Expression of *Wsb2* in the developing and adult mouse testis

M A Sarraj^{1,2,3}, P J McClive², A Szczepny^{1,3}, H Daggag², K L Loveland^{1,3} and A H Sinclair^{1,2}

¹The ARC Centre of Excellence in Biotechnology and Development, ²Murdoch Children's Research Institute and the Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Melbourne, Victoria, Australia and

³Monash Institute of Medical Research, Monash University, Clayton, Victoria, Australia

Correspondence should be addressed to A H Sinclair, Murdoch Children's Research Institute, Royal Children's Hospital, Melbourne, Victoria 3052, Australia; Email: andrew.sinclair@mcri.edu.au

Abstract

We present a detailed study of the expression pattern of WD repeat and SOCS box-containing 2 (*Wsb2*) in mouse embryonic and adult gonads. *Wsb2* was previously identified in a differential screen aimed at identifying the genes involved in male- and female-specific gonadal development. *Wsb2* expression was analysed during mouse gonadogenesis by real-time PCR, whole-mount and section *in situ* hybridisation and immunofluorescence. *Wsb2* mRNA expression was initially detected in gonads of both sexes from 11.5 days *post coitum* (dpc) until 12.0 dpc. By 12.5 dpc and thereafter, *Wsb2* expression rapidly decreased in the female, while persisting in the male gonads. In foetal, newborn and juvenile testes, *Wsb2* mRNA and protein were readily detected in the seminiferous cords within both Sertoli and germ cells. *Wsb2* mRNA was present in spermatogonia, spermatocytes and in Sertoli cells of the adult mouse testis. The differential expression of *Wsb2* in male versus female embryonic gonads suggests some male-specific role in gonad development, and its expression in the first wave of spermatogenesis indicates a role in germ cells. Real-time analysis of adult mouse testis tubules cultured in the presence of the Hedgehog signalling inhibitor, cyclopamine, showed a downregulation of *Wsb2* mRNA after treatment which suggests that *Wsb2* may be a target of Hedgehog signalling.

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Introduction

Sex determination and testis formation in most mammals is initiated through the expression of the sex-determining region on the Y chromosome (*SRY*) gene (Sinclair *et al.* 1990). The presence of *SRY* results in male differentiation; its absence leads to ovary formation and female differentiation. Suppression subtraction hybridisation was used to identify genes specifically transcribed in the male but not the female gonad at 12.5 days *post coitum* (dpc; McClive *et al.* 2003). One of the male-specific genes isolated was WD (tryptophan-aspartate) repeat and SOCS box-containing 2 (*Wsb2*), which belongs to the suppressor of cytokine signalling (SOCS) family. Two WSB proteins (WD-40 repeat-containing proteins with a SOCS box) have been identified, WSB1 and WSB2 (Hilton *et al.* 1998). These proteins are composed of an N-terminal region of variable length and amino acid composition, eight WD-40 repeats and a SOCS box. WSB1 and WSB2 share 65% similarity at the protein level and hence potentially share some functional homology. *WSB1* was identified in a screen for Sonic Hedgehog (*SHH*)-inducible genes and was originally named *SWIP1* (SOCS box and WD repeats in

Protein1). *WSB1* induction by *SHH* has been demonstrated in explant cultures of segmental plates from chick embryos, and it was one of the earliest markers to respond to *SHH* signalling in the limbs (Vasiliauskas *et al.* 1999).

Wsb2 was previously detected by northern analysis in most adult mouse tissues and also during embryogenesis between 9 dpc and 18 dpc (Hilton *et al.* 1998). The present study describes a detailed analysis of the expression pattern of *Wsb2* during mouse gonadogenesis.

Materials and Methods

Animals and preparation of mouse embryos

Outbred Swiss white CDI mouse embryos were obtained from the animal house at Royal Children's Hospital (RCH) or from Monash University Animal Services. All investigations conformed to the National Health and Medical Research Council/Commonwealth Scientific & Industrial Research Organization/Australian Agricultural Council Code of Practice for the Care and Use of Animals for Experimental Purposes and were approved by the RCH Animal Ethics Committee or the Monash

University Standing Committee on Ethics in Animal Experimentation.

The embryos were dissected from pregnant mice in ice-cold DEPC–PBS (diethyl pyrocarbonate–phosphate buffer solution). Heads, torsos and viscera overlying the urogenital systems (gonads, mesonephros, Müllerian and Wolffian ducts) were removed and the embryos containing the urogenital system were processed. Embryos were fixed in 4% paraformaldehyde (PFA) for whole-mount *in situ* hybridisation (WISH) analysis or fixed in Bouin's for section *in situ* hybridisation. For RT-PCR, gonads were dissected free from the mesonephros, frozen immediately on dry-ice and stored at -80°C until RNA extraction. For immunofluorescence, embryos were fixed in 4% PFA, left overnight in 20% sucrose at 4°C , embedded in OCT (Tissue Tek, USA) and snap frozen in isopentane/liquid nitrogen.

Busulfan was administered *in vivo* to deplete developing embryos of germ cells as described (McClive *et al.* 2003). Briefly, 9.5 dpc pregnant mother mice were injected intraperitoneally with busulfan (40 mg/kg; Sigma) in dimethyl sulphoxide (50% DMSO; Sigma) and mouse embryos were dissected at 13.5 dpc, as previously described for WISH.

Adult mouse seminiferous tubule culture

Dissected adult mouse testes were decapsulated and placed in Dulbecco's minimal eagle medium (DMEM). Tubules were dissociated using fine forceps and cut into 2–5 mm fragments. Up to five tubule fragments were cultured in 30 μl hanging drops in DMEM and 0.1% BSA at 32°C for 48 h with 5% CO_2 /95% air. Cyclopamine (final concentration 100 μM ; Calbiochem, Kilsyth, Victoria, Australia) was added to the culture medium to inhibit Hedgehog signalling (Chen *et al.* 2002). An equivalent volume of ethanol (the solvent for cyclopamine) was added to control samples. After culture, the tubules were collected and stored at -80°C prior to RNA extraction. All experiments were performed for a minimum of three times.

Section *in situ* hybridisation (ISH) and WISH

The *Wsb2* clone isolated during the differential screen described by McClive *et al.* (2003) was used as a template for production of antisense and sense riboprobes. This fragment comprised 610 bp mouse clone AF072881. Whole-mount *in situ* hybridisation was carried out as previously described on PFA-fixed embryos (Andrews *et al.* 1997). A minimum of two embryos per sex were sampled in at least two independent experiments.

Section ISH using digoxigenin-labelled cRNAs was used to localise *Wsb2* mRNAs in 4 μm sections of Bouin's fixed, paraffin-embedded mouse tissue using procedures previously described, with hybridisation and

washing temperatures up to 50°C (Meinhardt *et al.* 1998). Both antisense and sense (negative control) cRNAs were used on each sample in every experiment. A minimum of two independent sections were assessed in at least two independent experiments.

RT-PCR and real-time PCR

Three pools of seven to ten pairs of gonads of each sex were collected and total RNA was isolated using the Qiagen RNA isolation kit (Qiagen), from embryos at 12.5, 14.5, 16.5 dpc and 0 dpp (day of birth). RNA was treated with DNase I (IU/1 mg RNA) for 30 min at 37°C , and the enzyme inactivated by heating to 70°C for 5 min. DNase I-treated total RNA samples (500 ng) were then transcribed using Superscript II reverse transcriptase and a mixture of oligo d(T)₂₀ and random hexamer primers as previously described (Sarraj *et al.* 2005). RNA was quantified using the Quant-iT RiboGreen RNA Assay Kit (Molecular Probes, Invitrogen). For RT-PCR, samples were processed as described by Sarraj *et al.* (2005). For real-time PCR, samples were prepared in a final volume of 10 μl using either Roche Diagnostics SYBR-Green PCR master mix or the Applied Biosystems (ABI, Scorsby, Victoria, Australia) SYBR-Green PCR master mix, 300 nM forward and reverse primers. PCR was performed either on the Light Cycler 2.0 Instrument (Roche Molecular Biochemicals) or on the 7900 Applied Biosystem Fast real-time machine using the following thermocycler conditions: stage 1, 50°C for 2 min, 1 cycle; stage 2, 95°C for 10 min, 1 cycle; stage 3, 95°C for 15 s and 55°C for 1 min, 40 cycles. Melting curve analysis and agarose gel electrophoresis were used to monitor production of the appropriate PCR product.

Each PCR was performed in triplicate with negative controls, where water was used in place of the reverse-transcribed template included for each primer pair, to visualise PCR amplification of any contaminating DNA. The amount of *18S* mRNA was measured in each sample template to normalise between samples (*18S*, forward: 5' gtaaccgttgaacccatt 3'; *18S*, reverse: 5' ccatacaatcggtagtagcg 3' from accession number: NM_003278). For RT-PCR, β -actin primers used were as follows: β -actin forward: 5' aggtgtgtgtgtcctgtat 3' and β -actin reverse 5' aaggaaggctggaaaagagc 3' from accession number (AK075973). *Wsb2* primers used were: *Wsb2* forward: 5' gtaagcagatccaggtgttatccg 3' and *Wsb2* reverse: 5' ccagatcctgagcagcctgt 3'. *Wsb2* primers amplified a product of 401 bp. *Gli1* primers used were: *Gli1* forward: 5' ggaagtcctattcagccttga 3' and *Gli1* reverse: 5' caaccttctgtctcacatgta 3'. These primers amplified a product of 88 bp from accession number (NM_010296; Doles *et al.* 2006). All products were verified by subcloning and sequencing.

To correlate the cycle threshold (Ct) values from the sample amplification plot to target copy number, a standard curve was produced for each target using adult

mouse testis cDNA containing a starting material of 500 ng RNA. To control for overall gene expression, the average Ct for *18S* was subtracted from the average Ct value for *Wsb2* to generate the ΔCt for each sample. Fold change was calculated as 2 to the $-\Delta\Delta\text{Ct}$ power ($2^{-\Delta\Delta\text{Ct}}$) as previously described by Yao *et al.* (2006). Statistical analysis was performed using Student's unpaired *t*-test or one-way ANOVA ($P < 0.01$ is considered significant) to analyse statistical differences between samples. The data were calculated as the percentage mean $\pm$ S.E.M. The experiment was performed three times and each sample was done in triplicate.

Immunohistochemistry and immunofluorescence

Wsb2 antibody was kindly provided by Doug Hilton (Walter Eliza Hall Institute, Melbourne, Victoria, Australia) and was purified from crude rabbit antiserum using Protein A Sepharose according to the manufacturer's instructions (Bio-Rad) using a column connected to a Bio-Rad Econo pump. Individual fractions were analysed by SDS-PAGE to determine purity. Anti-Wsb2 was used at 1:2000 dilution (0.2 mg/ml) in Tris-buffered saline (TBS)/0.1% BSA/PBS. For immunohistochemistry, binding was detected using a biotin-labelled goat anti-rabbit IgG secondary (DAKO, Botany, Australia) applied

Figure 1 A protein alignment of WSB2 human, mouse and chicken sequences. Amino acid alignment of human WSB2 (accession number: NP_061109), mouse Wsb2 (accession number: NP_067514) and the available incomplete chick WSB2 (accession number: XP_415313). Conservative amino acid changes are represented in light grey and identities are represented by the dark grey shading. The light grey box represents the SOCS box sequence and the black boxes represent the WD40 repeats. The sequence corresponding to the coding region of the *Wsb2* probe is underlined with a black thick line.

Figure 2 Whole-mount *in situ* hybridisation analysis of the time course of *Wsb2* expression in the embryonic male and female mouse gonads. The top panel shows expression in the male gonads between 11.5 (A) and 13.5 (D) days *post coitum* (dpc). The lower panel shows expression in the female gonads between 11.5 and 12.0 dpc and a decreased signal at 12.5 (G) and 13.5 (H) dpc. Arrowhead, gonad; arrow, mesonephros. Scale bar = 50 µm.

to sections at the ratio of 1:500. For immunofluorescence, binding was detected using green-fluorescent Alexa Fluor 488 goat anti-rabbit IgG secondary (Molecular Probes, Invitrogen) applied to sections at the ratio of 1:200 in TBS/0.1% BSA/PBS.

Tissue was fixed in Bouin's fixative for 5 h, dehydrated in ethanol and embedded in paraffin. Sections (4 µm) were deparaffinised two times for 5 min each and rehydrated in an ethanol gradient (100% (two times), 95 and 70%) for 5-min cycles. Slides were then treated with 3% hydrogen peroxide to quench endogenous

peroxidases. For immunofluorescence, the cycle times were extended to 20 min for deparaffinising and 15 min for ethanol washes and no hydrogen peroxide was used. Antigen retrieval was performed in 50 mM glycine (pH 3.5; for 10 min at >90 °C) and slides were left to cool for 20 min before proceeding. Slides were incubated for 20 min with 10% normal serum (of the species in which the secondary antibody was raised) in TBS. Slides were rinsed with TBS (two times for 5 min each) between incubations. The primary antibody was applied at (1:2000 dilution) for overnight incubation in TBS/0.1%

Figure 3 Whole-mount *in situ* analysis of *Sox9*, *Oct4* and *Wsb2* expression in the foetal mouse testis at 13.5 dpc with and without busulfan treatment. (A, C and E) Testes from untreated animals. (B, D and F) Testes from pups exposed to busulfan *in utero* to deplete germ cells. (A and B) *Sox9* expression in testis. (C and D) *Oct4* expression in testis. (E and F) *Wsb2* expression in testis. *Wsb2* expression persists in the absence of germ cells (F). Scale bar = 50 µm.

Figure 4 *In situ* hybridisation analysis of 13.5 dpc mouse testis sections. (A) Antisense probe shows expression of *Wsb2* in the germ cells, Sertoli cells and some interstitial cells in a transverse section of the embryonic mouse testis. (B) A higher magnification of the cord marked with a dashed line in (A). White arrow, germ cell; asterisk, Sertoli cell cytoplasm. Scale bar = 50 µm.

BSA/PBS. The primary antibody binding was detected using the secondary antibody to sections, for 1 h at room temperature. Following application of the secondary antibody, sections were washed with TBS (two times for 5 min each). For immunohistochemistry, the Vectastain Elite ABC kit was used according to manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). Antibody binding was detected as a brown precipitate following development with 3,3-diaminobenzidine tetrahydrochloride, and Harris Hematoxylin was used as a counterstain. The sections were mounted under glass 22 × 55 mm coverslips (HD Scientific, Bayswater, Victoria, Australia) in Depex (BDH Laboratories, Poole, UK). For immunofluorescence, following secondary detection, sections were washed with TBS (two times for 5 min each) and mounted with FluorSave (Calbiochem) under glass coverslips.

Results

Sequence analysis of *Wsb2*

The *Wsb2* cDNA fragment isolated from the differential screen previously described (McClive *et al.* 2003), was used as a template to generate antisense and sense riboprobes for WISH analysis. This clone comprised 610 bp cDNA sequence encoding part of the sixth WD40 repeat, the seventh and eighth WD40 repeats, the SOCS box and 40 bp 3' UTR of the cDNA (accession number: AF072881). A comparative alignment of WSB2 sequences displaying the WD40 repeats and the SOCS box in human, mouse and chick is shown in Fig. 1. Human WSB2 is on chromosome 12q24.3 and mouse *Wsb2* on chromosome 5 (LocusLink, NCBI database).

Differential expression of *Wsb2* during the time of sex determination

The analysis of *Wsb2* expression indicated that the transcript is present in the male gonads at 11.5 dpc (Fig. 2). At 12.5 and 13.5 dpc, the staining is observed as a striped pattern across the organ, characteristic of expression in the seminiferous cords. In the female, staining is observed throughout the gonads at 11.5 and 12.0 dpc. At 12.5 and 13.5 dpc, the *Wsb2* signal in the female gonads is relatively reduced (Fig. 2). *Wsb2* was also found to be expressed in other sites in the mouse embryo by WISH analysis: forebrain, midbrain, the mantle layer of the neural tube, the dorsal root ganglia, the heart and the myotome-derived epaxial muscle mass (data not shown).

Figure 5 Protein localisation of *Wsb2* at 13.5 dpc in the developing mouse testis. (A) Transverse section showing immunohistochemical staining (in brown) to detect *Wsb2* in the 13.5 dpc male gonad, with a blue nuclear counterstain to enable cell-type identification. Yellow dashed lines mark cord borders. (B) A higher magnification of (A) showing cellular localisation of *Wsb2* protein. (C) Transverse section showing immunofluorescent staining for *Wsb2* protein in 13.5 dpc (green). Signal is prominent within the cords and is readily visible as a cytoplasmic protein. Yellow dashed lines mark cord borders. (D) A higher magnification of (C) showing cellular localisation of *Wsb2* protein. White arrow, a germ cell; yellow arrow, Sertoli cell nucleus; yellow arrowhead, interstitial cells; small white arrowheads, Sertoli cell cytoplasm; black arrowhead, endothelial blood cells. Inserts in (A) and (C) represent negative controls prepared without primary antibody. Scale bar = 50 µm.

Figure 6 (A) The relative levels of *Wsb2* mRNA when compared with levels of the housekeeping gene, *18S* from 12.5 dpc to the day of birth were measured by quantitative real-time PCR in mouse foetal gonads of both sexes. The Y-axis represents the relative expression of *Wsb2* normalised to *18S* levels in both sexes across the different ages of development. Upregulation of *Wsb2* mRNA is measured in gonads with the significant maximal differential increase at the day of birth. Asterisk (*) indicates a statistical difference ($P < 0.01$). Error bars represent standard errors of the mean. (B) RT-PCR amplification of *Wsb2* mRNA in postnatal mouse testis and ovary. A PCR product of the correct size (401 bp) was detected following amplification with *Wsb2* primers in testes and ovaries at each age tested. β -Actin mRNA amplification served as a positive control.

Expression of *Wsb2* mRNA and protein in both Sertoli and germ cells of the embryonic testis

To identify the specific cell types expressing *Wsb2* in the testis, WISH analysis was conducted on gonads from busulfan-treated (germ cell) depleted embryos, as well as on untreated controls. In the untreated controls, the expression of *Sox9* (Sertoli cells marker) and *Oct4* (germ cell marker) was readily detected in the testicular cords. Following busulfan treatment, as expected, the expression of *Sox9* was maintained and the expression of *Oct4* was lost. In busulfan-treated testes, *Wsb2*-staining remained detectable within the cords, which indicates that *Wsb2* is expressed at least in Sertoli cells (Fig. 3). Section *in situ* hybridisation analysis of foetal testis also revealed mRNA signal in germ cells, Sertoli cell cytoplasm and in some interstitial cells (Fig. 4).

To clearly identify the cell types containing *Wsb2* protein within the testis cord and its approximate subcellular localisation, both immunohistochemistry and immunofluorescence analyses were performed. From 12.5 to 18.5 dpc, *Wsb2* protein is detected in both germ and Sertoli cells as well as some interstitial cells. Figure 5 illustrates *Wsb2* protein expression at 13.5 dpc localised to the cytoplasm of germ and Sertoli and interstitial cells of the mouse testis. Expression is also evident in some cells with morphology similar to that of endothelial blood cells.

Wsb2 mRNA levels differ throughout male and female foetal gonad development

Quantitative PCR was used to compare *Wsb2* mRNA in foetal gonads of both sexes at 12.5, 14.5, 16.5 dpc and at

Figure 7 Section *in situ* hybridisation and immunohistochemical analysis on postnatal mouse testis. Transverse sections showing the presence of *Wsb2* mRNA and its protein in developing testes. (A) mRNA expression at the day of birth signal is present in the Sertoli cell cytoplasm and in the gonocytes. (C) mRNA expression at day 15 *dpp* signal is evident in spermatogonia through the pachytene spermatocytes and in Sertoli cells. Antisense (A and C), expression of *Wsb2* mRNA. Sense controls (B and D), no signal detected. (E) Protein expression at the day of birth signal is present in the Sertoli cell cytoplasm and in the gonocytes. (G) Protein expression at day 15 *dpp* signal is evident in spermatogonia through the pachytene spermatocytes and in Sertoli cells. Negative control (F and H), no expression of *Wsb2* protein in the absence of the primary antibody. Yellow arrowhead, Sertoli cell nucleus; asterisk, Sertoli cell cytoplasm; white arrowhead, spermatogonium; white double arrowhead, pachytene spermatocyte; black arrowhead, interstitial cells. Scale bar = 50 μ m.

Figure 8 Section *in situ* hybridisation showing cellular localisation of *Wsb2* mRNA within Sertoli cells, germ cells and some Leydig cells of the adult testis. The antisense probe (A, C and D) produces a strong signal corresponding to *Wsb2* mRNA in spermatogonia, and weaker expression in spermatocytes, and round spermatids. Sense control probes (B), no signal. (E) Staging diagram illustrates the relative levels of signal intensity observed in cells of adult testis. Adapted from Russell *et al.* (1990). White arrowhead, Sertoli cells; white arrow, spermatogonia; black double arrowhead, pachytene spermatocytes; double arrowhead, round spermatids; black arrow head, Leydig cell.

the day of birth (Fig. 6A). In males, an increase in mRNA expression was measured with increasing age, with a threefold increase between 12.5 and 14.5 dpc and sixfold increase between 12.5 and 16.5 dpc. A maximal significant increase of 6.6-fold ($P < 0.01$) was observed between 12.5 dpc and the day of birth. In female gonads, a threefold increase was seen between 12.5 and 14.5 dpc followed by a twofold increase in mRNA levels between 12.5 and 16.5 dpc. The highest sex-specific differential expression was observed at the day of birth with a significant sevenfold higher level of RNA detected in the newborn testis when compared with that in the ovary ($P < 0.01$; Fig. 6A).

Expression of *Wsb2* at the day of birth and in postnatal gonads

RT-PCR amplification of total RNA extracted from whole mouse gonads indicated that *Wsb2* levels also differ between male and female gonads after birth (Fig. 6B). *Wsb2* expression was not detected by RT-PCR at the day of birth in the ovary, but was detectable from 2 days post partum (*dpp*) and in the adult ovary. *Wsb2* expression was also detected in the adult mouse testis.

Section *in situ* hybridisation and immunohistochemistry indicated the presence of *Wsb2* mRNA and

protein within the cytoplasm of Sertoli cell, gonocytes and interstitial cells at the day of birth (0 *dpp*; Fig. 7A and E). At 15 *dpp*, signal was detected in Sertoli cells and germ cells, including spermatogonia and spermatocytes (Fig. 7C and G).

In the adult mouse testis, *Wsb2* was detected in some Sertoli cells, spermatogonia and spermatocytes but was absent from the elongating spermatocytes (Fig. 8A–F). In the adult ovary, signal was detected in the follicles within granulosa cells of primary throughout antral stages of follicle development. A weaker signal was also observed in the corpus luteum and the internal layer of thecal cells (Fig. 9A).

Evidence that *Wsb2* is a target of Hedgehog signalling

To test whether *Wsb2* may be a target of Hedgehog signalling, adult mouse testis tubules were cultured in the presence of the Hedgehog inhibitor, cyclopamine. Real-time PCR analysis revealed that *Wsb2* mRNA levels were 28% lower in cyclopamine-treated tubules when compared with untreated controls (Fig. 10A), while *Gli1* mRNA, an established downstream target of Hedgehog signalling pathway in the testis, was downregulated by 87.1% upon cyclopamine treatment of tubule fragments (Fig. 10B).

Figure 9 Section *in situ* hybridisation showing cellular localisation of *Wsb2* mRNA within the adult mouse ovary. *Wsb2* expression is detected in granulosa cells of the follicles, theca cells and corpus luteum. White arrow, granulosa cells; white double arrowhead, theca cells; asterisk, corpus luteum (A and C). Sense control probe (B). Scale bar = 50 μ m.

Figure 10 Quantitative real-time PCR analysis of *Wsb2* and *Gli1* expression in adult mouse seminiferous tubule cultures. Treatment with 100 μ M cyclopamine was compared with control cultures treated with ethanol. The Y-axis represents the relative levels of *Wsb2* mRNA when compared with the housekeeping gene *18S*. * $P < 0.01$ compared with control. Error bars represent standard errors of the mean from three replicate cultures.

Discussion

The present analysis demonstrates that the *Wsb2* mRNA is upregulated during development of foetal male gonads, extending previous findings from a subtractive hybridisation screen that was performed to identify genes expressed more highly in either male or female mouse gonads at 12.0–12.5 dpc (McClive *et al.* 2003). *Wsb2* expression was detected in the screen to be elevated in male gonads after the peak of *Sry* expression. This suggested that it acts downstream of the *Sry* switch and is involved in early testis differentiation.

The present study demonstrates that *Wsb2* is expressed in the developing male mouse gonads at 11.5 dpc, when the mRNA levels are roughly equivalent in both sexes. The signal is present in both sexes from 11.5 dpc until 12.0 dpc. The signal increases with age in the male gonads, while in the female gonad, a threefold increase was seen between 12.5 and 14.5 dpc followed by the downregulation in mRNA levels from 14.5 dpc and until the day of birth. At birth, a significant sevenfold higher level of *Wsb2* RNA was detected in the testis when compared with that in the ovary. *Wsb2* mRNA and its protein were detected in Sertoli, interstitial and germ cells throughout testis development.

Wsb2 contains WD repeats, highly conserved amino acid sequences found in a wide variety of cytoplasmic

proteins many of which are involved in signal transduction or cell regulatory functions (Neer *et al.* 1994). *Wsb2* also contains a SOCS box, a feature of other SOCS proteins involved in cytokine signalling and intracellular signal transduction. Although not much is known about the WSB family in testis development, WSB has been shown to be involved in Hedgehog signalling during limb and neural development. The Hedgehog pathway is also critical for foetal gonad development. Hedgehog signalling in the mammalian testis is involved in communicating between Sertoli and Leydig cells, germ cells (Kroft *et al.* 2001) and peritubular cells (Clark *et al.* 2000). The Hedgehog family member desert Hedgehog (Dhh) was found to be expressed in Sertoli cell precursors shortly after the activation of *Sry* and persists in these cells into adulthood (Bitgood *et al.* 1996, Szczepny *et al.* 2006). Male mice with a Dhh null mutation are sterile and exhibit a block in germ cell maturation at the pachytene primary spermatocyte stage (Kroft *et al.* 2001). Adult mouse testis tubules cultured *in vitro* for 48 h in the presence of the specific Hedgehog signalling inhibitor, cyclopamine, exhibited down-regulation of mRNA expression of *Wsb2*. The decrease in *Wsb2* mRNA levels after Hedgehog inhibition indicates that *Wsb2* is downstream of *Dhh* in the signalling pathway, however, further studies would be required to establish its nature. The structure and expression pattern of *Wsb2* suggest that it may have roles in cell–cell interactions in embryonic gonad development and later in spermatogenesis. The presence of *Wsb2* in the adult female ovarian follicles and in theca cells suggests that it may also be participating in the communication between granulosa and developing theca cells. A recent study showed the mammalian ovary to be a site of active Hedgehog signalling (Wijgerde *et al.* 2005), and further study will provide an insight on whether *Wsb2* participates in this signalling.

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