^{-2}	1.72×10^{-2}
SOX9 Targets Upregulated in E13.5 versus E11.5 M			
1	Sertoli cell-Sertoli cell junction signaling	6.03×10^{-3}	2.69×10^{-2}
4	Tight junction signaling	1.82×10^{-2}	2.53×10^{-2}

[a]IPA was performed from April 27 to April 30, 2013.

For those that showed minimal induction by a *Sry* transgene, inclusion of a cotransfected *Sf1* transgene could greatly stimulate the expression of some reporters directed by the respective target gene promoters (Figure 2H). Selected endogenous SRY and/or SOX9 targets were stimulated in MSC1 cells transiently transfected with either a *Sry* or a *Sox9* transgene (Figure 3E). Collectively, these studies confirmed the bindings and regulation/coregulation of the target genes of this canonical pathway by SRY and/or SOX9, similar to those previously observed (Figures 2 and 3).

Because the Sertoli cell-Sertoli cell junction signaling was initially established in adult testis pertaining to the establishment and physiology of the BTB, we postulated that perhaps only a subset of genes involved in this canonical pathway could be important for testis cord formation in the embryonic testis, in which spermatogenesis was absent. To explore this possibility, we had used the hierarchical clustering analysis on the SRY and SOX9 targets within this signaling pathway with respect to the expression patterns in the supporting cells at various stages of sex differentiation in the embryonic gonads. Our results showed that subsets of the respective targets were preferentially upregulated in the early M supporting cells, particularly those at E12.5 and E13.5 testes (Figures 3F and 3G; gene lists in Table S5). Significantly, based on the expression patterns of the

respective targets, the M and F supporting cells at E12.5 and E13.5 stages were clustered at opposite sides, whereas those for E11.5 stage were positioned in the middle of the dendrogram. These findings suggest that the expression patterns of both M and F supporting cells at the early stage, i.e., E11.5, were similar, but distinguishable. The M supporting cells took on a specific expression pattern toward later stages, i.e., E12.5 and E13.5, with the formation of testis cord, a process in which certain SRY and SOX9 targets were highly expressed and presumably exerted a positive function(s) (Figures 3F and 3G, boxed), whereas the F supporting cells showed less dramatic changes in their expression patterns from E11.5 to E13.5 stages. These expression patterns seem to parallel the morphological differentiation between M and F gonads at the respective stages, in which the M gonad shows profound changes with the testis cord formation, whereas the F gonad shows only slight phenotypic changes in its vasculature (Cool et al., 2012).

Because the SRY targets were identified at the E11.5 stage, whereas their differential expressions were noticeable at this stage but only became prevalent at the E12.5 and later stages, SRY must exert its regulatory functions at the E11.5 stage when its expression was at the peak. Currently, the exact regulatory mechanisms for such SRY actions are uncertain. SRY could play an initial and crucial role(s) in activating or repressing the respective target genes, whose regulations are then taken over by collaborating transcription activators or repressors at the later stages. One prime example of such a gene regulatory mechanism is the *Sox9* gene, which is initially activated collaboratively by SRY and SF1 and subsequently by SF1 and its own product beyond the active period of SRY. Alternatively, SRY could recruit chromatin regulators, such as the KAP1-HP1 gene-silencing complex and the chromatin modulator PARP1, and remodel the chromatin landscapes and accessibility to subsequent transcriptional regulators on its target genes beyond its own expression in the supporting cells. Nevertheless, our data support the notion that SRY plays a pivotal role in the initiation of the vasculature development of embryonic testis, and SOX9 assumes such functions at later stages of the differentiation.

DISCUSSION

SRY Plays Key Roles in Early Events in M Sex Determination

The Y-located *Sry* gene serves the indispensable function in M sex determination, initiated by its activation in supporting cells of E10.5 gonad and followed by cascades of molecular and cellular events in early stages (Svingen and Koopman, 2013). At present, the exact mechanisms by which *Sry*, *Sox9*, and other sex-determining genes mediate the early cellular events are uncertain. A current hypothesis suggests that SRY primarily switches on *Sox9*, the presumed master facilitator in testis differentiation (Sekido and Lovell-Badge, 2009). The present study challenges this paradigm and has provided evidence supporting the notion that SRY regulates a wide spectrum of targets, involved in early events in testis differentiation, some of which could precede the normal *Sox9* activation. These early cellular events include Sertoli cell fate determination, inhibition of ovarian development, Sertoli cell proliferation and recruitment, arrest of meiosis in germ cells and definition of their M lineage, and eventual formation of the testis cord, encapsulating the germ cells (Cool et al., 2012; Svingen and Koopman, 2013). At present, the exact order of their occurrence is uncertain; SRY could exert its regulatory actions in simultaneous and/or overlapping manners. We surmise that the initial *Sry* activation and its subsequent actions in the supporting cells determine the fate of these cells to Sertoli cell lineage. SRY binds and regulates *Ptgds*, whose product catalyzes the conversion of prostaglandin H2 to prostaglandin D2, critical for recruitment of supporting cells into Sertoli cells (Wilhelm et al., 2005). *Fgf9* and *Gdnf* are involved in Sertoli cell proliferation (Colvin et al., 2001) and M germ stem cell niche development (Oatley and Brinster, 2008), respectively. SRY, but not SOX9, binds in vivo to the promoters of various ovarian-differentiating genes and suppresses their activation by WNT/β-catenin signaling. SRY activates *Cyp26b1*, which encodes an enzyme degrading retinoid acid (RA), thereby arresting the RA-induced meiosis and maintaining the germ cells in the M lineage (Bowles et al., 2006). Other notable SRY targets, such as *Wnt5a* and *Dhh*, are respectively involved in primordial germ cell migration and testis differentiation (Chawengsaksophak et al., 2012), and peritubular cell development, seminiferous tubule formation, and Leydig cell differentiation (Clark et al., 2000; Yao et al., 2002). Gene inactivation or mutations of other SRY targets result in abnormalities in sex determination and differentiation (Table S4). Significantly, Gene Ontology analysis identified many of the SRY targets, encoding (1) various cell adhesion molecules, such as cadherins, claudins, and integrins, and (2) cytoskeletal proteins, such as actins, and (3) signaling molecules, such as platelet-derived growth factor β (*Pdgfb*) and its receptor (*Pdgfrb*), and (4) MAP kinases, which are important in the Sertoli cell-Sertoli cell junction signaling, and cell-cell adherens and tight junction, and actin cytoskeletal signaling pathways. Because Sertoli cell-Sertoli cell junction signaling and adhesion are critical in testis cord formation (Cool et al., 2012), these SRY targets must serve important functions in the process(es).

SRY and SOX9 Targets Share Significant Functional Roles in Testis Differentiation

SRY and SOX9 bind to the same promoter regions of a large number of common targets in the supporting cells of M gonads. Among the notable ones, *Ptgds*, *Dhh*, *Cyp26b1*, *Pdgfb*, *Pdgfrb*, and others play key roles in early developmental and cellular events in testis differentiation, as discussed above. Because the targets were identified with independent biological replicates at E11.5 for SRY and E12.5 for SOX9, respectively, such similarities in binding patterns suggest that SOX9 could bind to the same *cis* elements and assume the gene regulatory functions of SRY on their common targets. This postulation is supported by our data showing that SRY and SOX9 were capable of (1) regulating each other's target genes; (2) interacting with the same coactivators, e.g., SF1, in transactivating respective target genes; and (3) interfering with the WNT/β-catenin transactivation of ovarian-differentiating genes in an in vitro and transient transfection reporter system. We reasoned that the respective transcription factors were expressed at high levels in such in vitro assays, thereby increasing their ability in binding to likely

conserved *cis* elements and/or coregulators and regulate the expression of the respective promoter-directed reporter or endogenous target genes in Sertoli cells. Indeed, under diseased or transgenic conditions, a constitutively active *Sox9* could perform certain functions of SRY in testis differentiation without a functional *Sry* gene in the developing gonads (Bishop et al., 2000; Qin et al., 2004; Vidal et al., 2001). Importantly, SOX9 also regulates a distinct set of unique target genes and exerts its own gene regulatory program in testis differentiation. Mutation and gene inactivation of the *SOX9*/*Sox9* gene result in XY partial or complete sex reversal in both patients and transgenic mice (Chaboissier et al., 2004; Foster, 1996), confirming SOX9 being critical for the continuation of testis differentiation, past SRY actions. Hence, our study shows that under normal conditions, SRY regulates a variety of genes, which determine the fate of the Sertoli cells, define the germ cells in the M lineage, promote Sertoli cell proliferation and recruitment, and induce the newly differentiated Sertoli cells to form tight and adherens junctions, important for testis cord formation. Once accomplished, it passes on these functions to *Sox9*, which also activates its own gene regulatory program and continues the testis differentiation.

Although *Sry* plays a cardinal role in activating the M sex determination and early events in testis differentiation, the developmental pathway is complex and likely involved the actions of *Sox9* and other sex-determining genes essential in differentiation of a functional testis (Svingen and Koopman, 2013). As discussed, SRY likely requires interaction(s) with cofactors, such as SF1 and SP1, for its gene regulatory functions (Sekido and Lovell-Badge, 2008; Wu et al., 2009). Significantly, it also interacts with various chromatin modulators, such as PARP1 and KRAB-O-KAP1-HP1 gene-silencing complex (Li et al., 2006; Oh et al., 2005), capable of epigenetically remodeling chromatin landscape of its target genes into ''open'' or ''close'' domains (Oh and Lau, 2006), critical for the accessibility of transcription factors controlling their expression beyond SRY actions. At present, the exact mechanisms responsible for SRY and SOX9 regulation or chromatin modification of their common and unique targets are uncertain because the full spectrum of their interactive partners is still incomplete. The availability of the present data sets on SRY and SOX9 targets in the mouse embryonic gonads at the time of sex determination has provided significant opportunities for detailed investigations on the specific contributions of these transcription factors and their targets in mammalian sex determination and differentiation.

EXPERIMENTAL PROCEDURES

Single cells were isolated from E11.5 and E12.5 M gonads (Li et al., 2012; Taketo et al., 2005) and processed for ChIP (Cai et al., 2006). Microarray hybridization was performed by the Custom Array Service at NimbleGen Systems, using the 385K RefSeq Promoter Array of the mouse MM8 genome. All ChIP-Chip experiments were performed in biological replicates. Significant binding peaks were identified by the Mpeak program, version 2.0 (Zheng et al., 2013). The ChIP-Chip signals and differential gene expression patterns (at log2 scale) between M versus F and changes in expression levels in supporting cells at different stages of mouse gonadal development (Jameson et al., 2012) were visualized with the Genome Browser (Karolchik et al., 2011). The WIG files containing these results are provided in Folder S1. They can also be downloaded directly from http://labs.medicine.ucsf.edu/chrislau/DataSoftware.html. Gene Ontology analyses were performed with the knowledge base IPA (http://www.ingenuity.com) using the Core Analysis module and the human, rat, and mouse reference set with 587 canonical pathways.

Quantitative PCR was used to confirm SRY or SOX9 bindings on selected targets (Table S3). Promoters of selected SRY or SOX9 targets were inserted in front of a luciferase reporter and analyzed in the Sertoli cell line, MSC1, or the granulosa cell line, KK1 (Li et al., 2006; Oh et al., 2005). To determine the effects of SRY or SOX9 on endogenous target genes in Sertoli cells, mouse *Sry* or *Sox9* was transiently transfected in MSC1 cells and analyzed with qRT-PCR (Table S3).

See Supplemental Experimental Procedures for more details and references in Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.055.

AUTHOR CONTRIBUTIONS

Y.L. performed all experiments. M.Z. performed most of the computation and statistical studies. Y.-F.C.L. conceived the idea, performed the IPA Gene Ontology and Genome Browser analyses, and wrote the manuscript with valuable input from the coauthors.

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