(Clontech, Palo Alto, CA, USA). 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 *Eco*RI-*Mfe*I fragment of one vector into the *Eco*RI 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 *BspMI* site of piGENEhU6 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-AtA-GAG-gTT-GAA-GgT-CAA-CGT-GTG-CTG-TCC-GTT-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-TT-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 × TBE buffer. The DNA was blotted onto Hybond-N membranes (Amersham Bioscience, Amersham, UK). After blocking 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 chemoluminescence 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 blocking 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).

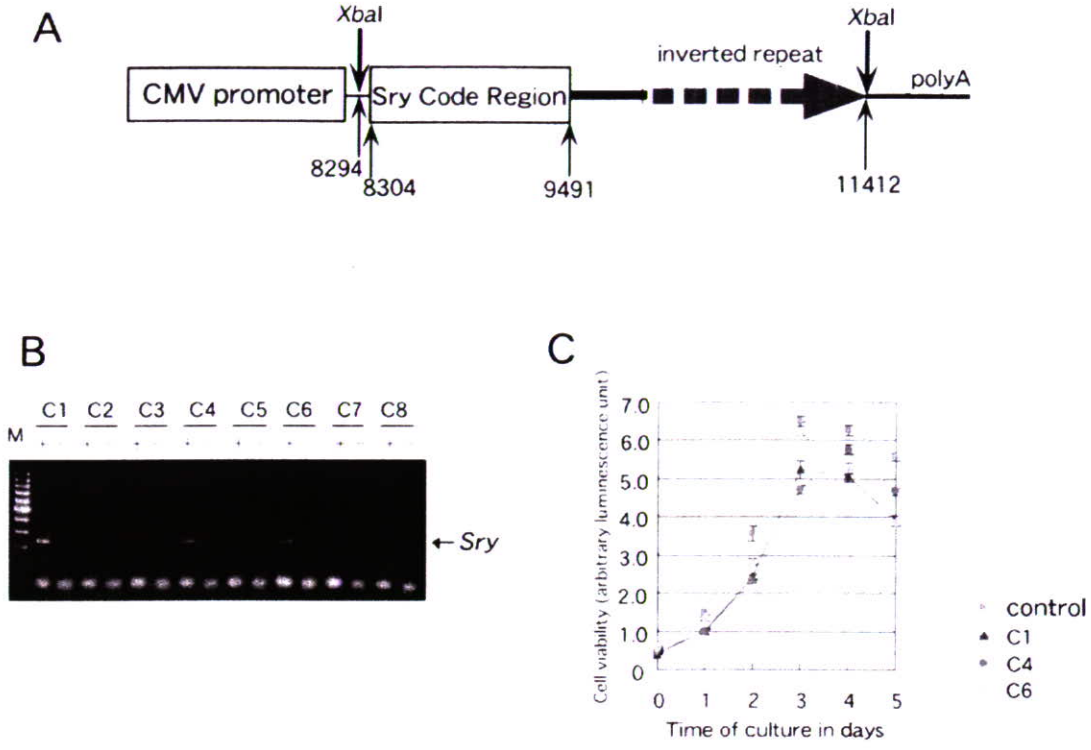

Fig. 1. Establishment of stably transformed *Sry*-expressing M15-derived cell lines. (A) Schematic illustration showing the structure of the CMV/mSry fusion gene used for transfection of M15 cells that endogenously express *Wt1*. Base position numbers are according to Gubbay *et al.* (1990). (B) The *Sry* expression in the cell lines transfected with CMV/mSry. The electrophoretogram shows a typical result from RT-PCR analysis of the transfected cells to confirm the transgene expression. An arrow indicates the position of the *Sry* transcripts. Eight cell lines were independently cloned and tentatively named C1 to C8. The transformed cell lines in which high levels of *Sry* expression were confirmed, i. e., C1, C4, and C6, were renamed Sry#1, Sry#2 and Sry#3 respectively and used in the subsequent experiments. + and – signs: the presence and absence of reverse transcriptase in the reaction mixture for RT-PCR amplification. M: Marker. (C) Comparison of proliferation rates of the 3 stably transformed cell lines with those of the control non-transformed M15 cells. The proliferation rates were assayed as described in Materials and Methods. No differences were detected in the proliferation rates as judged by changes in viable cell number among the cell lines examined.

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*

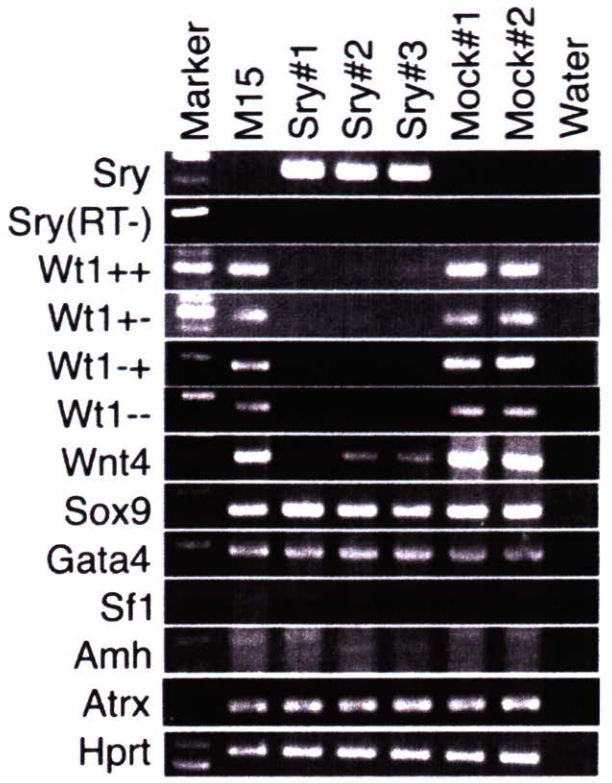

Fig. 2. RT-PCR analyses of the expression of genes putatively comprising the *Sry* cascade during sex differentiation in mammals. *Sry*, *Wt1*, *Wnt4*, *Sox9*, *GATA4*, *Sf1*, *Amh*, and *Atrx*: the names of genes examined. *Hprt*: a house-keeping gene used as a reference. Wt1++, *Wt1* mRNA isoform of theexon 5+ type and coding for +KTS. Wt1+-: *Wt1* mRNA isoform of the exon 5+ type and coding for -KTS. Wt1-+: *Wt1* mRNA isoform of the exon 5- type and coding for +KTS. Wt1--: *Wt1* mRNA isoform of the exon 5- type and coding for -KTS. M15: non-transformed M15 cell line. Sry#1, Sry#2, and Sry#3: the transformed *Sry*-expressing cell lines. Mock#1 and Mock#2: mock-transformed cell lines (see Materials and Methods for details regarding the mock-transformed cell lines). The expression levels of the 4 isoforms of *Wt1* mRNA and those of the *Wnt4* mRNA were strongly down-regulated in the *Sry*-expressing cell lines.

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

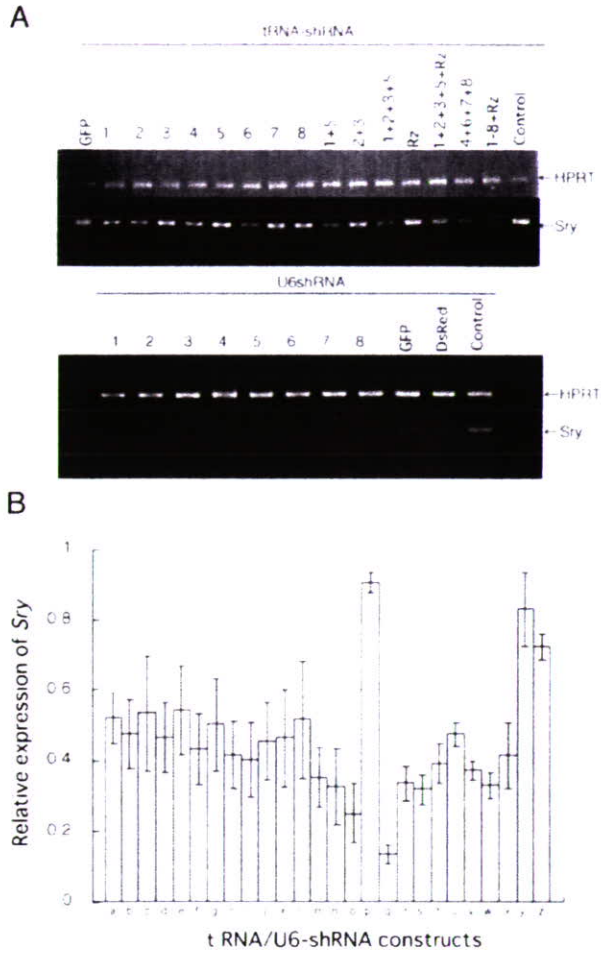

Fig. 3. Silencing effects of the shRNA expression vectors upon *Sry* expression in M15 cells. (A) Electrophoretograms of RT-PCR amplification products showing the expression levels of *Sry* mRNA in reference to the control house-keeping gene, *Hprt*, under the influence of the presently-designed shRNAs. The sequence of each shRNA is presented in Table 2. (B) Silencing effects of the shRNA vector constructs against *Sry* expression, relative to the control vectors, tRNA-empty or U6-T7 (a vector that generates no transcripts). In the following, n+m means that shRNAn and shRNAm are tandemly connected in the construct. Rz3 is the ribozyme sequence from tRNA-Rz3. a, tRNA-shRNA1; b, tRNA-shRNA2; c, tRNA-shRNA3; d, tRNA-shRNA4; e, tRNA-shRNA5; f, tRNA-shRNA6; g, tRNA-shRNA7; h, tRNA-shRNA8; i, tRNA-shRNA1+5; j, tRNA-shRNA2+3; k, tRNA-shRNA1+2+3+5; l, tRNA-Rz3; m, tRNA-shRNA1+2+3+5+Rz3; n, tRNA-shRNA4+6+7+8; o, tRNA-shRNA1-8+Rz3; p, tRNA-shRNAGFP; q, U6-shRNA1; r, U6-shRNA2; s, U6-shRNA3; t, U6-shRNA4; u, U6-shRNA5; v, U6-shRNA6; w, U6-shRNA7; x, U6-shRNA8; y, U6-shRNADsRed (shRNA construct targeted to a red fluorescent protein from coral, DsRed; used as a control); and z, U6-shRNAGFP (shRNA construct targeted to green fluorescent protein, GFP; used as a 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

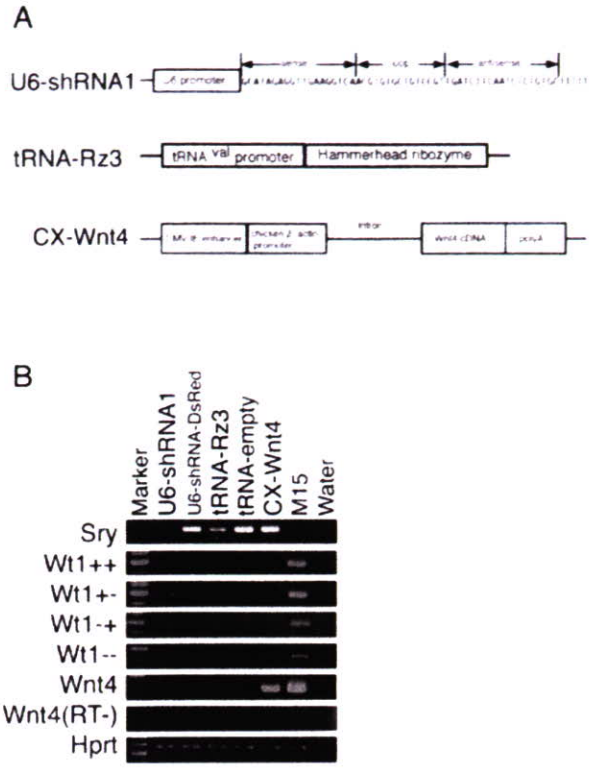

Fig. 4. Restoration of the reduced expression levels of *Wt1* and *Wnt4* in Sry#1 cells to normal levels by silencing *Sry*. (A) Schematic illustration of the expression vector constructs of the shRNA (U6-shRNA-Sry) and tRNA-ribozyme (tRNA-Rz3) against *Sry* mRNA as well as that of Wnt 4 (CX-Wnt4). (B) Results of RT-PCR analyses of the expression levels of *Sry*, *Wt1*, *Wnt4*, and the control house-keeping gene, *Hprt*, in Sry#1 cells with or without expression of shRNA or ribozyme against *Sry* mRNA. The expression levels of all 4 isoforms of *Wt1* tmRNA as well as *Wnt4* mRNA were elevated in the shRNA- and the ribozyme-expressing cells compared with those in the cells to which the shRNA-control (U6-shRNADsRed; see legends for Fig. 3) and tRNA-empty vectors (pPUR-tRNA; Koseki *et al.*,1999) were introduced. The expression levels of *Wt1* and *Wnt4* in the *Sry*-silenced Sry#1 cells were approximately 1/5 of those in the control M15 cells, and restoration was incomplete, probably owing to leaky inactivation of *Sry* mRNA.

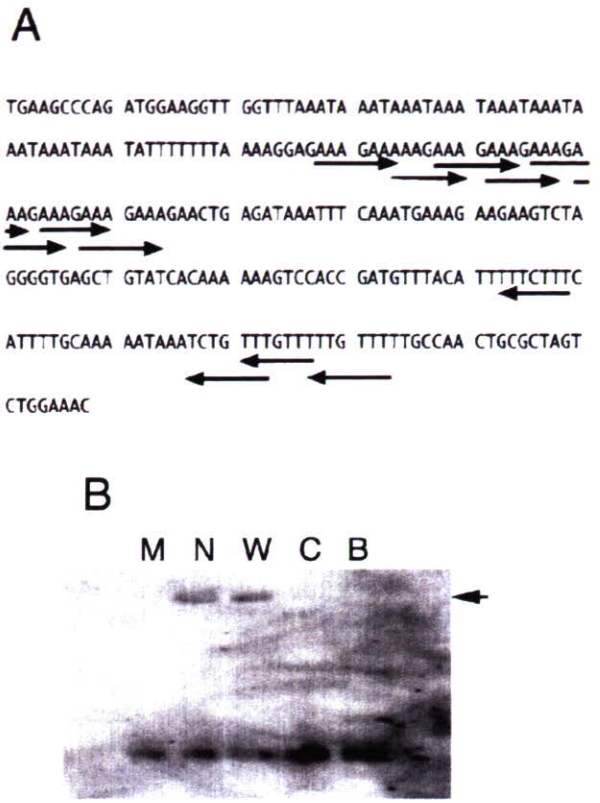

Fig. 5. EMSA analyses of the 5′ up-stream region of *Wt1*. (A) Positions of theoretically-predicted Sry binding sites in the region approximately −3.7 kb from the transcription initiation site of *Wt1*. The results of a computer search (TF-SERECH, Application TRC Laboratory, Japan). Arrows indicate the binding sites and their direction. (B) Electrophoretogram from EMSA analysis of the 5′ upstream region of *Wt1* carried out using the biotin-labeled PCR amplicons as the probe. The probe and the nuclear extacts of the cells were reacted and electrophoresed in 0.5X TBE buffer on agarose gels. The DNA was blotted for detection. An arrow indicates the position of retarded bands. M, nuclear protein extracts of M15 cells; N, nuclear protein extracts of Sry#1 cells; W, whole protein extracts of Sry#1 cells; C, cytoplasmic protein extracts of Sry#1 cells; B, BSA as a control. Only the nuclear protein extracts and whole cell protein extracts of Sry#1 formed a complex with the DNA probe.

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 the *Wt1* transcripts, since differential regulation of the expression levels of Wt1 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 *Wt1* mRNA coding for the Wt1 isoforms were equally affected by *Sry* expression, indicating that *Sry* did not regulate *Wt1* expression post-transcriptionally.

In our experiments, the causal relationship between the down-regulation of *Wt1/Wnt4* expression and *Sry* is of little doubt because 1) down-regulation of the expression of the 2 genes was observed in all 3 sta bly-transformed *Sry*-expressing cell lines (Sry#1, Sry#2, and Sry#3), which were cloned independently; 2) no down-regulation of *WT1/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 *WT1/Wnt4* expression (Fig. 5B). The fact that the restored expression levels of *WT1/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 *Wt1* expression levels (Fig. 5B), suggesting that inhibition of *Wt1* 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 *Wt1* (R394W) in addition to the endogeneous *Wt1*. The expression levels of *Wnt4* were significantly reduced in the presence of the DDS *Wt1* mutant, leading Sim *et al.* [41] to conclude that *Wnt4* is likely u nder the control of *Wt1*. 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 *Wt1*, 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 *Wt1* 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*-expessing 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 *Wt1* 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 *Wt1* expression.

As mentioned before, the *Sry* expression in normal mouse embryos is limited to a very narrow time window during the developmen t of indifferent gonads [24]. Balanced and well-timed expression of *Sry* and *Wt1* 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 *Wt1* 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 Wt1 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 Wt1 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

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 peculier 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 diametric 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 kown 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 know ledge 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.

Acknowledgments

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