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The human and mouse sex-determining SRY genes repress the Rspo1/ β -catenin signaling

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Abstract

The sex-determining region Y (*SRY*) is the gene on the Y chromosome responsible for switching on male sex determination during mammalian embryogenesis. In its absence, ovaries develop in the embryo. Hence, ovarian determination and differentiation is considered to be a default, or passive, developmental pathway. Recently this classical paradigm of sex determination has been challenged with the discovery of the R-spondin 1 (*RSPO1*) as an active ovarian determinant. Mutations of *RSPO1* cause a female-to-male sex reversal. *RSPO1* synergizes with *WNT4* in activating an ovarian development in the bipotential gonad *via* the canonical Wnt signaling. Early studies showed that *SRY* represses such Wnt signaling, but also generated discrepancies on whether only mouse *Sry* is capable of inhibiting such Wnt signaling and whether both human and mouse *SRY* proteins are able to interact with β -catenin, the intracellular messenger responsible for executing the Wnt signals. Our studies show that both human *SRY* and mouse *Sry* are capable of repressing the Rspo1/Wnt/ β -catenin signaling. However, the repression activities vary among different *SRY/Sry* proteins and paradoxically related to the presence and/or size of an acidic/glutamine-rich domain. The HMG box of human *SRY* could bind directly to β -catenin while the mouse *Sry* binds to β -catenin *via* its HMG box and glutamine-rich domain. The results clarify some of the initial discrepancies, and raise the possibility that *SRY* interacts with β -catenin in the nucleus and represses the transcriptional activation of the Rspo1/Wnt target genes involved in ovarian determination, thereby switching on testis determination.

Keywords: *SRY*; sex determination; R-spondin 1; canonical Wnt signaling; β -catenin; protein interaction

Introduction

In mammals, the sex of an individual is determined at conception. When a Y-bearing sperm fertilizes a X-bearing

egg, the sex of the embryo is male while a X-bearing sperm will produce a female embryo. In the presence of the Y chromosome, specifically the sex-determining region Y (*SRY*) gene on this chromosome, the fetus develops testes, which in turn synthesize male hormones that are critical for further development of male phenotypic characteristics (Sinclair et al., 1990; Lovell-Badge et al., 2002; Wilhelm et al., 2007; Sekido and Lovell-Badge, 2009). In the absence of the Y chromosome, the fetus develops ovaries and female phenotypes. Hence, *SRY* switches on the male sex determination and differentiation while female sex determination is considered to be the default, or passive, gonadal development. *SRY* encodes a nuclear protein

Abbreviations: *SRY*, sex-determining region Y; *Rspo*, R-spondin; *Sox9*, *SRY*-box 9; *Wnt4*, Wingless-type MMTV integration site family, member 4; *DKK1*, Dickkopf homolog 1 (*Xenopus laevis*); *TCF*, T-cell factor; *APC*, adenomatous polyposis coli protein; *GSK3*, glycogen synthase kinase 3; *LRP6*, low density lipoprotein receptor-related protein 6; *KRAB*, Krüppel-associated box; *KAP1*, KRAB-associated protein 1; *HP1*, heterochromatin protein 1.

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harboring a DNA-binding high mobility group (HMG) box and is a founding member of a family of transcription factors, termed SRY-box (SOX) proteins that contain a conserved HMG box highly homologous to that of SRY (Bowles et al., 2000; Kiefer, 2007). *SRY* and *SOX* genes play important roles in stem cell pluripotency and cell fate determination and differentiation during embryogenesis, among other functions (Wegner, 1999; Bowles et al., 2000; Schepers et al., 2002; Kiefer, 2007; Lefebvre et al., 2007). Although *SRY* is critical in determining the male sex, other sex-determining genes, particularly *SOX9*, are required to propagate the testis differentiation process, beyond the initial switch on male development (Sekido et al., 2004; Kobayashi et al., 2005). A recent report suggests that *SRY* is required for up-regulation of *SOX9*, which in turn specifies the fate of the pre-Sertoli cell differentiation, inadequacy of which results in hypoplastic testes or sex reversal (Sekido and Lovell-Badge, 2008). Although these findings support the notion that *SOX9* is an immediate target gene for *SRY*, the question remains on whether *SOX9* is the only and sufficient downstream gene essential for proper testis differentiation. Further, the exact mechanism(s) by which *SRY* preempts the ovarian differentiation needs to be defined in molecular terms.

Sex is determined actively by the antagonistic functions of respective testis and ovarian determinants

The traditional view that ovarian determination and differentiation is a default, or passive, developmental pathway has recently been challenged by studies of Camerino and colleagues (Parma et al., 2006), which showed that the human R-Spondin 1 (*RSPO1*) gene is disrupted in a recessive syndrome characterized by complete XX female-to-male sex reversal, palmoplantar hyperkeratosis and predisposition to squamous cell carcinoma of the skin. Such XX sex reversal, in the absence of *SRY*, suggests that *RSPO1* could be an ovarian determining gene whose mutations have provided opportunity for testis differentiating genes, downstream of *SRY*, in mediating a testicular differentiation of the bipotential gonad in the absence of *SRY* (Capel, 2006; Wilhelm, 2007; Chassot et al., 2008a; DiNapoli and Capel, 2008). Subsequent studies demonstrated that *Rspo1*^{-/-} mice showed partial XX male sex reversal (Chassot et al., 2008b; Tomizuka et al., 2008), and down regulation of *Wnt4* gene, another early ovarian differentiating gene (Vainio et al., 1999), in the developing gonads. Notably, *Sox9* gene is not significantly

up-regulated until late in embryo development, resulting in hermaphrodite genitalia. Such XX sex reversal is similar to those observed in *Wnt4*^{-/-} mice (Vainio et al., 1999; Jeays-Ward et al., 2004; Yao et al., 2004; Kim et al., 2006b; Bernard and Harley, 2007). Interestingly, both *RSPO1* and *WNT4* are located closely at bands p34.3 and p35-36 respectively on the short arm of chromosome 1 in humans, duplication of which results in XY sex reversal (Biason-Lauber et al., 2007; Biason-Lauber and Konrad, 2008; Maatouk et al., 2008). Such close physical linkage of both genes is also present in other mammals, such as on chromosome 4D2.2 and 4D3 respectively in the mouse and on the same chromosome 5q36 location for both genes in the rat. These observations suggest that *RSPO1* could be an ovarian determining gene that positively switches on the ovarian determination while *WNT4* could be an immediate downstream, or co-factor, gene whose expression overlaps with that of *RSPO1* and is either directly or indirectly regulated by *RSPO1*.

R-spondins are new players in Wnt/β-catenin signaling

Rspo1 is a member of a family of four secreted proteins, designated as *Rspo1*–4, which are ligands for the canonical Wnt signaling (Kamata et al., 2004; Kim et al., 2006a; Nam et al., 2006). They share 40%–60% protein identity and structural homologies. The *Rspo* proteins contain a N-terminal signal peptide, two adjacent cysteine-rich furin-like domains, a highly homologous thrombospondin (TSP-1) motif, and a basic charged C-terminal tail harboring a bipartite nuclear localization signal. They play important roles in various developmental and oncogenic pathways (Blaydon et al., 2006; Kim et al., 2006a; Aoki et al., 2007, 2008; Nam et al., 2007a, 2007b; Bell et al., 2008; Chassot et al., 2008a). *Rspo1* is expressed in mesenchymal cells of the diencephalons, forebrain and dorsal neural tube during mouse embryogenesis (Nam et al., 2007b). *RSPO1* ectopic expression induces intestinal crypt cell proliferation (Kim et al., 2005b). Mutations/dysregulations of *RSPO1* have been associated with palmoplantar hyperkeratosis, squamous cell carcinoma and sex reversal (Parma et al., 2006). *RSPO2* is down-regulated in colon, rectum, small intestine, lung and breast tumors. *Rspo2*^{-/-} mice develop malformations of laryngeal, tracheal, cartilages, limbs, kidney, and palate, and lung hypoplasia (Bell et al., 2008; Chassot et al., 2008a). *Rspo3* is essential for placental development in mouse (Kazanskaya et al., 2008),

while mutations of *RSPO4* are associated with anonychia, a malformation of toe and finger nails in humans (Blaydon et al., 2006). Expression of Rspo proteins overlaps with those of various partnering Wnt proteins (Nam et al., 2007b). Rspo proteins play key roles in synergizing with respective Wnt ligands in cell proliferation, cell fate determination and organogenesis. Rspo members mediate their biological functions by a common mechanism, resulting in activation of canonical Wnt signaling (Kim et al., 2008) (Fig. 1). Rspo ligands interact with the Frizzled receptors *via* high affinity for LRP6 co-receptor (Wei et al., 2007). They antagonize DKK1 activity by interfering with DKK1 mediated LRP6 and Kremen association and LRP6 internalization, resulting in increased levels of LRP6 co-receptor at the cell surface (Binnerts et al., 2007). Their canonical Wnt signaling activities are highly dependent on the presence of co-expressed Wnt ligands and are mediated by a stabilized β -catenin and TCF gene activation (Kim et al., 2006a, 2008). In the absence of Rspo/Wnt ligands, β -catenin is sequestered by a destruction complex consisting of APC, Axin and GSK3 and is phosphorylated by GSK3. The phosphorylated β -catenin is ubiquitinated by

E3 ubiquitin ligase and subjected to proteasomal degradation. Upon activation of Wnt signaling by Rspo and/or Wnt ligand bindings to their receptors, the destruction complex is disassembled and β -catenin is stabilized. It translocates to the nucleus, displaces Groucho from binding to TCF. The activated TCF/ β -catenin complex then transactivates the TCF target genes (Fig. 1) (Moon, 2005; Nusse, 2005, 2008; Gordon and Nusse, 2006; Coombs et al., 2008). Hence, Rspo activation of the canonical Wnt signaling requires LRP6 for activity and synergistic actions of other Wnt ligands for functions. Recent studies demonstrated that stabilization of β -catenin in mouse XY gonads induces male-to-female sex reversal both *in vitro* and *in vivo* (Maatouk et al., 2008). Thus, RSPO1 could positively switch on the canonical Wnt signaling and synergize with WNT4 in ovarian determination and differentiation (Tomizuka et al., 2008). This scenario is analogous to that of testis determination and differentiation, in which *SRY* switches on the male gonadal fate and requires the positive actions of *SOX9*, and other *SOX-E* genes, for a continuation of the developmental cascade (Schepers et al., 2003; Chaboissier et al., 2004; Cory et al., 2007).

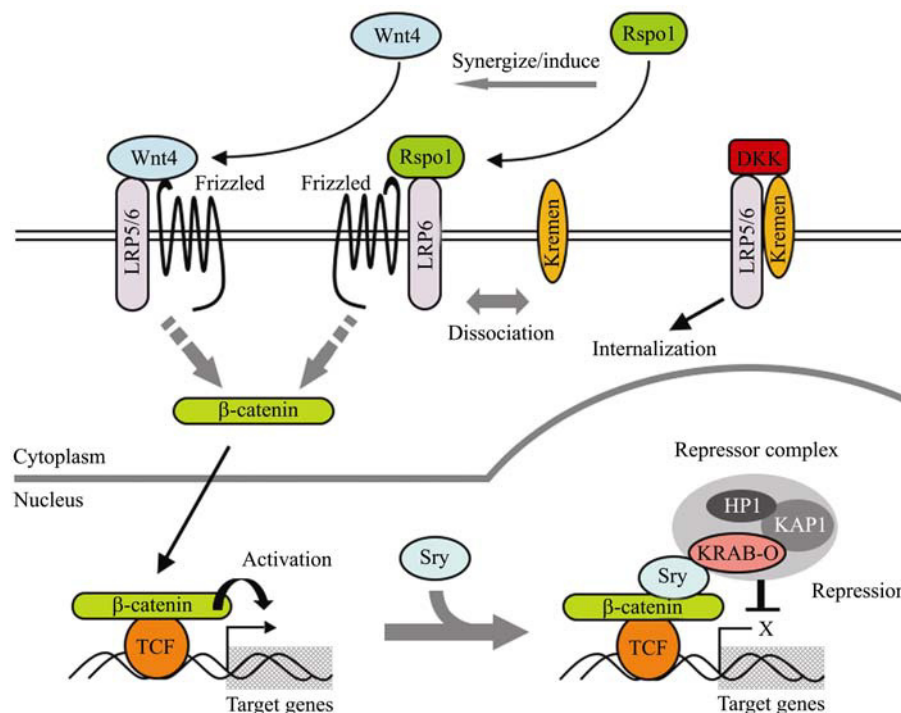


Fig. 1. A model of *Sry* action on *Rspo1*-activated Wnt signaling in sex determination. *Rspo1* functions as a primary ovarian determinant by activating the Wnt signaling in the bipotential gonads during embryogenesis. It has high affinity to and induces phosphorylation of the Wnt co-receptor, LRP6, thereby counteracting the DKK1-induced internalization of LRP6 and increasing the availability of Wnt receptors at the cell surface. It synergizes with available Wnt ligands, such as Wnt4, and stabilizes the intracellular β -catenin, which migrates to the nucleus and forms an active transcription complex with TCF. The β -catenin/TCF complex then binds to and transactivates the Wnt target genes, involved in ovarian development. In male gonads, *Sry* can repress such transactivation of the ovarian gene cascade through various means. One possible mechanism involves *Sry* binding and recruitment of gene silencing apparatus, including KRAB-O/KAP1/HP1 (Oh et al., 2005; Oh and Lau, 2006), to the β -catenin/TCF complex, thereby repressing the *Rspo1*-activation of the Wnt target genes.

Discrepancies in current studies of SRY functions on Rspo1/Wnt/ β -catenin signaling

The identification of two antagonistic sex determinants, i.e., *SRY* for testis and *RSPO1* for ovarian determination, respectively, raises the important question on how *SRY* counteracts the *RSPO1-WNT4* functions in order to switch on testis determination. Two recent reports suggest that *SRY* is capable of repressing Wnt/ β -catenin signaling in cultured cells (Bernard et al., 2008; Tamashiro et al., 2008). Both studies utilized the TCF dependent reporter, TOPflash that consists of a firefly luciferase gene directed by a minimal promoter and three binding sites (also known as Wnt response element or WRE, CTTGWWS) for TCF transcription factors (van de Wetering et al., 1997). Under normal conditions, TOPflash is inactive and is expressed at minimal levels. However, upon activation of the Wnt signaling, a stabilized β -catenin is translocated to the nucleus, where it interacts with and activates TCF transcription factors. The β -catenin/TCF complex, in turn, binds to its consensus site at the promoter of the luciferase reporter and transactivates its expression. The control construct, FOPflash, contains the same reporter gene, except that the TCF binding sites have been mutated, thereby is incapable of being activated by TCF transcription factors. Using this system, both groups of investigators have studied the effects of ectopically expressed *SRY* genes from human and mouse and have showed that *SRY* is capable of repressing such Wnt/ β -catenin signaling in cultured cells. Despite the common conclusion, these two reports differ significantly in terms of the probable mechanism(s) by which *SRY* mediates such signaling repression. The results and discrepancies of these two studies are summarized in Supplemental Table 1. The significant discrepancies are: 1) whether both the mouse and human *SRY* are capable of suppressing the Wnt/ β -catenin signaling, and 2) whether *Sry* activation of an unknown repressor gene or *SRY* interaction with β -catenin in the nucleus is important for such inhibitory function.

Preliminary studies in identifying the probable SRY action(s) on Rspo1/ β -catenin signaling

The discovery of a potential ovarian determinant challenges the classical paradigm of passive mode of differentiation for the female sex organ (Capel, 2006; Parma et al., 2006). The clarification of these initial discrepancies on

molecular actions of *SRY* on the *Rspo1*/Wnt signaling will be important for future research on the mechanism of sex determination. It is reasonable to argue that dissimilar findings could be results of differences in cell types and reagents utilized in these studies. To provide preliminary insights on these possibilities, we have examined the functions of human *SRY* and mouse *Sry* on *Rspo1*/ β -catenin signaling on a sensitive TOPflash system, utilizing the Super8XTOPflash reporter that harbors eight copies of the consensus TCF binding site on its promoter and the Super8XFOPflash control (Veeman et al., 2003). In addition to the human *SRY* gene, we have also evaluated two versions of the mouse *Sry* that are derived from the *musculus* and *domesticus* strains (Coward et al., 1994). The human *SRY* is a 24 kDa protein of 204 amino acids (Sinclair et al., 1990; Su and Lau, 1993), while the *musculus* *Sry* is a 49.5 kDa protein of 395 amino acids. The *domesticus* *Sry* is a truncated version of the *musculus* *Sry* with 230 amino acids and predicted molecular weight of 28.4 kDa, due to a premature translational termination generated by a C→T substitution at a CAG (glutamine) codon in the middle of the glutamine-rich domain (Coward et al., 1994). All three proteins harbor a conserved HMG box that is located at the middle and the N-termini of the human and mouse protein, respectively. Both versions of the mouse *Sry* protein harbor a glutamine-rich domain at their carboxyl termini, linked by a small bridge-domain to their N-terminal HMG box (see sketches in Fig. 3B). The glutamine-rich domain is capable of transactivating a reporter gene in cultured cells (Dubin et al., 1995) and is responsible for an apparently higher molecular weight (retarded) migration in SDS-PAGE (Coward et al., 1994). Transgenic studies demonstrated that the glutamine-rich domain is essential for proper male sex determination in mice (Bowles et al., 1999). The glutamine-rich domain of the *domesticus* *Sry* is polymorphic among different *domesticus* strains that show various degrees of XY sex reversal in the inbred *musculus* strain, C57BL/6 (B6), background (Coward et al., 1994; Taketo et al., 2005). The exact nature of such sex reversal, however, has not been fully resolved.

Using the human *SRY* and mouse *Sry* from *domesticus* and B6 strains, we have first addressed the β -catenin activation of the Super8XTOPflash reporter in human HEK293 cells. Minimal activities were detected in cells transfected with Super8XTOPflash reporter alone while significant induction of the luciferase activities were observed in cells co-transfected with the reporter and constitutively expressed β -catenin construct (Fig. 2A, left panel).

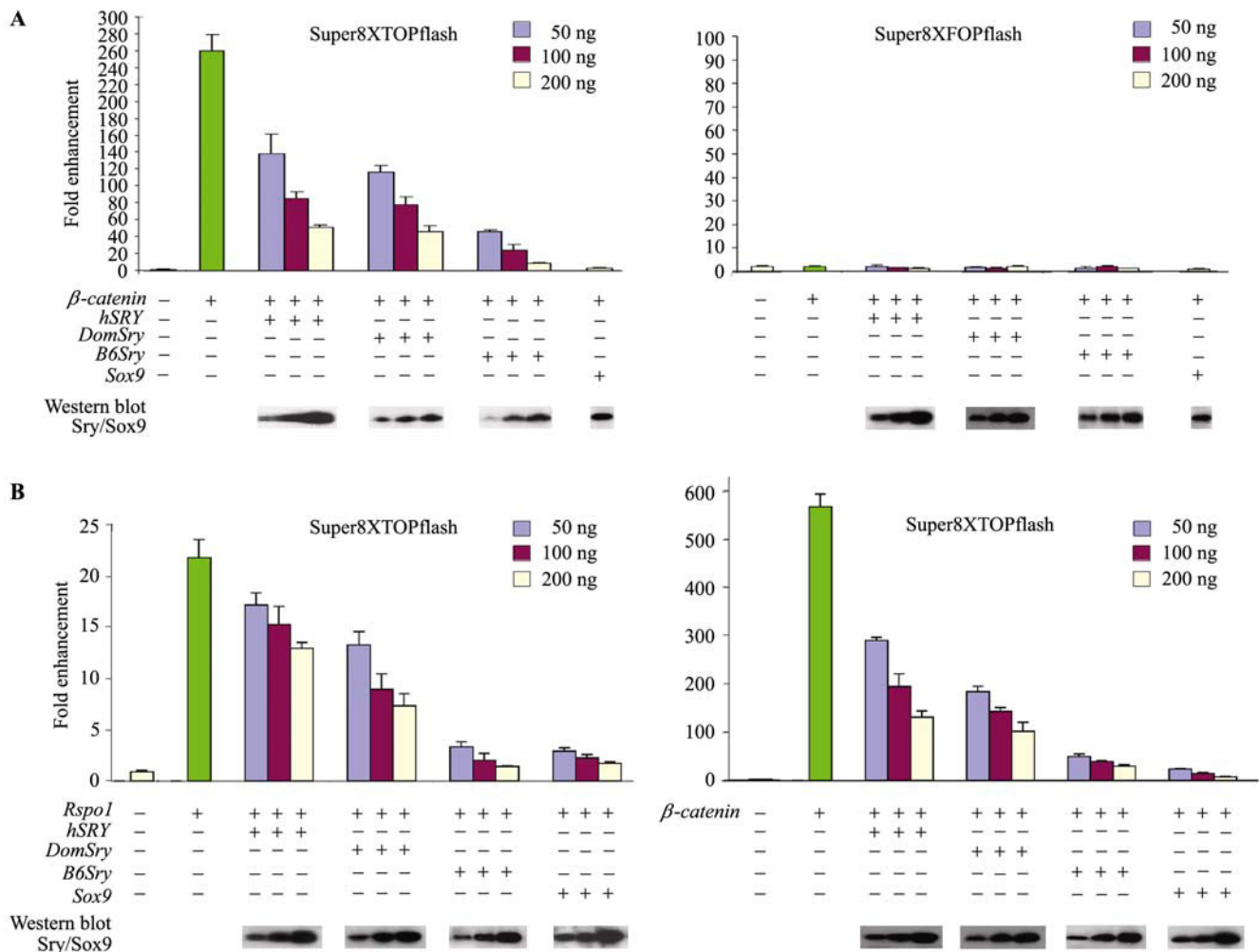


Fig. 2. SRY/Sry represses the Rspo1/Wnt/β-catenin signaling in cultured cells. Human HEK293 (A) or mouse Sertoli MSC-1 cells (B) were transfected in 24-well plates in triplicates with 200 ng of constitutively expressed β-catenin or Rspo1 gene (directed by a CMV promoter in pcDNA3.1), 100 ng of Super8XTOPflash (Veeman et al., 2003) and 50, 100 or 200 ng of respective expression vectors of human SRY (hSRY), mouse domesticus (DomSry) and musculus (B6Sry) Sry (Li et al., 2006), or mouse Sox9 (Oh et al., 2007), in the presence of 10 ng of Renilla luciferase vector (Promega Corp., Madison, WI, USA) as an internal control. The respective SRY cDNA was cloned in the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) and expressed under the control a CMV promoter. Super8XFOPflash (Veeman et al., 2003) was used as a negative control for Wnt/β-catenin signaling in these assays. Cells were harvested 48 h following transfection and analyzed for the firefly and Renilla luciferase activities with the dual-luciferase reporter assay system, as described before (Oh et al., 2005, 2007; Li et al., 2006). The triplicate samples of each assay point were combined and examined with Western blotting and corresponding antibody probes (Coward et al., 1994; Taketo et al., 2005; Li et al., 2006). The results show that both human SRY and mouse Sry, and Sox9 are capable of repressing a direct or Rspo1 activated β-catenin signaling in HEK293 and mouse MSC-1 cells in a dosage dependent manner.

Such β-catenin dependent transactivation of the reporter was significantly repressed by addition of human SRY and mouse Sry expression constructs in a dosage dependent manner. Interestingly, the potency of repression seemed to increase in the order of human SRY, DomSry and B6Sry, while SOX9 showed the highest repression. Parallel experiments conducted with the control Super8XFOPflash reporter showed minimal luciferase activities in all combinations of expression vectors (Fig. 2A, right panel). To evaluate the effects of SRY on Rspo1 in activation of Wnt/β-catenin signaling, we had performed similar ex-

periments with a mouse Sertoli cell line, MSC-1 that possesses properties resembling primary Sertoli cells (McGuinness et al., 1994; Griswold and Kim, 2001). Transfection of the mouse Rspo1 with Super8XTOPflash induced a significant expression of the luciferase reporter. Such induction was repressed with co-expressed human SRY and mouse Sry in a dosage dependent manner (Fig. 2B, left panel). Since Rspo1 expression construct was transfected in these assays, the activation of the Wnt/β-catenin signaling was presumably mediated in an autocrine manner. Rspo1 was synthesized in the cells and

secreted to the media where it stabilized the co-receptor LRP6 in the cell surface and activated the canonical Wnt signaling. Rspo1 also synergized with available Wnt ligands in mediating the β -catenin dependent activation of the Super8XTOPflash reporter. Parallel experiments in which Rspo1 expression vector was replaced with a β -catenin vector showed similar β -catenin induction of the reporter and repression of such activation by human and mouse SRY/Sry and Sox9 (Fig. 2B, right panel). Again, we observed a dosage dependent repression of the Rspo/Wnt/ β -catenin signaling in both sets of experiments in MSC-1 cells. The reporter activation and repression effects were generally higher with direct β -catenin expression in the MSC-1 cells than those with Rspo1 autocrine induction. Significantly, the magnitude of repression was similar to those observed in HEK293 cells and was in the order: human SRY < DomSry < B6Sry < Sox9. It is interesting to note that human SRY does not contain any glutamine-rich domain, while DomSry glutamine-rich domain is about half the size of that for B6Sry. The degrees of repression in this assay system seemed to be proportional to the relative size of the glutamine-rich/acidic domain of the respective SRY/Sry protein. Further, Sox9 harbors a relatively larger acidic domain at its carboxyl terminus and it exerted the most repression on the Wnt/ β -catenin signaling in this reporter system. Since the glutamine-rich/acidic domain of the mouse Sry has been demonstrated to be capable of transactivating a reporter gene *via* the consensus SRY binding site at the promoter of the reporter (Dubin et al., 1995), the glutamine-rich domain could possibly considered as a potential transactivation domain. These observations paradoxically suggest that gene transactivation of a yet-to-be determined repressor gene(s) could potentially be part of a mechanism(s) for SRY repression on Rspo/Wnt signaling in the transfected cells (Tamashiro et al., 2008).

Bernard and colleagues (2008) showed that the nuclear localization rather than the DNA binding property of the human SRY was important for its repression of the Wnt/ β -catenin signaling; and that the human SRY was capable of interacting with β -catenin. These observations suggest that SRY interfered with β -catenin function at the nucleus. Since only human SRY was analyzed in their studies, the question remains whether the mouse Sry is capable of binding to β -catenin. To explore this possibility, we had conducted a pulldown experiment (Oh et al., 2005; Li et al., 2006) using 35 S-labeled β -catenin and GST-fusion proteins containing human SRY, mouse Sry, or those with HMG box, bridging domain and glutamine-rich domain of

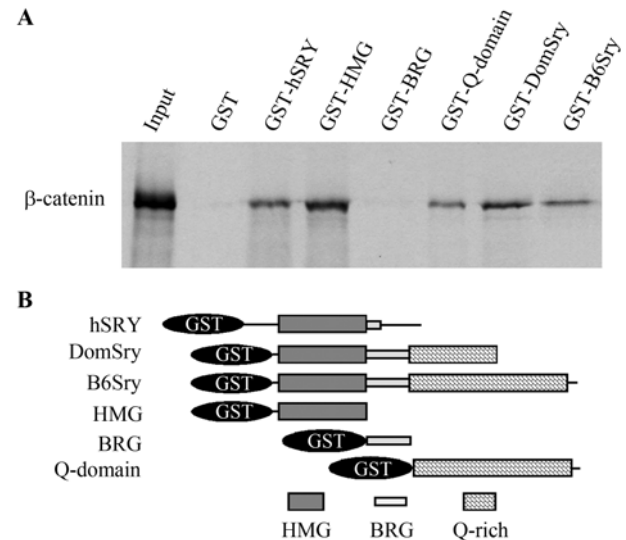


Fig. 3. β -catenin interacts directly with human SRY and mouse Sry (A). The open reading frames for human SRY, mouse *domesticus* (Dom) and *musculus* (B6) Sry and HMG box, bridge (BRG) domain and glutamine (Q)-rich/acidic domain of *musculus* Sry were subcloned in frame into the bacterial expression vector, pGEX2T (Amersham Biosciences, Piscataway, NJ, USA), as illustrated in (B), and were used to synthesize the respective GST fusion proteins in BL21(DE3) bacterial hosts (Oh et al., 2005; Li et al., 2006). They were individually reacted overnight at 4°C with 35 S- β -catenin, synthesized by a couple transcription-translation kit (Promega). After extensive washing, GST-resin bound β -catenin was analyzed by SDS-PAGE and detected by autoradiography (Oh et al., 2005; Li et al., 2006). The results showed that both human SRY and mouse Sry are capable of interacting directly with β -catenin. Further, the HMG box and glutamine-rich domain were identified as the β -catenin binding domains of the mouse Sry with the GST pulldown assay.

the *musculus* (B6) Sry, respectively. Our results demonstrated that both human SRY and mouse Sry were capable of binding to β -catenin while the control GST alone could not (Fig. 3A). Specifically, the HMG box and the glutamine-rich domain of the mouse Sry were responsible for such interactions with β -catenin. Although preliminary in nature, these results suggest that the human SRY could potentially bind to β -catenin *via* its HMG box while the mouse Sry could bind to β -catenin *via* its HMG box and glutamine-rich domain. These observations might account for the differential repression of the SRY/Sry on Wnt/ β -catenin signaling because the mouse Sry possesses additional β -catenin binding site(s).

How does the battle between the sex determinants shape up?

The demonstration of SRY/Sry function(s) on the

Rspo1/ β -catenin signaling of an ovarian determinant has greatly advanced our understanding on the molecular mechanism by which *SRY* switches on testis determination and differentiation. These early studies, however, have generated some discrepant results that might potentially hinder further research in the field (Bernard et al., 2008; Tamashiro et al., 2008). Our findings, although relatively preliminary, have clarified a few important points. First, the human *SRY* and two versions of the mouse *Sry* are capable of suppressing the signaling of *Rspo1* and its intracellular messenger, β -catenin, in activation of TCF dependent gene expression. We surmise that the Super8XTOPflash with 8 TCF binding sites is highly sensitive in the detection of effects affecting the *Rspo1*/Wnt/ β -catenin signaling (Veeman et al., 2003). We are uncertain, however, whether the cell types utilized in our studies also provide greater sensitivities, e.g., higher transfection efficiency or more compatible cellular environment, than those used by other investigators. Second, such *SRY*/*Sry* repression of *Rspo1*/ β -catenin signaling varies among *SRY*/*Sry* proteins of different origins. Currently, it is uncertain if such differential repression effects could be attributed to the presence and/or the size of the glutamine-rich/acidic domain in the respective *SRY*/*Sry* and *Sox9* proteins. Third, the HMG box of *SRY* binds directly to β -catenin while the glutamine-rich domain of the mouse provides additional binding to the same target. Again, it is unclear if HMG box and glutamine-rich/acidic domain bind to the same domain(s) of β -catenin or if such additional bindings might amplify the *Sry* repression of β -catenin function in the canonical Wnt signaling.

The present findings have raised several important questions. The most important one is how do these two antagonistic sex determinants, i.e., *SRY* and *RSPO1*, mediate the male and female sex development, respectively. As the ovarian determinant, *RSPO1* is postulated to switch on ovarian determination and differentiation by synergizing with specific Wnt ligands and activating the canonical Wnt signaling (Fig. 1), which in turn activates ovarian differentiating genes in the bipotential gonad. Further, it is conceivable that the same Wnt signaling could also repress the testicular differentiating genes, which are downstream of *SRY* and whose mutations contribute to some forms of XY sex reversal. Despite the popular use of the TOPflash system, which primarily demonstrates gene activation, Wnt signaling could also repress certain genes in various cell types (Jamora et al., 2003; Kahler and Westendorf, 2003; Kim et al., 2005a; Spencer et al., 2006; Delmas et al., 2007;

Hoverter and Waterman, 2008). Activated β -catenin/TCF complexes could bind to a different motif, AGAWAW (Blauwkamp et al., 2008), instead of the TCF consensus binding site or WRE, and could exert a repression on the target gene(s). Hence, *RSPO1* may possibly function as an activator and a repressor signaling ligand simultaneously during ovarian determination and differentiation. If *RSPO1* indeed switches on a repression program on testicular differentiating genes, it could be the postulated “Z” gene whose product is a negative regulator of the male sex determinant and is functional in wild-type females, proposed by McElreavy and colleagues (McElreavey et al., 1993).

Although McElreavey and colleagues (1993) predicted that the most important function of *SRY*/*Sry* is to repress or negatively regulate the “Z” gene, i.e., the *RSPO1*/*Rspo1*-activated Wnt signaling and to preempt the ovarian determination, the question remains as whether *SRY* can activate other testis differentiating genes and can repress the ovarian differentiating genes, downstream of *RSPO1*. Since in XX *Rspo1*^{−/−} mice, *Sox9* is not up-regulated until relatively late in embryo development, resulting in hermaphroditism (Chassot et al., 2008a, 2008b; Maatouk et al., 2008), *Sox9* must need a positive action(s) of *Sry* or other upstream genes to be activated during normal testis differentiation. A recent report suggests that *Sry*, in conjunction with *SF-1*, could regulate the proper gonadal expression of *Sox9* during mouse sex determination (Sekido and Lovell-Badge, 2008). Hence, as the primary sex determinants, *SRY*/*Sry* and *RSPO1*/*Rspo1* activate not only the gene programs of respective sex determination pathways but also mechanisms in repressing each other’s genetic cascades in order to properly propagate the differentiation of the testis and ovary, respectively.

Currently, the exact mechanism(s) by which *SRY* represses *Rspo1*/Wnt signaling is uncertain. One simple mechanism could be the degradation of β -catenin induced by its binding with *SRY*/*Sry* or *Sox9*. Indeed, a reduced level of β -catenin was observed in *SRY* transfected cells by Bernard and colleagues in their studies on the human *SRY* (Bernard et al., 2008) and others in studies on *Sox9* and β -catenin interactions (Akiyama et al., 2004). Alternatively, *SRY*/*Sry* could affect the TCF transactivation of Wnt targets by interacting with and interfering a β -catenin/TCF transactivation complex (Fig. 1). If repression of Wnt/ β -catenin signaling is at the TCF transcriptional level, the HMG box of *SRY*/*Sry* could compete with those of TCFs in binding to the TCF sites, resulting in a competition between an *SRY*/ β -catenin complex and a TCF/ β -catenin

complex in repression and transactivation respectively of the same target genes. Significantly, SRY/Sry has been demonstrated to interact with powerful chromatin modulators and/or transcriptional silencing complex(es), including KRAB only and KRAB zinc finger, KAP1 and heterochromatin proteins (Oh et al., 2005; Li et al., 2006; Oh and Lau, 2006), and its binding to β -catenin could potentially recruit these powerful repressors to the TCF/ β -catenin complex, thereby repressing the transactivation activities of the latter (Fig. 1). The demonstration of antagonistic functions between the two sex determinants has provided a new model(s) for gonadal differentiation. Many of these interesting questions raised here would likely be addressed and resolved experimentally in the future, thereby advancing our understanding of the elusive mechanism(s) of SRY function(s) in sex determination.

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Supplemental data

Supplemental Table 1 associated with the article can be found in the online version at www.jgentgenomics.org.

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