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Communication

Molecules and Cells

Expression of Murine *Asb-9* During Mouse Spermatogenesis

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We previously showed that *Asb-4* and *Asb-17* is uniquely expressed in developing male germ cells. A recent report showed that *Asb-9* is specifically expressed in the kidney and testes; however, detailed expression patterns in developing germ cells have not been shown. Northern blot analysis in various tissues demonstrated that *mAsb-9* was strongly expressed in the testes. Expression analysis by RT-PCR and Northern blot in developing mouse testes indicates that *mAsb-9* is expressed from the fourth week after birth to adulthood, with the highest expression in round spermatids. Expression sites were further localized by *in situ* hybridization in the testes. Pachytene spermatocytes and spermatids expressed *mAsb-9* but spermatogonia and generated spermatozoa did not. This study reveals that *mAsb-9* could be a specific marker of active spermatogenesis and would be useful for studies of male germ cell development.

INTRODUCTION

The ankyrin repeat-containing SOCS box proteins (Asbs) contain a protein interaction motif upstream from the SOCS box composed of a variable number of ankyrin repeats (Debrincat et al., 2007). The suppressor of the cytokine signaling (SOCS) box motif, extending approximately 40 amino acids, has been identified in a number of proteins. It has been shown that these proteins inhibit signaling pathways initiated by cytokines, hormones and growth factors through direct interactions with JNK kinases or activated cytokine receptors (Kile et al., 2001; Kohroki et al., 2005; Nicholson et al., 1999; Yoshimura et al., 1995). Ankyrin repeats are expressed in more than 400 different proteins that contain from 2 to more than 20 units arrayed as tandem repeats. The fact that ankyrin repeat structures are comprised of helix-turn-helix motifs linked together by loops provides evidence that ankyrin acts as a stable platform for sites of protein-protein interaction. Ankyrin repeats occur in molecules with a wide variety of functions including receptors, cell-cycle regulators, membrane skeletal proteins, secreted proteins, tumor suppressors, and transcription factors (Breeden and Nasmyth, 1987; Sedgwick and Smerdon, 1999; Serrano et al., 1993; Wharton et al., 1985).

We previously showed that *Asb-4* and *Asb-17* were uniquely expressed in developing male germ cells (Kim et al., 2004; 2008). Even though Asbs have been studied in a number of cellular systems, their functions in male germ cells are poorly understood. Formation of the male gamete occurs in sequential mitotic division of the spermatogonial stem cell, two meiotic divisions by spermatocytes, and unique morphological remodeling of the spermatid during spermiogenesis - a process that continues throughout adult life in most mammals, including humans. Many germ-cell-specific gene expressions have been observed during this process. Such expression is developmentally regulated and stage-specific. Understanding the genetic regulation of male gametogenesis including spermatocytogenesis and spermiogenesis, will provide insight into the cellular process of differentiation (Park et al., 2001; Rhee and Wolgemuth, 2002).

We report here a clarification of the characterization of a member of the Asb family, *mAsb-9* and its tissue- and stage-specific expression in mouse developing germ cells.

MATERIALS AND METHODS

Animals and isolation of spermatogenic cell population

Male ICR mice (5 days, 2, 4, 6, and 10 weeks old) and female ICM mice (15.5 dpc and 6 weeks old) were purchased from Daehan Biolink Co. Ltd. (Korea). All tissue samples were dissected under a microscope and were processed immediately. A mixed population of spermatogenic cells were obtained from mouse testes using the collagenase dissociation method (Bellve et al., 1977). A purified population of spermatogenic cells were isolated following collagenase treatment of the testes and trypsin digestion of isolated seminiferous tubules using unit gravity sedimentation velocity in a bovine serum albumin (BSA) gradient (Bellve et al., 1977; Romrell et al., 1976). Tubes containing each cell population (pachytene spermatocyte (PS), > 90% pure; round spermatid (RS), > 90% pure; and condensing spermatid (CS)-residual body mixture) were selected by phase-contrast microscopy. All of the animal care procedures and management followed standard operating procedure (SOP) of Hanyang University, College of Medicine, Animal Care and Use Committee.

STO and mouse ESC culture

Mouse STO fibroblast feeder cells (CRL-1503; American Type

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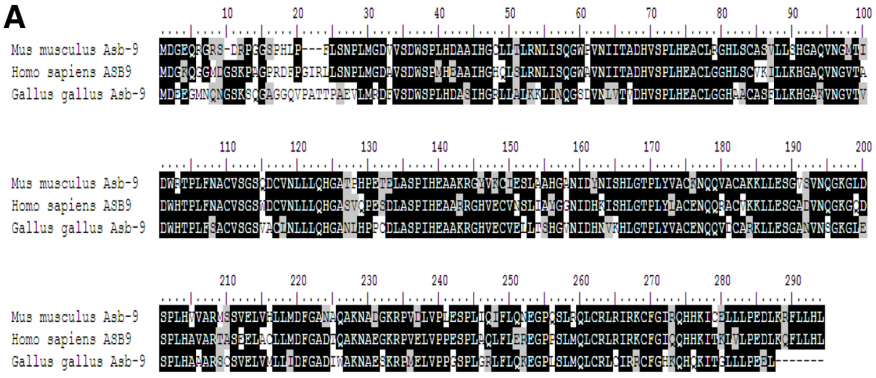

Fig. 1. (A) Alignment of amino acid sequences for mouse Asb-9 (NP_081303), human Asb-9 (NP_001026909), and gallus Asb-9 (NP_001006262) using BioEdit. (B) Domain composition of mouse Asb-9, indicating two ankyrin repeats (ANK: amino acids 59-183 and 132-234) and one SOCS box domain (SOCS: amino acids 249 and 292).

Table 1. The nucleotide and amino sequence identities of *mAsb-9* with other members of the Asb-9 family

Mouse (mAsb-9)	Nucleotide sequence identity (%)	Amino acid sequence identity (%)
Human	78%	64.4%
Chick	72%	64.1%

Culture Collection [ATCC], USA http://www.atcc.org) were grown in DMEM (GIBCOBRL, USA) supplemented with 10% FBS (HyClone, USA) and 1% penicillin-streptomycin (GIBCOBRL, USA). These cells were mitotically inactivated with 10 μg/ml mitomycin-C (Sigma, USA) for 1.5 h. HS-3 mouse ESCs were grown under standard conditions. Both cells were grown in 5% CO_2, 95% air and were routinely passaged every 4-5 days.

RNA isolation and RNA expression analysis

Total cellular RNA was extracted from mouse testes and mouse tissue by using TRIzol (GIBCOBRL, USA). Whole mouse testes were obtained from 5 day-, 2-, 4-, 6-, and 10-week-old mice. PS, RS and CS were separated from the whole testes of 10-week-old mice by STA-PUT (Bellve et al., 1977). The 1 μg of total RNA was treated with DNase I (Sigma, USA). Expression of the *Asb-9* mRNA was analyzed in mouse tissue and indicated developmental stage testes by Northern blot analysis. All RNA samples (20 μg each) were electrophoresed and blotted onto Hybond-N+ membranes (Amersham Biosciences, England). The probe used for Northern blot analysis was labeled by random primers with [α-^{32}P] dCTP (Amersham Biosciences, England). Hybridization was performed in a bag containing ExpressHyb™ hybridization solution (Clontech, USA) at 68°C overnight. Hybridized membrane was washed at room temperature in 2× SSC and 0.1% SDS, and then in 0.1× SSC and 0.1% SDS. The membrane was exposed to AGFA medical X-ray film blue with an intensifying screen for 20 h at -70°C.

In situ hybridization

Male ICR mice (5 days, 6 weeks old) testes were fixed overnight in 0.1% DEPC/4% PFA at 4°C and processed for paraffin embedding. The 5 μm thick paraffin sections were prepared and mounted on slides. DIG-labeled sense and anti-sense riboprobes were prepared by *in vitro* transcription using T7 and SP6 polymerase (Promega, USA). The *in situ* hybridization of testis sections was carried out as described by Yoshimura et al. (Yoshimura et al., 2007). DIG-labeled sense and anti-sense probes were detected using anti-DIG-AP and NBT/BCIP phosphate reagents (Roche, USA). Then, tissues were counterstained with Fast Red.

RESULTS AND DISCUSSION

Alignment of amino acid sequences for mouse, human and gallus Asb-9

The mouse *Asb-9* is 873 bp in length, with an open reading frame encoding a protein of 290 amino acids (Fig. 1). Figure 1A shows the amino acid sequences of Asb-9 family members searched for in GenBank aligned using the Clustal method with the BioEdit program. The amino acid sequence for mouse Asb-9 revealed a 64.4% and 64.1% identity with that of the human and gallus, respectively (Table 1). Computer analysis (http://smart.embl-heidelberg.de) revealed that the deduced amino acid sequence of the mouse Asb-9 contains two ankyrin repeats (59-183, 132-234) and one SOCS box domain between amino acids 249 and 292 (Fig. 1B).

Expression analysis of the *Asb-9* in mouse tissue

To characterize the expression of *mAsb-9*, Northern blot analysis was performed in various tissues obtained from 6-week-old mice (Fig. 2). Although weak expression was detected in the stomach and kidney, *mAsb-9* was strongly expressed in the testes. This result suggests that *mAsb-9* could be involved in male germ cell development.

Expression analysis of *Asb-9* mRNA during mouse testis development and spermiogenesis

To understand the expression of *mAsb-9* during male germ cell differentiation, we analyzed the *mAsb-9* mRNA level by Northern blot (Fig. 3). Murine spermatogenic cells were obtained from testes using unit gravity sedimentation velocity in a BSA gradient (Bellve et al., 1977; Romrell et al., 1976). This result indicated that *mAsb-9* has not shown until the fourth week of

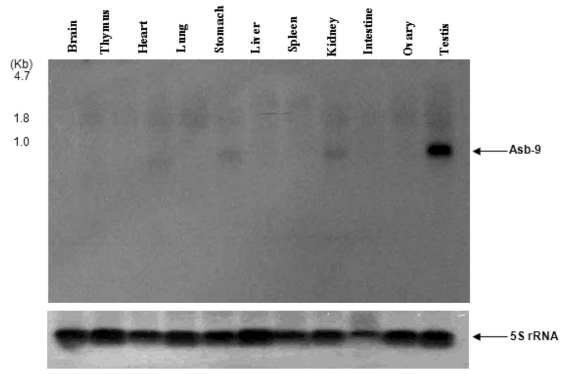

Fig. 2. Expression of *mAsb*-9 mRNA in mouse organs. Total RNA was prepared from the brain (lane 1), thymus (lane 2), heart (lane 3), lung (lane 4), stomach (lane 5), liver (line 6), spleen (lane 7), kidney (lane 8), intestine (lane 9), ovary (lane 10), and testes (lane 11) of a 6-week-old mice. Strong expression was shown in the testes. The 5s RNAs were used as loading controls.

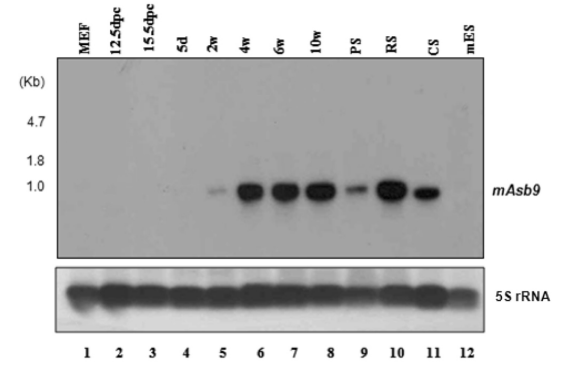

Fig. 3. *mAsb-9* expression during mouse testes development and spermatogenesis, shown by Northern blot performed on RNA extracted from harvested embryos, and from indicated times. Northern blot hybridization of *mAsb-9* mRNA from 12.5 and 15.5 dpc fetuses (lane 2, 3), 5-day-old, 2-, 4-, 6-, and 10-week-old mouse testes, pachytene spermatocytes (PS), round spermatids (RS), and condensing spermatids (CS) during spermatogenesis. Lane 1 is mouse embryonic fibroblast (MEF) cell and line 12 is HS-3 mouse embryonic stem cell.

mouse development. The *mAsb-9* gene was expressed in pachytene spermatocytes, round spermatids, and condensing spermatids, but was not expressed in mouse embryonic stem cells (mES) (Fig. 3). Interestingly, *mAsb-9* expression was relatively less in pachytene spermatocytes and then dramatically increased in round spermatids and sustained its expression in condensing spermatids. This result suggests that *mAsb-9* may play a role in spermiogenesis, or in other words, may be involved in reshaping cells as spermatozoa. Even though we used highly purified spermatogenic cell fractions from testes by unit gravity sedimentation velocity in a BSA gradient, we further confirmed localization of *mAsb-9* expression by *in situ* hybridization analysis in mouse testes from 5 days and 6 weeks old. Six-week-old testis sections were hybridized with an antisense probe which revealed a strong expression in round and condensing spermatids and no labeling in interstitial, peritubular, and Sertoli cells (Figs. 4A-4C). *mAsb-9* was barely expressed

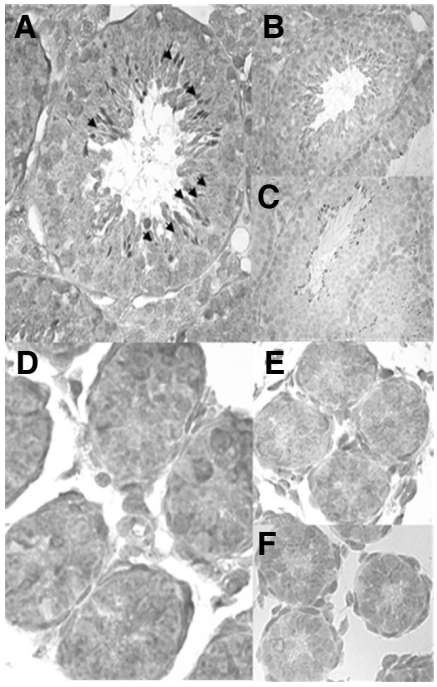

Fig. 4. *mAsb-9* expression pattern in 5-day- and 6-week-old mouse testes analyzed by *in situ* hybridization. *In situ* hybridization using an anti-sense *Asb-9* RNA probe in 6-week- (A) and 5-day-old (D) testes. *In situ* hybridization using a sense *Asb-9* RNA probe in 6-week- (B) and 5-day-old (E) testes. Counterstaining using Fast Red in 6-week- (C) and 5-day-old (F) testes. Black arrows indicate round spermatids (RS) and condensing spermatids (CS).

in the 5-day-old testis, which has immature germ cells and does not undergo spermatogenesis (Figs. 4D-4F).

Mice reach puberty at 4-6 weeks of age. The testes descend into the scrotum and produce sperm after the fifth week and male mice develop sexual maturity between 6 and 8 weeks of age (Guo et al., 2004). *mAsb-9* began to be expressed near puberty and was continuously expressed thereafter. The expression pattern of Asb-9 is very well correlated with those of spermatogenesis. In particular, the expression of Asb-9 was increased in pachytene spermatocytes and secondary meiosis occurred in this stage. Round spermatids are haploid cells for producing spermatozoa. Thus, we suggested that *mAsb-9* could play a role in spermiogenesis, a precisely controlled cellular process (Guo et al., 2004; Kim et al., 2004). Although Debrincat et al. have reported that Asb-9 acts as a specific ubiquitin ligase regulating CKB abundance in the 293T cells, its role in mouse testes has not been studied (Debrincat et al., 2007). In order to better understand the developmental role of Asb-9 in mouse testes, the functional roles of the ankyrin repeat and SOCS domains during testes development and spermatogenesis should be elucidated.

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