Down-Regulation of Endogenous Wt1 Expression by Sry Transgene in the Murine Embryonic Mesonephros-Derived M15 Cell Line

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Abstract. Wt1 is one of numerous candidate genes comprising the hypothetical chain of gene expression essential for male sex differentiation of the bipotential indifferent gonads during embryogenesis. However, the evidence in the literature is ambivalent regarding the position of Wt1 relative to Sry in this scheme; Wt1 might act either upstream or downstream of Sry. In the present study, the effects of Sry expression upon Wt1 were investigated using M15 cells (XX karyotype), which are derived from murine embryonic mesonephros and express endogenous Wt1. In 3 stably-transformed Sry-expressing M15 cell lines, we showed that the expression levels of the mRNAs coding for all 4 isoforms of the WT1 proteins were down-regulated. Similarly, Wnt4 expression was down-regulated in these cell lines. Silencing of Sry in the transformed cell lines using ribozymes or short hairpin RNAs (shRNAs) resulted in elevated levels of Wt1 and Wnt4 expression. These results strongly indicate that Wt1 might be under the control of Sry during gonadal differentiation in the mouse. In electrophoretic mobility shift assays (EMSA), we demonstrated that the 3.7 kb 5’-upstream DNA stretch of Wt1 containing potential Sry binding sites was capable of forming molecular complexes with nuclear protein(s) from Sry expressing cells but not with those from control non-Sry expressing cells. In summary, our present results support the notion that Wt1 is located downstream of Sry and down-regulated by the sex determining gene. Although the precise biological meaning of the present findings have yet to be clarified, it is possible that Wt1 plays a dual role during gonadal differentiation, i.e., turning on Sry expression on one hand, and being down-regulated by its product, Sry, on the other, possibly forming a type of negative feedback mechanism. Further work is needed to substantiate this view.

Key words: Sex determination, Sry, Wt1, shRNA, Ribozyme

SRY (Sry in the mouse) [1, 2] is the ‘master gene’ for initiating male sex differentiation in the bipotential indifferent gonads of mammals [3]. The Sry-encoded protein, SRY (Sry in the mouse), possesses an HMG domain and acts as a transcription factor, triggering a cascade of gene expression, i.e., the Sry cascade [4, 5].

Numerous genes have been suggested in the literature as candidate genes comprising the hypothetical chain of gene expression essential for male differentiation of the primordial gonads during embryogenesis, e.g., Wt1 [6–9], Sf1/Ad4BP [10–12], Wnt4 [13], Amh (MIS) [14, 15] and Sox9 [16–18] among many others (see [19, 20] for recent reviews).

As a part of our effort to experimentally identify the target genes of Sry action, we introduced an Sry expression vector driven by CMV promoter into a non-Sry expressing TM-4 cell line derived from cultured mouse Sertoli cells [21], and a mouse ES cell line with the XX karyotype, TMA-18 [8]. In the TMA-18 cells, the Sry transgene stably incorporated into their genomes induced expression of endogenous Wt1 [8]. On the other hand, in TM-4 cells, the Sry transgene induced expression of endogenous Cyp19a1 (synonymous to P-450arom), and Wt1 expression was not induced in these cells. These results suggested that the effects of Sry expression might vary according to the differentiation status of the cells and that the appropriate combinations of cells of different differentiation statuses and Sry transgene could give us clues toward understanding the molecular mechanisms underlying sex differentiation in the mouse.

It has been shown that WT1 is expressed in the lateral mesenchyme and that it is involved in genitourinary development in humans [22]. Subsequently it was demonstrated in the mouse that WT1 is expressed from 9.0 dpc [23] before the onset of the surge of Sry expression, i.e., 10.5 dpc [24]. It was hypothesized, therefore, that WT1 might be located upstream, rather than downstream, of Sry in the chain of gene expression leading to sexual differentiation of the primordial gonads in the mouse. Concomitantly, on the other hand, it has also been shown that the WT1 promoter contains a consensus DNA binding sequence for Sry [25], suggesting the possibility that WT1 might be regulated by Sry.

Human WT1 expression vector constructs transfected into 3 different types of cultured cell lines, i.e., murine TM4, human HeLa, and human NT2D1, either transiently or stably have been shown to be capable of activating the promoter of the co-transfected full-length human SRY DNA construct [26]. However, the WT1 binding site present in the human SRY promoter is not conserved in the mouse [26], raising a question about simple generalization of the findings made in the human WT1-SRY system to the murine equivalent.

A mouse mesonephric cell line with the XX karyotype, M15, has been known to express high levels of the 4 wild-type WT1 protein isoforms, i.e., with or without the 17 amino acids encoded by exon 5 (+ or –exon 5) and with or without KTS (+ or –KTS) between the 3rd and 4th zinc fingers [27]. It should serve, therefore, as an appropriate cell line for investigation of the effects of Sry expression on the endogenous expression of Wt1. In the present study, we attempted to examine the effects of Sry expression upon the endogenous expression of Wt1 in M15 cells by employing ribozyme and RNAi technology to inactivate the Sry transcripts.

Materials and Methods

Cell culture

The M15 cell line (XX karyotype) originally derived from mesonephric epithelial cells of the mouse embryo [27] was kindly provided by Dr. T. Noce, Mitsubishi-kagaku Life Science Institute, Machida-shi, Tokyo, Japan. The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma, St. Louis, MO, USA) containing heat inactivated FBS [10% (v/v); Gibco, Rockville, MD], penicillin (10,000 U/l; Sigma), and streptomycin (10 mg/l; Sigma) (this medium will be referred to as DMEM/10 below) under a humidified atmosphere of 5% CO2 in air at 37 C.

Transfection of plasmids

Plasmids were transfected to the cell cultures using PolyFect Reagent (Qiagen, Valencia, CA, USA) according to manufacturer’s instructions. For establishment of stably transformed cell lines, cells transfected with linearized plasmids were cultured for 48 h in DMEM/10. Then, the medium was changed to fresh DMEM/10 containing G418 (500 mg/ml; Sigma) and the cells were cultured for an
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additional 10 days. The surviving cell colonies were randomly picked up, expanded in fresh DMEM/10, and established as stably transformed cell lines. Cellular proliferation rates were determined using a CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA).

Reverse transcriptase mediated polymerase chain reaction (RT-PCR)

Two micrograms of total RNA isolated from the cell cultures using Isogen (Wako, Osaka, Japan) were heat-denatured and reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Promega). The primer pairs used for PCR amplification of the DNA sequences of the relevant target genes are listed in Table 1. The optimal conditions for PCR amplification were determined for each primer pair prior to the experiments. The size of the target amplicons was confirmed by electrophoresis of the PCR products on 2% agarose gels in TAE buffer. Densitometric analysis of the electrophoretograms was conducted using NIH ImageJ (NIH, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/).

Expression vector constructs

Sry and Wnt4: The Sry expression vector used in the present series of experiments, pCMV/mSry, was described previously [8, 21]. An empty vector for the control mock-transformation experiments was constructed by removing the Sry coding region from pCMV/mSry by XbaI digestion and self-ligating the linearized plasmid. Wnt4 cDNA was generated by reverse transcription of the total RNA extracted from the gonads of C57BL/6J female mice on 13.5 dpc as described previously [28] and was PCR-amplified using the primer pair 5’-ACGGCACCATGAGCCCCCGTTC-3’ (forward) and 5’-AGGCCACACCTGCTGAAGAG-3’ (reverse). The 1 kb segment of Wnt4 cDNA.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sets</th>
<th>Sequences</th>
<th>Annealing temperature</th>
<th>Size of products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sry</td>
<td>Sry-F</td>
<td>5’-GGGACTGGTGAGCAATGTCT-3’</td>
<td>56 C</td>
<td>440 bp</td>
</tr>
<tr>
<td></td>
<td>Sry-R</td>
<td>5’-ATCAACAGGGCTTGGCAAATAA-3’</td>
<td>56 C</td>
<td>488 bp</td>
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<tr>
<td>Wt1++</td>
<td>Wt1-F exon5+</td>
<td>5’-GCTTGGAGCTCCAGCTGAT-3’</td>
<td>56 C</td>
<td>456 bp</td>
</tr>
<tr>
<td></td>
<td>Wt1-R KTS+</td>
<td>5’-GAGGCTTTTCACCTGTTTAC-3’</td>
<td>56 C</td>
<td>450 bp</td>
</tr>
<tr>
<td>Wt1--</td>
<td>Wt1-F exon5-</td>
<td>5’-TACCTTAAAGGGCCAAGGTAT-3’</td>
<td>56 C</td>
<td>437 bp</td>
</tr>
<tr>
<td></td>
<td>Wt1-R KTS-</td>
<td>5’-GAAGGGCTTTTCACCTGTTTAC-3’</td>
<td>56 C</td>
<td>437 bp</td>
</tr>
<tr>
<td>Wnt4</td>
<td>Wnt4-F</td>
<td>5’-ACGGCACCATGAGCCCCCGTTC-3’</td>
<td>56 C</td>
<td>374 bp</td>
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<tr>
<td></td>
<td>Wnt4-R</td>
<td>5’-AGGCCACACCTGCTGAAGAG-3’</td>
<td>56 C</td>
<td>374 bp</td>
</tr>
<tr>
<td>Sox9</td>
<td>Sox9-F</td>
<td>5’-GTGGCAAGTATGGTCCCA-3’</td>
<td>50 C</td>
<td>321 bp</td>
</tr>
<tr>
<td></td>
<td>Sox9-R</td>
<td>5’-GAACAGACACTCACACCTCT-3’</td>
<td>50 C</td>
<td>321 bp</td>
</tr>
<tr>
<td>Gata4</td>
<td>Gata4-F</td>
<td>5’-ATAACCCGGCCTCATTAAAG-3’</td>
<td>58 C</td>
<td>472 bp</td>
</tr>
<tr>
<td></td>
<td>Gata4-R</td>
<td>5’-GACAAGCTCAGAGACAGAC-3’</td>
<td>58 C</td>
<td>472 bp</td>
</tr>
<tr>
<td>Sfi1</td>
<td>Sfi-F</td>
<td>5’-TGTTGTCAGTGTCACCCCTAT-3’</td>
<td>60 C</td>
<td>212 bp</td>
</tr>
<tr>
<td></td>
<td>Sfi-R</td>
<td>5’-TCCGTCAGCTCTGTAATTGCT-3’</td>
<td>60 C</td>
<td>212 bp</td>
</tr>
<tr>
<td>Amh</td>
<td>AMH-F</td>
<td>5’-TCCTACATCTGCTGAAATGCTATGGGAGC-3’</td>
<td>65 C</td>
<td>276 bp</td>
</tr>
<tr>
<td></td>
<td>AMH-R</td>
<td>5’-GGAAGGCTTTTCACCTGTTTGAGC-3’</td>
<td>65 C</td>
<td>276 bp</td>
</tr>
<tr>
<td>Atrx</td>
<td>Atrx-F</td>
<td>5’-AGCAAGATGATGGTGAACTCCG-3’</td>
<td>65 C</td>
<td>236 bp</td>
</tr>
<tr>
<td></td>
<td>Atrx-R</td>
<td>5’-TGTTCTCTGTAATCTCCTCTC-3’</td>
<td>65 C</td>
<td>236 bp</td>
</tr>
<tr>
<td>Hprt</td>
<td>Hprt-F</td>
<td>5’-GAAAGACTCTTCAGAGATCTGAT-3’</td>
<td>56 C</td>
<td>570 bp</td>
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<tr>
<td></td>
<td>Hprt-R</td>
<td>5’-TGGCAACATCAAAGAGACTCTTCG-3’</td>
<td>56 C</td>
<td>570 bp</td>
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</table>
obtained was inserted into pGEM-T Easy vector (Promega). To obtain a mammalian expression vector for \textit{Wnt4} (pCX-Wnt4) the EcoRI/EcoRI fragment of \textit{Wnt4} cDNA (Fig. 4) was inserted into pCX-blank, which was made by removing the EGFP sequence from pCX-EGFP [29] (kindly provided by Dr. M. Okabe, Osaka University, Osaka, Japan) by EcoRI restriction. Similarly, the mammalian expression vector for \textit{Sry}, pCX-Sry, was constructed by inserting the blunted XbaI/SpeI fragment of the \textit{Sry} coding region into a blunted linearized pCX-blank. Because pCX-Sry has an artificial intron, the primer set for RT-PCR amplification of the transcript was positioned so that they contained the intron as a marker specific to the transcript; the primer pair used consisted of forward: 5’-CTG-ACC-GCG-TTA-CTC-CAA-CA-3’ and reverse: 5’-GGT-ATT-TCT-CTC-TGT-GGA-3’.

\textbf{Table 2.} List of sense strand DNA sequences for shRNA targeted to \textit{Sry} (see text for details)

<table>
<thead>
<tr>
<th>Name</th>
<th>Sense strand DNA sequences for shRNA</th>
<th>Target site*</th>
</tr>
</thead>
<tbody>
<tr>
<td>tRNA-shRNA1</td>
<td>TACAACCTTCTTCGAGTGAATTGAGAAAAAGGG</td>
<td>295–323</td>
</tr>
<tr>
<td>tRNA-shRNA2</td>
<td>TACAGGCGAAGACCTTGAGTAGCAGTGAATGAAAAGGT</td>
<td>337–365</td>
</tr>
<tr>
<td>tRNA-shRNA3</td>
<td>CCTGTTGATATCCCAATTTGGGACTGAGTGAAGAAATGACGGT</td>
<td>403–431</td>
</tr>
<tr>
<td>tRNA-shRNA4</td>
<td>TACTTATAACAGCTGAAATGGAATGAGAAAAATCACC</td>
<td>1105–1133</td>
</tr>
<tr>
<td>tRNA-shRNA5</td>
<td>ATCAGTGTGAGCAATCAAAAGAGAAAATGCTGAGTCAAAATGAT</td>
<td>1123–1151</td>
</tr>
<tr>
<td>tRNA-shRNA6</td>
<td>ATACACCATAACCCAGAGAACATGAAATTGCTGAGTCAAAATGAT</td>
<td>1136–1164</td>
</tr>
<tr>
<td>tRNA-shRNA7</td>
<td>CACCTGCAACAGCTTGGGAGTGAAGAAATGCTGAGTCAAAATGAT</td>
<td>1153–1181</td>
</tr>
<tr>
<td>tRNA-shRNA8</td>
<td>GCACAGGCCCTGTTGGGAGTGAAGAAATGCTGAGTCAAAATGAT</td>
<td>1160–1188</td>
</tr>
<tr>
<td>U6-shRNA1</td>
<td>GCAATAGAGGTTGGAAGTCAAGTCGTGCACTGTTTAGATCTTCAATCTTTCTCTTTTT</td>
<td>172–190</td>
</tr>
<tr>
<td>U6-shRNA2</td>
<td>GATGACATCTACAAAGCTGAGTCTGCCGTTGATCTTCAATCTTTCTCTTTTT</td>
<td>179–197</td>
</tr>
<tr>
<td>U6-shRNA3</td>
<td>AGGGAATAACCCCAATTATATCAGCTGCTGCCGTTATAGTTGTGATTTTTCTCTTTTTTT</td>
<td>198–216</td>
</tr>
<tr>
<td>U6-shRNA4</td>
<td>GAGGAAATACCCCAATTATTAACGCTGCTGCCGTTATAGTTGTGATTTTTCTCTTTTTTT</td>
<td>199–217</td>
</tr>
<tr>
<td>U6-shRNA5</td>
<td>ACICAAATTTAAATGTCAAGCTGCTGTCGCTTTATAGTTGTGATTTTTCTCTTTTTTTTT</td>
<td>206–224</td>
</tr>
<tr>
<td>U6-shRNA6</td>
<td>GTGTAAGCTGTCAGGAGAACGTGCTGCTGCCGTTCTCTGCGACAATTGATTTTTCTCTTTTTTTTT</td>
<td>237–255</td>
</tr>
<tr>
<td>U6-shRNA7</td>
<td>ACACAGCGCGCTACATACAGGTAGAGTATAGTTGTGAGTCAAAATGAT</td>
<td>314–332</td>
</tr>
<tr>
<td>U6-shRNA8</td>
<td>GCAGAGCAGCCCTGTTGGGAGTGAAGAAATGCTGAGTCAAAATGAT</td>
<td>828–846</td>
</tr>
</tbody>
</table>

*The base numbers indicate the target sites in the coding region of \textit{Sry} (NM_011564).

\textit{tRNA-ribozyme complexes}: Vectors for expression of \textit{tRNA-ribozyme complexes} targeted to \textit{Sry} was constructed using pPUR-tRNA plasmid [30], which includes a promoter for the human \textit{tRNA\textsuperscript{Val}} gene between the EcoRI and BamHI sites of pPUR
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The expression vector constructs of the ribozymes targeted to Sry, originally designed and confirmed to cleave Sry mRNA in vitro and in cells by our group have been described elsewhere [31, 32]. Among these constructs, the most effective one, pPUR/tRNARz3 [31], was used for the present series of experiments.

ShRNAs: Short hairpin-RNA (shRNA) expression vectors driven by a tRNA promoter were constructed by inserting annealed sense and antisense oligo-DNA strands (the sequence of the sense strands is identical to that of the encoded RNA if T is replaced by U) into the SacI and KpnI sites of pPUR-tRNA. The core sequences of each vector are shown in Table 2. Tandemly connected (2 to 9) shRNA-coding oligo-DNA constructs were made by inserting an EcoRI-MfeI fragment of one vector into the EcoRI site of another vector. Similarly, shRNA expression vectors with a human U6 promoter were constructed by inserting annealed sense and antisense oligo-DNA into the BspM1 site of piGENeU6 vector (iGENE Therapeutics, Tsukuba, Japan; http://www.iGENe-therapeutics.co.jp). The predicted favorable shRNA sequences were obtained using an algorithm originally developed by our group [33, 34]. The sequence of U6-shRNA1, a representative example of the U6-shRNA series presently designed, is as follows: sense, 5'-CAC-CGC-A1A-GAG-gTT-GAA-GgT-CAA-CGT-GTG-CTG-TCC-GT-T-GAT-CTT-CAA-TCT-CTG-TGC-TTT-TT-3', and antisense, 5'-GCA-TAA-AAA-GCA-CAG-AGA-TTG-AAG-ATC-AAC-GGA-CAG-CAC-ACG-TTG-AcC-TTC-Aac-CTC-TaT-GC-3'. The construct contains a loop sequence (underlined) flanked by a mutated sense and an antisense sequence specific to Sry. Introduction of multiple C to T (or A to G) mutations (indicated by lowercase letters) into the sense strand rendered the plasmids genetically stable and did not affect silencing activity [35]. The core sequence of each vector is shown in Table 2. U6-T7 was used as the control for the vectors of U6 series.

Electrophoretic Mobility Shift Assay (EMSA)

For EMSA of nucleoprotein binding to the Wt1 promoter region, the procedures described by Rodgers et al. were essentially followed [36]. Briefly, the 3.7 kb 5' upstream region of Wt1 from the transcription initiation site was amplified by PCR method. The following primer pair was employed for this purpose: 5'-TGA-AGC-CCA-GAT-GGA-AGG-3' (forward) and 5'-GTT-TCC-AGA-CTA-GCG-CAG-TT (reverse). Biotin-labeled forward primers were used for production of a PCR-generated probe for EMSA analysis [36]. The biotin-labeled PCR products and protein to be assayed were incubated for 30 min at room temperature in solution containing 1.5 µg of poly(dI-dC), 25 mM HEPES-KOH (pH 7.9), 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 8% glycerol. The solution was transferred to the wells of 1.2% agarose gel plates and electrophoresed in 0.5 x TBE buffer. The DNA was blotted onto Hybond-N membranes (Amersham Bioscience, Amersham, UK). After blotting with 5% ECL (electrochemiluminescence) blocking reagent (Amersham Bioscience), the membrane was probed with streptavidin-alkaline phosphatase conjugate which are bound to the biotinylated DNA. For the chemiluminescence detection, ECL Plus (Amersham Bioscience) was employed. Photons were detected with a CCD camera (Model LAS-3000mini; Fuji Film, Tokyo, Japan).

Immunoblotting

Transfection of pCMV/mSry-FLAG into the M15 cell culture was done at 80% confluence in a 60 mm non-surface-treated plastic culture dish (#1010-060; IWAKI, Tokyo, Japan) using PolyFect reagent (QIAGEN). Forty-eight hours after transfection, proteins were extracted from the cytoplasm and the nuclei using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA). The extracts were separated by SDS-PAGE and electrophoretically transferred (Trans-blot SD Cell, cat #170-3940, BioRad, Mississauga, ON, USA) at 200 V for 90 min onto a polyvinylidene difluoride membrane (Hybond-P; Amersham Bioscience). After blotting with 5% ECL blocking reagent, the membrane was incubated with anti-FLAG M2 monoclonal antibody (#F3165, Sigma, St. Louis, MO, USA; 1:1000 dilution) for 1h at room temperature. After washing, the blot was incubated with horseradish peroxidase-conjugated anti-mouse IgG (#NA931, Amersham Bioscience), and bands were detected using ECL Plus (Amersham Bioscience).
Results

Establishment of transformed M15 cell lines expressing Sry

We established 8 cloned cell lines of M15 stably transformed with pCMV-mSry (Fig. 1A) as described in Materials and Methods. Among these lines, the expression of Sry transgene was confirmed by RT-PCR analysis in 3 lines, i.e., clone 1 (C1), clone 4 (C4), and clone 6 (C6) (Fig. 1B). The proliferation rates in the transformed cell lines were not affected by expression of the foreign gene compared with those of non-transformed M15 cells (Fig. 1C). The Sry-expressing transformed cell lines, i.e., C1, C4, and C6, were renamed Sry#1, Sry#2, and Sry#3, respectively and used in the following experiments. As controls, 2 cell lines (mock#1 and mock#2) were established by stably transforming with the empty plasmid construct (pCMV-blank).

Expression of putative Sry-cascade genes in transformed cell lines

The effects of expression of the Sry transgene upon the expression levels of 7 genes that are putatively under either direct or indirect control of Sry, i.e., Wt1, Wnt4, Sox9, Gata4, Sf1, Amh (MIS), and Atrx, were examined in the Sry#1, Sry#2, and Sry#3 cell lines by RT-PCR method. We found that the expression levels of all 4 isoforms of the Wt1
mRNA and those of the Wnt4 mRNA were lower in the 3 Sry-expressing transformed cell lines compared to those in the non-transformed M15 cell line and control cell lines (mock#1 and mock#2). On the other hand, Sox9, Gata4, and Atrx were expressed at equal levels in Sry#1, Sry#2 and Sry#3 as well as in mock#1 and mock#2. The transcripts of Amh and Sf1 were not detected in the Sry-expressing cell lines and control cell lines (Fig. 2).

**Restoration of Wt1 expression levels by silencing Sry**

In order to confirm that Wt1 expression is indeed under the control of Sry and is down-regulated in our experimental systems, the shRNAs intended to silence Sry action were designed as listed in Table 2, and the expression vectors coding for these shRNAs were constructed. The effect of each construct upon Wt1 and Wnt4 expression was assayed by co-transfecting the shRNA expression vectors with pCX-Sry into M15 cells. Seventy-two hours after transfection, total RNAs were extracted from the cells and RT-PCR analyses of the Sry expression levels were performed (Fig. 3A). The relative silencing effect of each shRNA construct was evaluated and presented as shown in Fig. 3B. Among the constructs examined, U6-shRNA1 exhibited the highest silencing effect toward Sry (Fig. 3B). This construct was exclusively employed for further experiments.

When the Sry#1 cells were transfected with the silencing vectors (U6-shRNA1 and tRNA-Rz3) (Fig. 4A), the expression levels of all 4 isoforms of Wt1 mRNA and the levels of Wnt4 transcripts were clearly elevated, indicating that Wt1 and Wnt4 are regulated by Sry. It must be pointed out, however, that the elevated levels reached were approximately 1/5 of the control M15 levels (Fig. 4B) and restoration was not complete, probably owing to the leaky silencing action of the ribozyme and shRNA employed.

The questions that arise are whether the suppressing effects of Sry on Wt1 expression might possibly be mediated by Wnt4 and whether the decreased levels of Wt1 might be the result of reduction of Wnt4 expression. Therefore, we carried out experiments, in which Sry#1 cells were transfected with Wnt4 expression vector. No changes were observed in the expression levels of all the isoforms of the Wt1 transcripts before and after transfection of the cells with Wnt4 vector (Fig. 4B).

**EMSA analysis of Wt1 promoter region**

The results of the aforementioned experiments strongly pointed to the possibility that Wt1 might be regulated directly by Sry, thereby locating the gene downstream, as suggested earlier by Toyooka et al. [8], rather than upstream of Sry in the chain of gene expression leading to differentiation of the gonads during embryogenesis in the mouse.

To further clarify the problem of possible control
of Wt1 by Sry, we analyzed the base sequence of the 5' region of Wt1 spanning 3.7 kb upstream from the transcription initiation site for the possible presence of Sry binding motifs using TFSEARCH ver. 1.3 (the program developed by Y. Akiyama is available on-line at http://mbs.cbrc.jp/research/db/TFSEARCHJ.html). The potential Sry binding sites identified in the region are shown in Fig. 5A.

A part of the 5' region containing the theoretically-predicted Sry binding sites was amplified by PCR method using a primer pair with the 5' end of the forward primer is labeled with biotin and used as the probe. EMSA analyses were performed as described in Materials and Methods. Mobility shift of the probe DNA was clearly detected when the nuclear protein extracts from the Sry#1 cells were added to the probe, but not when similar extracts from non-transformed M15 cells were used (Fig. 5B). These results strongly indicated that the Sry protein present in the nuclear extract was indeed capable of binding to the 5' upstream region of Wt1.

**Discussion**

Targeted gene disruption by homologous recombination of Wt1 showed that Wt1 is essential for gonadal development [37] and for differentiation of germ cells as well as the somatic cell lineages in the testis [38]. It has been known, furthermore, that mice lacking +KTS isoforms of Wt1 show a complete XY sex reversal due to dramatic reduction of Sry expression levels [39]. In humans, WT1 recombinant protein was demonstrated to interact with the promoter region of human SRY and increases the promoter activity in vitro [26]. Although these results strongly indicate that WT1 is located upstream of SRY in the chain of events leading to gonadal differentiation in
mammals, they also suggest that in the mouse, \(Wt1\) expression might in fact be regulated by \(Sry\) under the pluripotent conditions of cells [8]. The latter notion is supported by the fact that no \(Wt1\) binding site appears to be conserved in the murine \(Sry\) promoter region [26].

In the present study, we investigated the effects of unscheduled expression of \(Sry\) stably incorporated into the genome upon endogenous \(Wt1\) expression in M15 cells. Our results clearly demonstrated in these cells that both \(Wt1\) and \(Wnt4\) expression was down-regulated by the \(Sry\) transgene expressed at high levels. We investigated the expression levels of the 4 isoforms of \(Wt1\) and found that their expression levels were reduced in \(Sry\)-silenced cells. This was confirmed by EMSA analyses of the 5' upstream region of \(Wt1\). The results showed that the expression levels of \(Wt1\) and \(Wnt4\) in the \(Sry\)-silenced cells were approximately 1/5 of those in the control M15 cells, and restoration was incomplete, probably owing to leaky inactivation of \(Sry\) mRNA.
of the WTI transcripts, since differential regulation of the expression levels of WTI protein isoforms might play important roles in the differentiation of embryonic cells (for a review, see [40]). Our results confirmed that the expression levels of all the WTI mRNA coding for the WTI isoforms were equally affected by Sry expression, indicating that Sry did not regulate WTI expression post-transcriptionally.

In our experiments, the causal relationship between the down-regulation of WTI/Wnt4 expression and Sry is of little doubt because 1) down-regulation of the expression of the 2 genes was observed in all 3 stably-transformed Sry-expressing cell lines (Sry#1, Sry#2, and Sry#3), which were cloned independently; 2) no down-regulation of WTI/Wnt4 expression took place in the mock-transformed cell lines (mock#1 and mock#2); and 3) silencing Sry in a transfomed cell line (Sry#1) using either the ribozyme or shRNA expression vector up-regulated the levels of WTI/Wnt4 expression (Fig. 5B). The fact that the restored expression levels of WTI/Wnt4 did not fully reach those in normal M15 cells might be explained by leaky inactivation of Sry mRNA by the ribozyme or shRNA.

Furthermore, introduction of Wnt4 expression plasmids into the Sry-expressing cells caused no change in the WTI expression levels (Fig. 5B), suggesting that inhibition of WTI expression was not mediated by reduced expression of Wnt4. Sim et al. [41] reported establishment of M15 cells that expressed the Deny-Drash syndrome (DDS) mutant form of WTI (R394W) in addition to the endogeneous WTI. The expression levels of Wnt4 were significantly reduced in the presence of the DDS WTI mutant, leading Sim et al. [41] to conclude that Wnt4 is likely under the control of WTI. Therefore, the reduction in the expression levels of Wnt4, as observed in the present study, might have been a result of changes in the expression levels of WTI, rather than vice versa. The results obtained by transfection experiments of Wnt4 expression vector support this inference.

Interestingly, while the lack of Wnt4 gives rise to masculinization of XX gonads [13], the same gene is required for Sertoli cell differentiation downstream of Sry and upstream of Sox9 and Dhh [42]. Sox9 is up-regulated by transient expression of Sry specifically in the precursors of Sertoli cells [43]. In our experiments, Sox9 was expressed continuously when Wnt4 was down-regulated by the Sry expression (Fig. 2), implying that there might be another regulatory pathway for Sox9 expression in M15 cells.

EMSA analysis of the 3.7 kb DNA fragment of the 5' upstream region of WTI where the predicted Sry binding motifs are present, revealed that the region is capable of binding to the nuclear protein of Sry-expressing cells. Sry is known to be localized in the nucleus and absent in the cytoplasm of Sry-expressing cells [44, 45]. We carried out experiments using a plasmid coding for FLAG-labeled Sry, pCMV/mSry-FLAG (see Materials and Methods), and confirmed the localization of Sry in the nucleus and absence in the cytoplasm of transfected M15 cells (data not shown). Our results strongly indicate that Sry binds to the 5' upstream region of WTI and might influence directly its expression, although the possibility cannot be excluded that other yet unidentified transcription factors under the control of Sry might indirectly regulate WTI expression.

As mentioned before, the Sry expression in normal mouse embryos is limited to a very narrow time window during the development of indifferent gonads [24]. Balanced and well-timed expression of Sox9 and WTI in topographically-restricted regions during embryogenesis might be crucial in assuring the normal development of not only gonads but also other fetal organs. Although the precise biological meaning of the present findings have yet to be clarified, it is possible to speculate that WTI might play a dual role during gonadal differentiation, i. e., turning on Sry expression on one hand, and being down-regulated by Sry on the other, possibly forming a type of negative feedback mechanism. Further work is certainly needed to substantiate this view.

In this context it is pertinent to point out that a DNA region was identified in the 5' upstream stretch of Sry that forms complexes with the nuclear proteins of 11.5-dpc gonads, but not with those from 12.5 and 13.5-dpc [46]. This region, however, does not contain the conserved WTI binding sites in mice [26], as mentioned in the Introduction, and no solid experimental evidence has so far been available in the literature to suggest either the presence or absence of WTI in the Sry-binding protein of 11.5-dpc gonads.

In fact, the profiles of gene expression during gonadal differentiation are so complex, that no simple scheme for the chain of molecular events
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underlying the phenomenon may be clearly envisaged with the knowledge currently available to us in the literature. The seemingly contradictory actions of a gene in the mesh of gene interactions underlying gonadal differentiation are not peculiar to Wt1 as suggested in the present report. A case in point is Sf1/Ad4bp which has been known to play a central role in gonadal development. While it contributes to transcriptional activation of several relevant genes, not just those required for male development, including Sox9 and Amh, it also does so to the activation of genes that can have an antagonistic effect on Sertoli cell differentiation, such as Dax1 [47]. Furthermore, evidence is available in the literature indicating that Sf1 expression may be up-regulated by Sox9, suggesting that the former might in fact serve as a target of the latter [48].

Toyooka et al. [8] reported that the Sry transgene up-regulated Wt1 expression in a transformed ES cell line. The diametrical difference between the results obtained using 2 cell lines, i.e., M15 derived from mesonephric epithelial cells and pluripotent ES cells, might reflect the different context of the genomic gene expression in the respective cell lines and might have possibly been caused by the difference in the co-factor(s), which might work cooperatively with Sry protein. For example, Sry contains a glutamine-rich (Q-rich) domain known to play an essential role in sex determination [49], and 3 proteins that are capable of interacting with this Q-rich domain of Sry and influence the sex determination process, have so far been identified and reported in the literature [50]. Further careful studies of the proteins that interact with Sry in cells, either in model systems of cultured cells or in embryos, would provide us with further insight into understanding the molecular mechanisms of sex determination. At present, it is not clear which one of the 2 different genomic situations represented more closely than the other the molecular phenomena taking place in the developing gonad.

As stated before, our current knowledge regarding the complex profiles of gene expression and the mesh of gene interactions during gonadal differentiation [47, 51], does not allow us to construct simple schematic models of the molecular mechanisms underlying the gonadogenesis. Detailed analysis of the proteins interacting with Sry in cells are urgently needed to fill the present gap of our knowledge and to further obtain essential clues to clarify the molecular mechanisms of sex determination.

An important point of concordance between our present results and those reported by Toyooka et al. [8] is the fact that both support the notion that the Sry binding site in the regulatory region of Wt1 is in fact functional and might play a role yet to be identified in the chain of the genomic sequence of events during sex determination in mouse embryos.

In the present study, we used a ribozyme and shRNAs to inactivate Sry mRNA. The results proved that the ribozyme and shRNA are valuable tools for investigating the effects of silencing Sry expression either in vitro or in vivo. If these ribozyme and shRNA constructs could be introduced into the embryonic genomes of animals as a transgene and effectively expressed, it might be possible to produce phenotypic sex reversals in those transgenic animals. Such transgenic animals would be able to serve not only as useful models for investigating the pathological sex reversals in mammals, including humans, but also as a basis for the practical purposes of developing genetic control systems for sex ratios at birth in livestock animals, where females are in greater demand than males.

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