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Ectopic expression of mouse Sry interferes with Wnt/ β catenin signaling in mouse embryonal carcinoma cell lines

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Abstract

In mammals, Sry is the master regulator of male sex determination, although how it functions is still unclear. By contrast, female sex determination depends on the action of Rspo1 and Wnt4, the regulators of Wnt/ β -catenin signaling. To seek a possible interaction between male and female sex determination mechanisms, we examined whether Sry affects Wnt/ β -catenin signaling. Using the TOPFLASH reporter system to measure Lef/Tcf-dependent transcriptional activity, we showed that ectopic expression of mouse Sry strongly suppressed Wnt/ β -catenin signaling in mouse embryonal carcinoma and human embryonic kidney cell lines. This inhibition occurred downstream of β -catenin but upstream of Lef/Tcf, and depended on both the HMG-box and the C-terminal transcriptional activation domain. By contrast, TOPFLASH was not inhibited by human SRY, which apparently lacks a transcriptional activation domain. However, a fusion construct consisting of human SRY attached to the C-terminal domain of mouse Sry was able to inhibit TOPFLASH effectively. Furthermore, Sry constructs carrying point mutations equivalent to those in human sex reversal mutations were less effective in inhibiting Wnt/ β -catenin signaling. Also, we showed that the action of Sry as a transcriptional activator was both necessary and sufficient to inhibit Wnt/ β -catenin signaling, suggesting that the transcriptional targets of Sry are responsible for the inhibition of signaling. Sox9 is a potential transcriptional target of Sry, although quantitative RT-PCR analysis indicates that the expression of Sox9 was not up-regulated by the ectopic expression of mouse Sry in mouse embryonal carcinoma cells. While the present study demonstrates an impact of mouse Sry on Wnt/ β -catenin signaling at an *in vitro* level, it requires further investigations to assess whether such action also takes place *in vivo* to regulate male sex determination.

Keywords

Sox; HMG-box; transcription factor; Rspo1; sex reversal; clinical mutation

Introduction

Male sex determination during mammalian development is initiated by the expression of Sry (sex-determining region on the Y chromosome) gene, which encodes an HMG-box DNA-binding protein. In mice, the expression of Sry initiates in the bipotential gonads before overt sex differentiation, and its action is both necessary and sufficient to direct testis determination.

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In the absence of Sry, the XY gonad develops along the ovarian pathway [1]. XX mice carrying the Sry transgene develop as males with normal testes [2]. Thus, Sry acts as the master regulator of male sex determination. However, the molecular nature of the downstream events elicited by the action of Sry is still not well-understood [3,4].

Recent studies revealed that female sex determination is also actively controlled by specific mechanisms, which may involve the Wnt/ β -catenin signaling pathway [5,6]. In this evolutionarily conserved pathway, a secreted glycoprotein of the Wnt family binds to the cell-surface receptors Frizzled and Lrp5/6, leading to the stabilization of β -catenin protein in the cytoplasm. β -catenin then moves to the nucleus, where it interacts with a DNA-binding protein of the Lef/Tcf family to activate the transcription of target genes [7]. In human, XX sex reversal (female-to-male) is mostly caused by translocations of the Sry gene to the X chromosome or to an autosome. However, in some cases, XX sex reversal also occurs independently of Sry. R-spondin1 (Rspo1) has been identified as the gene disrupted in a recessive syndrome characterized by complete XX sex reversal in human [8]. Rspo1 belongs to a family of ligands that interact with Frizzled and Lrp5/6 to activate the Wnt/ β -catenin signaling pathway [9,10]. The significance of Rspo1 in female sex determination is also evident in the mouse, as Rspo1-null XX mice exhibit masculinized features including pseudohermaphroditism in genital ducts, depletion of fetal oocytes, coelomic vessel formation, and ectopic testosterone production in the ovaries [11,12]. In addition, Wnt4, a member of the Wnt family, is involved in female gonad development, as its mutation results in partial XX sex reversal in mouse [13]. These cases suggest that the activation of Wnt/ β -catenin signaling is required for female sex determination.

Interestingly, Wnt/ β -catenin signaling can be inhibited by many Sox proteins. Sox proteins are a class of transcription factors containing an HMG-box that is similar to Sry, and are involved in regulating many developmental processes, including neurogenesis and skeletogenesis [14, 15]. Inhibition of Wnt/ β -catenin signaling by Sox proteins occurs in various cellular contexts (e.g., Sox1, Sox2, Sox7, Sox9, and Sox17 in mammalian cultured cells [16–20]; Xsox3 and Xsox17 in frog embryos [16]; and SoxNeuro in *Drosophila* embryos [21]). Several Sox proteins are shown to interact physically with β -catenin protein [18,20] and with Lef/Tcf [22,23], although the mechanisms of the inhibitory action on Wnt/ β -catenin signaling is still unclear.

Here, as a possible link between the male and female sex determination mechanisms, we investigated whether Sry also has the ability to inhibit Wnt/ β -catenin signaling.

Materials and methods

Cell culture and transfection

P19 and F9 mouse embryonal carcinoma cells were obtained from American Type Culture Collection (Manassas, VA), and HEK293 human embryonic kidney cells (293-H) were from Invitrogen (Carlsbad, CA). P19 cells were cultured in MEM Alpha Medium with 2.5% fetal bovine serum (FBS) plus 7.5% calf serum. F9 and HEK293 cells were cultured in DMEM with 10% FBS. Plasmid transfection was performed using Lipofectamine2000 (Invitrogen) according to the vendor's instruction. The total amounts of transfected DNA were adjusted with the control plasmid, pEF-GFP (pEF/myc/cyto/GFP; Invitrogen), to be equal among experimental treatments.

Plasmids

TOPFLASH and FOPFLASH were obtained from Upstate (Charlottesville, VA), and pRL-TK and pG5-Luc were from Promega (Madison, WI). The structures and properties of β -catenin [24], Axin [25], β eng [26], and Δ NTcf3 [27] were described previously. DNA encoding the

full-length ORF of mouse *Sry* and human *SRY* were isolated by PCR from genomic DNA. cDNA encoding *Rspo1* was isolated by RT-PCR from mouse embryonic RNA. The details on how all deletion, mutation, and chimeric constructs were generated are described in Supplementary Materials.

Luciferase assay

The luciferase assay was conducted using the Dual-Luciferase Reporter Assay System (Promega) with Gene Light 55 Luminometer (Microtech, Chiba, Japan), according to the vendors' instructions. Cell lysates were examined for the activities of firefly luciferase (encoded by TOPFLASH, FOPFLASH, pG5-Luc, and p3SBS-Luc) and *Renilla* luciferase (encoded by pRL-TK). Typically, 150 ng/well of the firefly luciferase plasmids and 50 ng/well of pRL-TK were co-transfected in a 24-well plate format, unless stated otherwise in the figure legends. All experiments were repeated independently at least three times, and the results are shown as mean $\pm$ standard deviation.

Immunocytochemistry

To activate Wnt/ β -catenin signaling, P19 cells were treated with 2 μ M BIO ([2',3',5'-6-bromoindirubin-3'-oxime; EMD Biosciences, San Diego, CA). Cells were fixed in 4% paraformaldehyde/PBS, permeabilized in 0.5% Triton X-100, and blocked with 10% heat-inactivated goat serum. β -catenin protein was detected with mouse monoclonal anti- β -catenin antibody (E-5; Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa Fluor 488 goat anti-mouse antibody (Invitrogen). Myc-tagged mSry protein was detected with rabbit polyclonal anti-c-myc antibody (A-14; Santa Cruz Biotechnology) and Alexa Fluor 568 goat anti-rabbit antibody (Invitrogen). Nuclei were counter-stained with DAPI (4',6-diamidino-2-phenylindole, dilactate; Invitrogen).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Extraction of RNA and synthesis of cDNA were described previously [28]. qRT-PCR was conducted using iCycler with MyiQ Single Color Detection System (Bio-Rad, Hercules, CA). The following primers were used: Sox9 (Fw: CAC AAG AAA GAC CAC CCC GAT TAC; Rv: GCT TCA GAT CAA CTT TGC CAG CTT) and Gapdh (Fw: GCA TGG CCT TCC GTG TTC CT; Rv: CCC TGT TGC TGT AGC CGT ATT CAT). cDNA was amplified using iQ SYBR Green Supermix (Bio-Rad) with the following conditions: the initial denaturation at 94°C (5 min) followed by up to 45 cycles of 94°C (15 sec), 60°C (20 sec) and 72°C (40 sec). The identity of PCR product was verified by sequencing.

Results

The expression of mouse *Sry* interferes with Wnt/ β -catenin signaling

We examined whether Wnt/ β -catenin signaling is inhibited by the expression of mouse *Sry* (mSry), using the TOPFLASH reporter plasmid. The TOPFLASH plasmid contains a luciferase reporter gene under the control of Lef/Tcf-response elements, and is widely used as an indicator of active Wnt/ β -catenin signaling [29]. In P19 cells, TOPFLASH was effectively activated by Lrp5 Δ N (Fig. 1A), which is a constitutively active form of Wnt receptor Lrp5 [30]. The Lrp5 Δ N-induced activation of TOPFLASH was strongly inhibited by the co-expression of mSry in a dose-dependent manner (Fig. 1A). mSry also inhibited the activation of TOPFLASH that was induced by pt- β -catenin (Fig. 1B), which is a stable form of β -catenin [24]. *Rspo1*, which is known to be critical for female sex determination in human [8] and mouse [11,12], activated TOPFLASH in P19 cells, confirming that *Rspo1* acts as an activator of Wnt/ β -catenin signaling (Fig. 1C). Notably, mSry also inhibited the activation of TOPFLASH that was induced by *Rspo1* (Fig. 1C), although it appears that a higher amount of mSry was required to

antagonize Rspo1 compared to Lrp5 Δ N and β -catenin. The reason for such differences in the inhibitory strength is unclear. However, mSry may act more effectively against cell-autonomous activators, such as Lrp5 Δ N and β -catenin, rather than against a non-cell-autonomous agent Rspo1, which is secreted to activate Wnt/ β -catenin signaling not only in Rspo1-expressing cells but also in the surrounding cells.

By contrast, mSry did not inhibit luciferase expression from the FOPFLASH control plasmid, which is identical to TOPFLASH except for mutations in the Lef/Tcf-response elements (Fig. 1D). Instead, mSry appeared to cause a slight but consistent elevation in the FOPFLASH activity (Fig. 1D), which suggests that mSry weakly activates the basal promoter in the reporter plasmids. Nonetheless, because mSry strongly inhibited the activation of TOPFLASH, the inhibitory effect of mSry is specifically on the Lef/Tcf-mediated transcriptional regulation. Furthermore, the inhibition of Wnt/ β -catenin signaling by mSry was observed in other cell lines. Namely, mSry inhibited the TOPFLASH activation by Lrp5 Δ N or β -catenin in F9 cells, and by β -catenin in HEK293 cells (Fig. 1E–G).

β -catenin is constitutively stable in the absence of Wnt ligands [24]. Nonetheless, mSry was able to inhibit the action of β -catenin (Fig. 1B,F,G). Thus, the inhibitory effect of mSry is likely to take place downstream of β -catenin. To support this idea, we examined the effect of mSry on the stability of the β -catenin-luciferase fusion (β -cat-luc) protein in P19 cells (Fig. 2A). The activity of β -cat-luc protein was substantially increased by the expression of Lrp5 Δ N (Fig. 2B), indicating that the stability of the fusion protein is regulated in a manner similar to normal β -catenin. The Lrp5 Δ N-induced stabilization of β -cat-luc was not inhibited by the co-expression of mSry (Fig. 2B). We also examined the effect of mSry on the stability of endogenous β -catenin protein. (2'Z,3'E)-6-bromoindirubin-3'-oxime (BIO) has been shown to stabilize β -catenin protein by inhibiting a negative regulator, glycogen synthase kinase 3 [25]. Consistently, the treatment of P19 cells with BIO resulted in substantial stabilization of β -catenin protein, as demonstrated by immunocytochemistry (Fig. 2C). However, ectopic expression of mSry did not interfere with the stabilization of β -catenin protein by BIO (Fig. 2C). These results indicate that mSry inhibits Wnt/ β -catenin signaling without destabilizing β -catenin protein.

Both mSry and Lef/Tcf contain an HMG domain. This raises the possibility that mSry blocks Wnt/ β -catenin signaling by competing for the Lef/Tcf-response elements. To test this possibility, we generated the reporter system for Wnt/ β -catenin signaling that is independent of HMG domains. The N-terminus of Lef1, which encodes the β -catenin binding domain (CBD; [27]), was fused to the DNA-binding domain (DBD) of yeast Gal4 protein to generate L1CBD-G4DBD (Fig. 3A). In the pG5-Luc plasmid, the luciferase gene is located downstream of the Gal4 DBD-binding sites. Thus, the system operates in such a way that L1CBD-G4DBD interacts with β -catenin, and activates the luciferase expression from pG5-Luc in response to active Wnt/ β -catenin signaling (Fig. 3A). First, this system was validated by the co-expression of Lrp5 Δ N and various inhibitory agents of Wnt/ β -catenin signaling that act at distinct levels along the cascade (Fig. 3B). Both the TOPFLASH and L1CBD-G4DBD/pG5-Luc systems were effectively inhibited by Axin (a negative regulator of the pathway that act upstream of β -catenin; [25]) and β eng (a dominant-negative form of β -catenin; [26]). However, Δ NTcf3 (a dominant-negative form of Tcf3; [27]) inhibited only the TOPFLASH but not L1CBD-G4DBD/pG5-Luc system (Fig. 3B). This confirms that the L1CBD-G4DBD/pG5-Luc system can be used to monitor the activity of Wnt/ β -catenin signaling in an HMG domain-independent manner. We demonstrated that mSry inhibited the activation of the L1CBD-G4DBD/pG5-Luc system (Fig. 3C). Thus, the inhibitory effect of mSry is not due to the competition with Lef/Tcf through the HMG domain, suggesting that Wnt/ β -catenin signaling is inhibited upstream of Lef/Tcf.

The C-terminal region of β -catenin acts as a transcriptional activation domain [31,32]. Thus, we further examined whether mSry inhibits the transcriptional activation activity of β -catenin protein. We generated the fusion construct G4DBD- Δ N β -cat, which contains Gal4 DBD and most of β -catenin except for the N-terminal region that controls the stability of the protein. G4DBD- Δ N β -cat strongly activated the pG5-Luc (Fig. 3D), confirming that the Δ N β -cat portion possesses transcriptional activation activity. However, the activation of pG5-Luc by G4DBD- Δ N β -cat was not inhibited by mSry (Fig. 3D), indicating that mSry does not interfere with the transcriptional activation action of β -catenin. These results altogether suggest that mSry inhibits Wnt/ β -catenin signaling at a level downstream of β -catenin but upstream of Lef/Tcf.

The histidine and glutamine-rich region of mSry is critical for the inhibition of Wnt/ β -catenin signaling

To examine which regions in mSry are required for the inhibition of Wnt/ β -catenin signaling, we generated deletion constructs (Fig. 4A). mSry Δ C lacks the C-terminal portion encoding the histidine and glutamine-rich (HQ-rich) sequences, while mSry Δ N lacks the N-terminal portion of mSry, including the HMG domain. Neither construct inhibited the TOPFLASH activation induced by Lrp5 Δ N (Fig. 4B). Also, the expression of mSry Δ C together with mSry Δ N did not inhibit the TOPFLASH activation (Fig. 4C). Thus, to inhibit Wnt/ β -catenin signaling, both the HQ-rich region and the HMG domain are required to be present in the same molecule.

The HQ-rich region of mSry has been shown to act as a transcriptional activation domain, and the length of the HQ-rich sequence correlates with the potency of transcriptional activation [33,34]. Furthermore, the *Mus musculus domesticus* Y-chromosome carries a polymorphic allele of Sry with a shorter HQ-rich region, and its introduction into the C57BL/6J inbred strain results in a high frequency of XY sex reversal [34,35]. These observations suggest that the length of the HQ-rich sequence is critical for the action of mSry. Here, we examined whether the length of the HQ-rich sequence also correlates with the effectiveness of mSry to interfere with Wnt/ β -catenin signaling. We created two types of truncated forms of mSry: mSry[1–345] and mSry[1–265] (Fig. 4D). mSry[1–265] is similar in length to the *M. m. domesticus* polymorphic allele, which encodes approximately 230 amino acids due to the premature stop codon within the HQ-rich sequence [34]. Compared to normal mSry, both truncated forms were less effective in suppressing TOPFLASH activation by Lrp5 Δ N, and mSry[1–265] was less effective than mSry[1–345] (Fig. 4D). These results indicate that the potency of mSry to inhibit Wnt/ β -catenin signaling is affected by the length of HQ-rich sequence.

The necessity of the HQ-rich region to inhibit Wnt/ β -catenin signaling prompted us to investigate the activity of human SRY (hSRY), because Sry homologs of non-rodent mammals, including human, lack the sequences corresponding to the HQ-rich region of mSry (Fig. 4A; [3,4]). hSRY was unable to inhibit the TOPFLASH activation that was induced by Lrp5 Δ N (Fig. 4E). To test whether the inability of hSRY to inhibit Wnt/ β -catenin signaling is solely due to the lack of the HQ-rich sequence, we generated the chimeric construct (hSRY-HQ) that fused hSRY to the HQ-rich region of mSry (Fig. 4A). hSRY-HQ inhibited the TOPFLASH activation as effectively as mSry (Fig. 4F). This result indicates that the absence of the HQ-rich sequence is responsible for the inability of hSRY to inhibit Wnt/ β -catenin signaling.

Amino acid substitutions equivalent to the human clinical mutations impair the ability of mSry to inhibit Wnt/ β -catenin signaling

Because hSRY was unable to inhibit Wnt/ β -catenin signaling, we questioned whether the inhibitory effect of mSry bears any biological significance, particularly for male sex determination. In human, several clinical mutations have been identified, in which distinct point mutations in the hSRY gene cause male-to-female sex reversal [36]. Many of these point

mutations lead to a substitution of specific amino acids in the HMG domain that are highly conserved among species, indicating that these particular amino acids are critical for the sex determination function of Sry. To test whether these critical amino acids are also essential for mSry to inhibit Wnt/ β -catenin signaling, we introduced point mutations that are equivalent to the human clinical mutations into mSry (Fig. 5A). Four types of human clinical mutations, namely V60L, M64I, G95R, and F109S, were examined in this study, because each of these mutations affects differently the biochemical properties of the HMG domain [36]. The corresponding mutations in mSry, namely V5L, M9I, G40R, and F54S, reduced the inhibitory effect on TOPFLASH (Fig. 5B), indicating that the point mutations that cause sex reversal in human also impair the ability of mSry to inhibit Wnt/ β -catenin signaling.

While the sequences of the HMG domain are highly conserved between mSry and hSRY, they are not identical (Fig. 5A). Thus, it is imperative to assess whether the point mutations in the human HMG domain sequence also affect the ability to inhibit Wnt/ β -catenin signaling. Because hSRY by itself was unable to inhibit Wnt/ β -catenin signaling (Fig. 4E), we took advantage of the hSRY-HQ chimeric construct to examine the effect of the clinical mutations. We focused on two point mutations, V60L and G95R, because the flanking amino acid sequences of these two sites differ between mSry and hSRY (Fig. 5A). Both the hSRY-HQ [V60L] and hSRY-HQ[G95R] mutant constructs were less effective than hSRY-HQ in suppressing the TOPFLASH activity (Fig. 5C). Thus, the clinical mutations impair the HMG domains of both mSry and hSRY such that they are unable to inhibit Wnt/ β -catenin signaling effectively.

The action of mSry as a transcriptional activator is responsible for the inhibition of Wnt/ β -catenin signaling

The HMG domain is essential for mSry to inhibit Wnt/ β -catenin signaling (Fig. 4B), raising the possibility that the action of mSry as a transcription factor is critical for the inhibition of Wnt/ β -catenin signaling. Consistent with the previous study [36], mSry acted as a transcriptional activator on the reporter plasmid p3SBS-Luc, which contains three consensus binding sites for the HMG domain of Sry (Fig. 6A). The activation of p3SBS-Luc was dependent on the HQ-rich region, as neither mSry Δ C nor hSRY was able to activate it (Fig. 6A). Thus, the activity as a transcriptional activator coincides with the ability to inhibit Wnt/ β -catenin signaling.

To directly test the role of transcriptional activation in the inhibition of Wnt/ β -catenin signaling, we generated three chimeric constructs (Fig. 6B). mSry Δ C-GFP contains the green fluorescent protein (GFP) sequence in place of the HQ-rich region, mSry Δ C-VP16 contains the transcriptional activation domain of the herpes simplex virus VP16, and mSry Δ C-EnR contains the transcriptional repression domain of *Drosophila* Engrailed. The Lrp5 Δ N-induced activation of TOPFLASH was significantly inhibited by mSry Δ C-VP16, but not by mSry Δ C-GFP, indicating that the action of mSry as a transcriptional activator is sufficient to inhibit Wnt/ β -catenin signaling (Fig. 6C). By contrast, mSry Δ C-EnR promoted the TOPFLASH activation by Lrp5 Δ N (Fig. 6C). These results suggest that Wnt/ β -catenin signaling is inhibited by the factors whose expressions are up-regulated by mSry and mSry Δ C-VP16, but are down-regulated by mSry Δ C-EnR.

The identity of the mSry target genes that are responsible for inhibiting Wnt/ β -catenin signaling remains unknown. In the male gonad, Sox9 plays critical roles in male sex determination, and its transcription is thought to be activated by Sry [3,4]. Also, Sox9 can inhibit Wnt/ β -catenin signaling in mammalian cells [18,37]. Thus, we tested whether mSry activates the Sox9 gene, which in turn inhibits Wnt/ β -catenin signaling in P19 cells. However, quantitative RT-PCR analysis showed that the expression of Sox9 was not up-regulated by the expression of mSry

(Fig. 6D), suggesting that Sox9 is not the mSry target gene that is responsible for the inhibition of Wnt/ β -catenin signaling.

Discussion

In this study, we demonstrated that the expression of mouse Sry inhibits the activation of Wnt/ β -catenin signaling in mammalian cultured cells. However, whether mSry also exhibits the same activity *in vivo*, particularly in the bipotential gonadal cells, has not been determined. Nonetheless, because the activators of Wnt/ β -catenin signaling, namely Rspo1 and Wnt4, are essential for the development of the female gonad [8,11–13], it is possible that one of the Sry actions as the master regulator of male sex determination is to interfere with Wnt/ β -catenin signaling. Previous studies of over 100 cases of human XX sex reversal patients support a regulatory cascade model, in which Sry acts by repressing a hypothetical factor, Z, which negatively regulates male sex determination [38]. Our finding may fit this model if the activation of Wnt/ β -catenin signaling takes on the role of Z as the negative regulator of male sex determination. Further investigations are required to determine whether the inhibition of Wnt/ β -catenin signaling in the bipotential gonad is sufficient to induce testis development, and/or whether the activation of Wnt/ β -catenin signaling is necessary for female sex determination.

We also showed that human SRY, which does not have a C-terminal HQ-rich domain, is incapable of inhibiting Wnt/ β -catenin signaling. By contrast, the fusion construct hSRY-HQ, in which human SRY is attached to the HQ-rich domain of mouse Sry, is able to inhibit Wnt/ β -catenin signaling effectively. This suggests that the action of the HQ-rich domain, possibly as a transcriptional activation domain, is crucial for the inhibition of Wnt/ β -catenin signaling, although the HQ-rich domain may also affect other properties of Sry, such as the stability of Sry protein. Importantly, it was reported that the transgene encoding human SRY ORF can cause sex reversal in XX mice, raising the possibility that the HQ-rich domain is not essential for male sex determination [39]. Paradoxically, however, it was also reported that mouse Sry with a truncation in the HQ-rich domain fail to cause XX sex reversal, implicating that the HQ-rich domain is essential for the proper action of mouse Sry [40]. One possibility is that human SRY may form a complex with proteins in the bipotential gonadal cells that possess properties similar to the HQ-rich domain, which together act like mouse Sry. It is plausible that these co-factors of human SRY are under-expressed or non-functional in P19, F9 and HEK293 cell lines, which may be the reason why hSRY is unable to inhibit Wnt/ β -catenin signaling in these cell lines. While the existence of such co-factors of human SRY is hypothetical, several proteins have been identified that physically interact with human SRY protein, such as SIP-1 [41], KRAB-O [42], Calmodulin [43], and WT1 [44]. In particular, WT1, which is a transcriptional activator, has been shown to interact with human SRY to activate the promoters containing Sry-binding sites [44]. Because the HQ-rich region acts as a transcriptional activator domain [36], it is of particular interest to examine whether the co-expression of WT1 and human SRY can inhibit Wnt/ β -catenin signaling.

Many of the point mutations in human SRY that cause male-to-female sex reversal have been mapped within the HMG domain [36]. In this study, we introduced analogous mutations in the HMG domains of mouse Sry and human SRY, and found that those mutations diminished the ability to inhibit Wnt/ β -catenin signaling. Among the mutations examined, G95R appeared to diminish the inhibitory activity most strongly (Fig. 5B,C). The previous study shows that binding of human SRY to the target DNA sequence is undetectable when the G95R mutation is introduced, while slight binding is associated with the V60L mutation [45]. The present study showed that V60L appeared to have less impact on the ability to inhibit Wnt/ β -catenin signaling compared to G95R (Fig. 5B,C), which suggest that the DNA binding ability of Sry is critical

for the inhibition of Wnt/ β -catenin signaling, possibly through the transcriptional activation of Sry target genes.

Although we showed that the expression of mSry effectively inhibited the TOPFLASH activation by β -catenin in three different cell lines, the intensity of inhibition appeared to be less in HEK293 cells compared to P19 and F9 cells. For example, 100ng of the mSry plasmid was required to reduce the TOPFLASH activity by 60% in HEK293 cells, while 10ng was sufficient in P19 and F9 cells (Fig. 1). It is unclear why a higher dose of mSry is required to inhibit Wnt/ β -catenin signaling in HEK293 cells. The effectiveness of the transfected mSry plasmid may be affected by various factors depending on cell lines, namely the transfection efficiency, the stability of protein products, the interactions with other proteins, and the epigenetic status of the Sry target genes. Further investigations using a wide range of cell lines from various animals are crucial to elucidate molecular mechanisms of how mSry inhibits Wnt/ β -catenin signaling and their potential differences across various cellular contexts.

The effect of mSry Δ C-EnR on Wnt/ β -catenin signaling was opposite to that of mSry, such that it further enhanced the TOPFLASH activity in P19 cells (Fig. 6C). Fusion proteins with EnR, which is a transcriptional repressor domain of the *Drosophila* Engrailed, have been constructed to investigate the actions of various transcription factors in a variety of animals [46–48]. mSry Δ C-EnR is likely to bind to the transcriptional regulatory elements of the mSry target genes through its HMG domain. Possibly, these target genes are expressed at a basal level in P19 cells by mSry-independent mechanisms, because the expression of the endogenous mSry was undetectable in P19 cells (data not shown). The expression of these genes, at least one of which we hypothesize to be an inhibitor of Wnt/ β -catenin signaling, may be further up-regulated by the overexpression of mSry and down-regulated by mSry Δ C-EnR.

Sox9 is a critical regulator of male sex determination that acts downstream of Sry. Namely, (1) the expression of Sox9 is up-regulated in the male gonad after Sry [49], (2) Sox9 is indispensable for male sex determination even in the presence of Sry [50,51], and (3) the Sox9 transgene is sufficient to induce testis development even in the absence of Sry [52]. Furthermore, a very recent study shows that the upstream regulatory element of the Sox9 gene contains multiple binding sites for Sry, and that the expression of Sox9 can be directly up-regulated by the cooperative action of mSry with another transcriptional activator SF1 [53]. As shown in the present study, however, the overexpression of mSry did not up-regulate the expression of Sox9 in P19 cells, while it inhibited Wnt/ β -catenin signaling. This indicates that the inhibition of Wnt/ β -catenin by mSry is not mediated by Sox9. Interestingly, another recent study shows that the knockout of the Rspo1 gene results in an elevation of the Sox9 expression in the XX gonad [11]. This raises the possibility that the expression of Sox9 may be up-regulated by the suppression of Wnt/ β -catenin signaling even in the absence of Sry.

Because the Sox9 transgene is sufficient for male sex determination in the absence of Sry [52], the following question arises. Are the activities associated with Sry, especially the inhibitory effect on Wnt/ β -catenin signaling, necessary for male sex determination? While the answer to this question requires further investigations, it is of particular importance to point out that Sox9 also inhibits Wnt/ β -catenin signaling in various cellular contexts [18,37]. Thus, it is possible that the Sox9 transgene also substitutes for the action of Sry as an inhibitor of Wnt/ β -catenin signaling, which may be essential for male sex determination.

Various Sox proteins have been shown to inhibit Wnt/ β -catenin signaling in mammalian cells, and frog and fruitfly embryos, which implicates that the inhibition of Wnt/ β -catenin signaling may be one of the fundamental and ancestral functions of Sox proteins, including Sry. Nonetheless, the molecular mechanisms of this inhibition are still unclear, and may vary depending on the Sox proteins and cellular contexts. For example, in frog embryos, Xsox17

appears to inhibit Wnt-induced axis formation without the HMG-box, while in mammalian cells, it requires the HMG-box to inhibit TOPFLASH [16]. The inhibition of Wnt/ β -catenin signaling by Sox9 may be caused by blocking Lef/Tcf from binding to β -catenin, and/or by inducing the degradation of β -catenin protein [18]. Sox9 is also suggested to inhibit Wnt/ β -catenin signaling by activating the transcription of target genes [37]. The latter mechanism may be similar to how mouse Sry inhibits Wnt/ β -catenin signaling, as shown in the present study. Our present study also showed that the inhibition of Wnt/ β -catenin signaling by mouse Sry occurs downstream of β -catenin but upstream of Lef/Tcf. This information can be used as a criterion to search for the Sry target gene that is involved in the inhibition of Wnt/ β -catenin signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Wnt/ β -catenin signaling is inhibited by the expression of mSry in mammalian cells
 In P19 cells, mSry represses the activation of TOPFLASH by Lrp5 Δ N (A), $\text{pt-}\beta$ -catenin (B) and Rspo1 (C), but not the basal expression of FOPFLASH (D). mSry represses the activation of TOPFLASH by Lrp5 Δ N (E) and $\text{pt-}\beta$ -catenin (F) in F9 cells, and by $\text{pt-}\beta$ -catenin (G) in HEK293 cells.

Fig. 2. mSry does not inhibit the stabilization of β -catenin protein in P19 cells

(A) The structure of β -cat-luc in comparison with normal β -catenin. (B) The stabilization of β -cat-luc by Lrp5 Δ N is not inhibited by mSry. (C) The stabilization of endogenous β -catenin protein induced by BIO treatment is not diminished by the expression of mSry. Note that the β -catenin staining in the cell that is most strongly expressing mSry (arrowheads) is as intense as in the neighboring cells that are not expressing mSry.

Fig. 3. mSry inhibits Wnt/ β -catenin signaling at a level downstream of β -catenin and upstream of Lef/Tcf in P19 cells

(A) The structure of L1CBD-G4DBD in comparison with normal Lef1 (left), and the schematic drawings illustrating the mode of action of L1CBD-G4DBD (right). (B) Comparison between the TOPFLASH and L1CBD-G4DBD/pG5-Luc reporter systems in response to known inhibitors of Wnt/ β -catenin signaling. 200 ng/well of L1CBD-G4DBD and 400 ng/well of the plasmid encoding an inhibitor were co-transfected. (C) mSry inhibits the L1CBD-G4DBD/pG5-Luc system that is activated by Lrp5 Δ N. (D) mSry does not inhibit pG5-luc that is activated by G4DBD- Δ N β -cat.

Fig. 4. The structures of mSry and hSRY in comparison with their deletion and chimeric constructs (A). mSry Δ C and mSry Δ N, whether transfected individually (B) or together (C), are unable to inhibit the activation of TOPFLASH by Lrp5 Δ N. mSry mutants carrying a truncated HQ-rich domain, mSry[1-345] and mSry[1-265], are less effective in inhibiting the TOPFLASH activation (D). The activation of TOPFLASH is not inhibited by hSRY (E), but effectively inhibited by the hSRY-HQ chimeric construct (F). All experiments were conducted using P19 cells.

Fig. 5. The point mutations in the HMG domain that are equivalent to human clinical mutations impair the ability of mSry and hSRY-HQ to inhibit Wnt/ β -catenin signaling in P19 cells
 (A) The locations and sequences of the four human clinical mutations, and the corresponding amino acid substitutions that are introduced into mSry. (B) The mutated mSry are ineffective in inhibiting the activation of TOPFLASH. (C) The mutations introduced into the HMG domain in the hSRY-HQ chimeric construct diminish the ability to suppress the TOPFLASH activation.

Fig. 6. The action of mSry as a transcriptional activator is responsible for the inhibition of Wnt/ β -catenin signaling

(A) The p3SBS-Luc reporter is activated by mSry but not by mSry Δ C or hSRY. (B) The structures of the mSry chimeric constructs, in which the HQ-rich region is replaced with GFP, VP16, and EnR. (C) The activation of TOPFLASH by Lrp5 Δ N is inhibited by mSry Δ C-VP16 but is promoted by mSry Δ C-EnR. (D) qRT-PCR analysis indicates that the transcription of Sox9 is not up-regulated by mSry. The graph shows the mean $\pm$ standard deviation of four independent experiments. All experiments were conducted using P19 cells.