SALL1 expression in the human pituitary–adrenal/gonadal axis

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Abstract

SALL1 was originally identified on the basis of its DNA sequence homology to the region-specific homeotic gene *Sal*, in *Drosophila melanogaster*, which acts as a downstream target of hedgehog/tumor growth factor- β -like decapentaplegic signals. The *SALL1* gene has been associated with the Townes–Brocks Syndrome (TBS), a disorder characterized by multiorgan dysgenesis including renal and genital malformations. In this study, *SALL1* message production was evaluated in association with the tissue localization of the protein product of *SALL1*, p140. *SALL1* protein expression was observed in various adult and fetal tissues which elaborate reproductive endocrine hormones. The p140 was localized in specific micro-anatomic sites of the pituitary, adrenal cortex and the placenta. In the human pituitary, *SALL1* protein expression was limited to the adenohypophysis, where it

colocalized to those cells producing GH and the gonadotropins, LH and FSH. *SALL1* expression was also found in most of the fetal and adult adrenal cortex in addition to the trophoblastic cells of the placenta. This pattern of expression complements prior studies demonstrating p140 in testicular fetal Leydig cells, adult Leydig and Sertoli cells, and granulosa cells of the ovary. The *SALL1* protein was also shown here to be highly expressed in trophoblast tumors, which overproduce sex hormones. The expression patterns of *SALL1* at multiple levels of the reproductive endocrine axis and the phenotypic effects associated with TBS suggest that *SALL1* may have an important role in the interaction of the pituitary–adrenal/gonadal axis during reproduction.

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Introduction

The temporal and site-specific production of gonadal hormones plays an essential role in fetal sexual differentiation and normal reproductive function. Testosterone is a key hormonal agent that influences the undifferentiated fetal sex organs, including internal and external genitalia, to develop into the male phenotype. The placenta regulates the production of fetal testosterone by the stimulatory effect of human chorionic gonadotropin- β (hCG β) on Leydig cells at an early stage of gestational age, including the critical 8th–12th weeks for male genitalia development (Winter *et al.* 1977). The fetal pituitary begins to regulate the production of testosterone at midgestation by the secretion of luteinizing hormone (LH). The production of gonadal hormones in the normal adult is tightly regulated through complex interactions within the pituitary–adrenal/gonadal axis (Treier & Rosenfeld 1996). Follicle-stimulating hormone (FSH) and LH are key regulators of gonadal hormones and are both produced by a sub-

population of cells called gonadotropes in the anterior pituitary. Gonadotropin release is controlled in turn by hypothalamic gonadotropin-releasing hormone.

Several lines of investigation have suggested that transcription factors are critical in regulating differentiation of steroidogenic tissues and reproductive functions. Among these genes, Wilms' tumor-1 (*WT1*) (Nachitgal *et al.* 1998, Kim *et al.* 1999), steroidogenic factor-1 (*SF-1*) (Ikeda *et al.* 1993, Hatano *et al.* 1994, Luo *et al.* 1994, Shen *et al.* 1994), and orphan nuclear receptor (*DAX-1*) (Muscatelli *et al.* 1994, Zanaria *et al.* 1994, Swain *et al.* 1998) have been identified, and are associated with impaired differentiation of steroidogenic tissues, gonads and/or adrenal glands. Although dissimilar phenotypes have occasionally been displayed by natural human or constructed mouse gene knockouts, these genes are essential for gonadogenesis. In addition, evidence has accumulated to suggest that these factors are positioned in series within a sex determination cascade. *DAX-1* and *SF-1* gene products are orphan nuclear receptors that were

initially thought to be essential for adrenal development as shown by their knockout phenotypes. Recent evidence indicates that the effects of SF-1 and DAX-1 are multifactorial and exerted at multiple levels of the hypothalamic–pituitary–adrenal/gonadal axis (Ikeda *et al.* 1996, Chau *et al.* 1997, Crawford *et al.* 1997, Sadovsky & Crawford 1998).

The human *SALL* gene family, *SALL1*, *SALL2* and *SALL3* (refer to Buck *et al.* (2000) for nomenclature), were originally cloned based on DNA sequence homology to the *Drosophila* spalt gene (*sal*), which is an important component of the hedgehog (hh)/tumor growth factor- β (TGF β)-like decapentaplegic (Dpp) signaling pathways (Kohlhase *et al.* 1996). In *Drosophila*, *sal* is a region-specific homeotic gene and is an essential component required for development of posterior head and anterior tail segments (Kuhnlein *et al.* 1994). Its expression is regulated by hh activity, which makes *sal* an hh target gene (Koster *et al.* 1997). In addition, the *Dpp* homologue to TGF β also acts upstream of *sal* (de Celis *et al.* 1996). The *Dpp* and *hh* signal pathways are highly conserved throughout the animal kingdom and are critical for pattern formation. Most genes in the *Dpp* and *hh* pathways have been shown to play essential roles in development (Ingham 1995, Perrimon 1995). Interestingly, a remarkable number of these genes in the human are either tumor-suppressor genes or oncogenes.

The components of the *SALL* gene family have been suggested to be important in developmental pathways and to function as either an oncogene (*SALL1*) (Ma *et al.* 2001a) or tumor-suppressor gene (*SALL2*) (Li *et al.* 2001, Ma *et al.* 2001b). The predicted transcript codes are 1325 amino acids in length for *SALL1* and 1008 amino acids for *SALL2*, based on translation of the open reading frames (Kohlhase *et al.* 1996, Ma *et al.* 2001b; Genbank database accession no. AF465630). A full-length cDNA for *SALL3* has not yet been isolated although partial sequence information is available. Mutations of the *SALL1* gene are associated with Townes–Brocks syndrome (TBS) with clinical features that include urogenital, limb and anal abnormalities (Kohlhase *et al.* 1998). Hypospadias occurs in approximately 30% of patients with TBS (Powell & Michaelis 1999) while the exact underlying mechanism is not known. It has been proposed that hypospadias is the result of a fetal endocrinopathy caused by extra- or intrauterine factors resulting in a disruption of the testosterone balance during the embryonic or fetal periods (Glatzl 1984, Stamper *et al.* 1999). The disruption of testosterone balance may arise from the mother's placenta or the fetal pituitary–adrenal/gonadal axis.

As an initial step towards understanding the pathophysiological basis associated with alterations in the *TBS* gene that give rise to TBS, sites of *TBS* gene expression coincident with TBS phenotypes were examined. These

studies were undertaken to provide insight into the function of the *TBS* gene in human development as has been achieved in prior studies evaluating *SALL1* expression in fetal and adult renal tissue (Ma *et al.* 2001, Nishinakamura *et al.* 2001). Prior studies utilizing multiple biochemical and genetic approaches have clearly indicated a role for the *SALL1* gene in renal development and subsequent function. Similar evaluations of the expression patterns of WT1, DAX1 and SF-1 in specific tissues have been correlated with their functions during human development (Harris & Mellon 1998, Kim *et al.* 1999). We report here that *SALL1* was frequently observed in specific cell types in tissues with known secretions of reproductive endocrine hormones. To extend our studies, we also investigated the expression of *SALL1* in a variety of sex hormone-producing tumors. Our results provide the first evidence that *SALL1* may act in several components of the reproductive axis including gonadotropes of the pituitary.

Materials and Methods

Antibody generation and affinity purification

The peptide TGNLERLQNSEPN, as previously described (Ma *et al.* 2001a), was derived from the sequence of the C-terminus of the deduced *SALL1* protein (amino acids 1280–1292) and used for preparing anti-peptide antibodies in rabbits in collaboration with Zymed, Inc. (San Francisco, CA, USA) (see Fig. 1). Sera from immunized rabbits bound to synthetic peptide with titers >1/100 000 in enzyme-linked immunosorbent assay. The C-terminal synthetic peptide of *SALL1* was subsequently utilized for antibody affinity purification using sulfolink chromatography columns (Pierce Chemical Co., Rockford, IL, USA). The anti-peptide antibodies utilized in the present studies, as previously described (Ma *et al.* 2001a), met several criteria supporting their specificity. First, antibodies were raised against a synthetic peptide containing a sequence unique to the *SALL1* isoform. Second, affinity-purified anti-peptide antibodies bound a defined *SALL1* recombinant fusion protein derived from *E. coli* lysates bearing the sequence used to immunize the rabbits (see Fig. 1), but not to a 66 kDa recombinant fusion protein of *SALL2*. Third, antibodies that had been pre-incubated with *SALL1* C-terminal peptide-coupled resin failed to recognize a *SALL1* fusion protein or *SALL1* protein in the immunohistochemistry studies. Similar negative results were seen when IgG-purified pre-immune sera was used. Fourth, a second antibody raised against a *SALL1* fusion protein (see Fig. 1) further confirmed the findings with the anti-peptide antibody (Ma *et al.* 2001). Evaluation of the immunoprecipitation and Western analyses, as previously demonstrated, experimentally confirmed specificity (Ma *et al.* 2001a). Anti-human LH β , anti-human adrenocorticotropin (ACTH) and

Figure 1 Structure of SALL1 protein and position of the peptide for antibody production. The boxed segments represent the regions associated with amino acids characteristic of zinc finger domains. SALL1 bears multiple characteristic C_2H_2 zinc finger domains predicted to bind DNA, acting as a transcription factor. The open triangles designate hot spots for mutation in TBS, the upper vertical arrow indicates the location of peptide residues within SALL1 used to generate the anti-peptide antibody while the horizontal lower arrow indicates the location of the SALL1 fusion protein.

anti-human growth hormone (GH) antibodies were purchased from Biogenex (San Ramon, CA, USA).

Case material and immunohistochemistry

Material for histological evaluation was retrieved in the form of formalin-fixed or formalin-fixed, paraffin-embedded tissues from both surgical pathology and autopsy files. The histological materials utilized were those available subsequent to complete pathologic evaluation. Material for use in the evaluation of message production was either retrieved in saline, subsequent to complete pathological study, or obtained as total RNA from Clontech (Palo Alto, CA, USA) and Ambion (Austin, TX, USA). The tissue sections were acquired according to institutional guidelines. For tissue and tumor classification, sections were also stained with hematoxylin–eosin. Immunohistochemistry was performed on fixed, paraffin-embedded tissues following a standard avidin–biotin complex (ABC) method. Briefly, sections were deparaffinized in xylene and dehydrated in graded ethanol. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. The sections were then incubated for 1 h with the primary antibody diluted in phosphate-buffered saline (PBS) plus 1% bovine serum albumin. Following washes in PBS, the sections were sequentially incubated with biotinylated goat anti-rabbit antibody. The ABC reagent and 3,3'-diaminobenzidine were used as chromogen. Sections were then counterstained, dehydrated and coverslipped. For double-labeling immunohistochemistry, paraffin-embedded sections of human pituitary glands were deparaffinized by incubating at room temperature twice (for 10 min each) in xylene, once (for 3 min) in 100% ethanol and once (for 3 min) in 95% ethanol. The slides were allowed to dry, and sections were circled with a Pap pen (G. Kisker Biotech, Steinfurt, Germany) to allow small incubation volumes. After a 20-min incubation in PBS, the tissue sections were covered with 5% normal

goat serum and placed in a humidified chamber at 37 °C to block for 90 min. The blocked tissue sections were briefly washed and then incubated in a humidified chamber at 4 °C overnight with both an affinity-purified rabbit anti-SALL1 (8 µg/ml) and a monoclonal anti-human LHβ or anti-human ACTH or anti-human GH. For control, pituitary gland tissue sections were also incubated with an anti-SALL1 antibody that had been pre-incubated with peptide-coupled resins in which the specific antibody moieties were removed. After incubation of the tissue sections with the primary antibodies, sections were washed three times for 15 min each at room temperature in PBS and incubated for 1 h at 37 °C in a dark humidified chamber, with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:50) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), and a Texas Red-conjugated goat anti-mouse secondary antibody (1:50). Sections were washed in PBS three times for 5 min each at room temperature in the dark and then once in PBS. Slides were covered with mounting medium and examined using a confocal microscope.

PCR analysis for transcript expression

RT-PCR was used to evaluate expression patterns of *SALL1* in selected tissues, as previously described (Ma *et al.* 2001). PCR amplifications were performed in a 50 µl reaction volume containing 5 µl cDNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs and 1.25 U Taq-DNA polymerase (Perkin-Elmer, Norwalk, CT, USA). After an initial denaturation at 94 °C for 10 min, amplification was performed for 30–35 cycles under the following conditions: 30 s, denaturation at 94 °C, 30 s annealing at 55 °C and 30 s extension at 72 °C. The last cycle was followed by final extension for 7 min at 72 °C. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used to control

Figure 2 RT-PCR of selected tissues for mRNA expression. GAPDH was amplified as an internal control with the reaction extended to 28 cycles. *SALL1* amplification was extended through 35 cycles. The adrenal, ovary and testis were from adult specimens, the placenta was from a term pregnancy, the brain and liver were fetal in origin.

for template concentration loading. The primer pair selected was specific for *SALL1* with sense primer (ATTG GCACCGGCAGTTACCACC) and antisense primer (AGTACTCGTGGGCATATTGTC). PCR products were electrophoretically separated on 1% agarose gels and visualized with ethidium bromide.

Cell culture and immunofluorescence

Human renal epithelial cells (HEK 293) were grown and seeded at a very low density on slides, and then fixed with methanol–acetone (1:2) for 10 min at 4 °C and washed with PBS. Primary antibodies for *SALL1* were used at 10 µg/ml in PBS. Secondary antibodies were FITC-labeled goat anti-rabbit antibody (1:50). Gray-scale images (12 bit) were acquired separately using the FITC and/or rhodamine wavelengths with a Nikon E800 fluorescent microscope (Nikon, Inc., Mellville, NY, USA) using a 20× or 40× Planfluor objective. Images were acquired with a Spot II digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) using the camera's internal filters to increase image contrast.

Results

SALL1 mRNA expression in selected tissue

Prior experimentation had demonstrated a relatively selective expression pattern for *SALL1*. RT-PCR revealed that fetal brain and kidney demonstrated the highest level of expression, fetal thymus was just detectable, with fetal spleen, skeletal muscle, lung and liver being negative (Ma *et al.* 2001a). For the present studies, which focus on the role of *SALL1* in endocrine production, additional tissues were evaluated. As seen in Fig. 2, *SALL1* mRNA was clearly detected in the multiple endocrine tissues tested. Adult testis, ovary and placenta demonstrated relatively

intense bands, although less than detected in the brain. Adult adrenal was clearly positive, although exhibiting a pattern less intense than that of the other tissues.

SALL1 expression in placenta

Given the fact that abnormalities of the external genitalia are frequently seen in TBS, we previously investigated the expression of *SALL1* p140 in selected tissues from adult and fetal testis (Ma *et al.* 2001a). In adult testis, *SALL1* was persistently present in Leydig cells and extended to testosterone target cells, the spermatogonia and its supporting network, the Sertoli cells. For the present studies, photomicrographs of fetal testis were included to expedite comparison of the gonadal tissue with endocrine-producing cells evaluated herein. Immunohistochemistry of tissue from a 12-week gestational fetus showed that *SALL1* was strongly expressed in the Leydig cells between the seminiferous tubules, whereas the background fibrous stroma was negative (Fig. 3A and B), commensurate with prior observations (Ma *et al.* 2001a). Considering that human placenta is an important source of both steroids and hCG, this system was next evaluated. During fetal development, testosterone secretion is initially regulated by the placenta through the production of hCGβ from the cyto- and syncytiotrophoblast. The placenta consists of numerous villi, which have fibrous stroma and blood vessels in the center and hCGβ-producing cells, i.e. the cyto- and syncytiotrophoblast, in the periphery. Evaluation of a 12-week placenta revealed strongly positive immunoreactivity in the trophoblasts, in contrast to the background staining of the central mesenchymal cells (Fig. 3D and E).

SALL1 expression in adrenals

The expression of *SALL1* in the adrenals was also analyzed using immunohistochemistry. The adrenal gland is another minimal source for testosterone. The fetal adrenal consists of an outer subcapsule, a small dark primitive layer and an inner fetal cortex. *SALL1* was expressed in specified cell types throughout development of the adrenals. With the exception of the outer primitive layer and the adrenal medulla, which were consistently negative, *SALL1* was observed to be expressed in most of the fetal adrenal cortex (Fig. 3G). Examination of the adult adrenal revealed that the three layers of the cortex were almost equally positive for *SALL1* expression (Fig. 3H; note labeling of individual zones) as expected given the fact that all of these three layers of cells are derived from the same cellular origin.

Expansion of *SALL1* expression cells in the developing pituitary

After midgestation, the regulation of testosterone secretion is taken over by the pituitary. Initial expression of *SALL1* was observed in a small cellular subpopulation of the

Figure 3 Expression of SALL1 protein in the developing fetal testis, adrenal gland and placenta. Immunohistochemical identification of SALL1 in fetal testis, adrenal gland and placenta using affinity-purified rabbit SALL1 antibodies. In 12-week fetal testis (A and B), SALL1 was highly expressed in the testosterone-producing Leydig cells (between the seminiferous tubules). Strong immunoreactivity for SALL1 was observed in the cyto- and syncytiotrophoblast but not for mesenchymal villous cells in the 12-week placenta (D and E). Fetal adrenal (G) and adult adrenal (H, from outer layer to inner layer, GL=zona glomerulosa, FA=zona fasciculata, RE=zona reticularis) demonstrating that all three zones of adult adrenal cortex express SALL1, in contrast to the medulla and the subcapsular primitive cell layer in the fetal adrenal. Negative controls for each tissue are also shown in (C), (F) and (I) respectively.

12-week fetal pituitary (Fig. 4A), and this population progressively expanded during the continuation of pituitary development (Fig. 4B). By 40 weeks of gestation, SALL1 expression clearly increased in the anterior pituitary and eventually occupied a large portion of the lateral lobe (Fig. 4C). The posterior lobe did not show any reactivity for SALL1 (Fig. 4G–I). In all cases of fetal or adult pituitary, staining was abolished when tissues were exposed to an anti-SALL1 antibody preparation that had been pre-incubated with peptide-coupled resins to remove the specific antibody moieties (Fig. 4D, E, F and J).

SALL1 is selectively expressed in pituitary

To gain insight into the potential role of SALL1 expression in specific cell lineages, double-label immunofluorescence

was utilized. Human pituitary gland tissue sections were simultaneously incubated with a rabbit affinity-purified anti-SALL1 antibody and a monoclonal mouse anti-human GH, anti-ACTH or anti-FSH β antibody, followed by incubation with Texas Red-conjugated goat anti-rabbit serum and an FITC-conjugated goat anti-mouse serum respectively. The tissue sections were viewed using a confocal microscope with the appropriate filters to detect red or green fluorescence. As shown in Fig. 5, FITC fluorescence demonstrated that GH (Fig. 5B), ACTH (Fig. 5E) and FSH β (Fig. 5H) proteins were present in distinct populations of cells scattered throughout the anterior pituitary gland. Examination of the Texas Red fluorescence in the same tissue sections showed that the SALL1 protein partially colocalized to the population of cells expressing GH, ACTH and FSH β proteins. A double

Figure 4 Expression of SALL1 in the pituitary gland. Immunohistochemical identification of SALL1 expression was undertaken on developing pituitary and adult pituitary using affinity-purified rabbit SALL1 antibodies. Only a few cells expressed SALL1 early in pituitary development ((A) 12 week and (B) 17 week), but more cells became positive for SALL1 expression with the advance of pituitary development such that positive cells comprised a large portion of the lateral lobe of the pituitary at 40 weeks ((C) 40 week). In adult pituitary, (G–H), SALL1 was mainly confined to the lateral lobe and, to a lesser extent, the middle lobe, but not detected in the posterior lobe. (I) area of positive cells shown at high magnification. Sections stained with antiserum pre-incubated with peptide-coupled resins, which served as negative controls, are shown in (D–F) and (J).

Figure 5 Immunocolocalization of SALL1 with GH, ACTH and FSHβ in adult pituitary. Utilizing affinity-purified rabbit antibodies against SALL1 with Texas Red-labeled secondary antibody (red) and mouse antibodies against pituitary hormones with FITC-conjugated secondary antibody (green), double-label staining was performed to demonstrate that SALL1 was expressed in certain subpopulations of the pituitary. GH-producing cells (A–C), ACTH-producing cells (D–F) and FSHβ-producing cells (G–I). (J) and (K) are negative controls for absorption and pre-immune serum respectively.

exposure photograph with FITC and Texas Red filters confirmed these results. Evaluation of the colocalization of SALL1 expression with a particular hormone phenotype was replicated on four anterior pituitary samples. As noted in Fig. 5, the ability to detect single SALL1-positive (red) cells in the presence of dual staining SALL1-positive and hormone-positive (yellow) cells is dependent upon the specific location of the hormone production being evaluated. The detection of SALL1-positive, GH-positive and doubly positive cells is clearly evident when

examining those fields being studied for GH-producing variants. In these microanatomic locations almost all cells are doubly labeled with only a scarce SALL1-negative variant (Fig. 5A–C). Evaluation of ACTH-producing moieties revealed that essentially only those cells capable of producing ACTH are present in these sites and the cells invariably colocalize with SALL1. It is of interest that the background cells, which demonstrate only marginal staining and are essentially negative for both the GH and the transcription factor, are the predominant type in these foci

Figure 6 SALL1 expression in sex hormone-producing tumors. Immunohistochemical identifications of SALL1 in Leydig cell tumor (A), hydatidiform mole (C), Sertoli–Leydig cell tumor (E) and steroid cell tumor (G) of the ovary using affinity-purified rabbit antibodies against SALL1. SALL1 was only detected in the Leydig cell component of the Sertoli–Leydig cell tumor. Negative controls for each tumor are also shown in (B), (D), (F) and (H).

(Fig. 5D–F). With regard to FSH, multiple hormone-producing variants are evidently located in the micro-anatomic site as SALL1-positive, FSH-negative variants (i.e. cells possibly making other hormones) are present

(Fig. 5G–I). This non-random distribution of immunohistochemical localization suggests that the results are a function of the anatomic distribution of the hormone-producing cells and reflect a physiologically relevant

finding. Of further interest, no detectable SALL1 was present in the posterior region of the pituitary gland (data not shown). To control for specificity of the SALL1 antibodies, pituitary gland tissue sections were simultaneously incubated with GH or ACTH or FSH β antiserum and anti-SALL1 antibody pre-incubated with peptide-coupled resins. The cells were FITC positive for GH or ACTH or FSH β protein, but showed no specific Texas Red staining for SALL1 protein (data not shown).

SALL1 expression in sex hormone-producing tumors

SALL1 expression was next examined in sex hormone-producing tumors. These included Leydig cell, hydatidiform mole, steroid cell and mixed Sertoli–Leydig cell tumors. It is known that these tumors produce a variety of sex hormones dependent on the type of tumor cells extant within the neoplasm. In most tumor tissues examined in the present study, SALL1 was observed to be over-expressed, as seen in Fig. 6, in which tumors stained more strongly than the non-tumorous counterparts. This was most evident in mixed Sertoli–Leydig cell tumors (Fig. 6E), which were strongly immunoreactive in the Leydig cell component while the Sertoli cells were negative. The trophoblast tumor cells, with the same hCG secretion function as that of the non-tumorous counterparts of the placenta, exhibited strong staining (Fig. 6C). Similar findings were seen in steroid cell tumors of steroid cell origin (Fig. 6G).

Cytoplasmic versus nuclear location of SALL1 is dependent on cell type

To explore the subcellular localization of the SALL1 protein, indirect immunofluorescence was performed with both affinity-purified antibody and a control antibody preparation on tissue sections of the anterior pituitary gland (Fig. 7A and B) and on the HEK 293 cell line derived from human renal epithelial cells (Fig. 7C–E). In the anterior pituitary gland, SALL1 was shown to be present diffusely in the cytoplasm and occasionally in the nucleus. In contrast, SALL1 protein was localized mainly in the nucleus of HEK 293 cells, although very weak cytoplasmic staining was also observed (Fig. 7C–E). Staining was not detected after pre-incubation with antiserum with SALL1 peptide resins (Fig. 7F). This study demonstrates that the pattern of staining of SALL1 varies among different cell types.

Discussion

The identification of mutations in the *SALL1* genes as the putative cause of TBS provides an opportunity to gain new insight into the molecular pathophysiology of this disorder. Although little is known at present about

the function of *SALL1*, it is notable that its *Drosophila* homologue is an important component of the hh/TGF β -like signaling pathway. Alterations in the *hh* gene are shown to be associated with a broad spectrum of cerebral midline defects, including pituitary and male gonadal defects. TGF β -like signals are additionally present in the anterior pituitary and regulate normal anterior pituitary cell functions (Treier *et al.* 1998, Coya *et al.* 1999). Given the activation of *Sal* in response to hh and TGF β -like signals and given their highly conserved function from an evolutionary aspect, it is conceivable that *SALL1* may play a role in pituitary development and function. In developing pituitary, we have observed that cells expressing SALL1 increased in association with maturation. It is still unclear whether or not these SALL1-expressing cells are precursors of functional endocrine cells.

To determine whether SALL1 protein is expressed in human gonadotropes, we performed additional immunohistochemistry using purified anti-peptide antibody preparations against SALL1. The antibodies have previously been shown to be specific for SALL1 but not for SALL2 (Ma *et al.* 2001a). Our results demonstrated that SALL1 was expressed in the anterior pituitary, but not in the posterior lobe responsible for the production of vasopressin and oxytocin hormones. The anterior pituitary gland contains five discrete cell types that secrete a variety of hormones including gonadotropins. By immunolocalization, we have shown that SALL1 colocalized with ACTH, FSH and GH in the anterior pituitary tissue. However, SALL1 protein was not detected in all those cells that were immunopositive for these hormones. It is possible that the gene for SALL1 is not expressed in every gonadotrope, or that it is expressed transiently. Alternatively, some ACTH- or FSH-containing cells lacking SALL1 expression may not be actively secreting. This finding raises the possibility that SALL1 may act in a cell-specific manner to regulate gene expression in the human gonadotrope.

The human placenta, as opposed to the pituitary, is another important source of gonadotropin. During the first 12 weeks of pregnancy, the maternal chorionic gonadotropins, e.g. hCG β , influence genital development by stimulating Leydig cells to produce testosterone. Our data showed that SALL1 was expressed in both normal human placenta and in trophoblastic tumors.

The cell lineages for both the adrenals and the gonads arise from a common embryonic origin. Our studies have shown that SALL1 was expressed in the adrenals from the early stages of their development. Expression levels were slightly down-regulated coincident with overt differentiation of the adrenal, yet expression persists in the adult. The expression was restricted to the cortex. The primitive layer of the fetal adrenal is thought to contain stem cells and has been shown to lack endocrine function. The bright staining of the fetal adrenal cortex suggests

Figure 7 Immunohisto- and cytochemical subcellular localization of SALL1. By immunofluorescent microscopy, the precise locations of SALL1 expression were studied with affinity-purified antibodies against SALL1. Diffuse cytoplasmic and occasionally weak nuclear staining for SALL1 were observed in pituitary (A and B). In the human HEK 293 kidney cell line (C–E), SALL1 was localized in the nucleus. Negative control stained with serum pre-incubated with peptide coupled resins is shown in (F).

that SALL1 may be required for maintenance of the differentiated state.

To investigate whether SALL1 is over-expressed in the malignant counterparts of cell types that produce sex

hormones, studies were extended to include a variety of sex hormone-producing tumors. SALL1 expression levels were greater in specific tumor cells capable of producing sex hormones as opposed to those tumor cells which

have not been reported to produce such hormones, exemplified by Sertoli cells. Given the origins of sex hormone-producing tumors, our observations suggest that SALL1 might have a role in the regulation of sex hormone production.

Since the primary structure of SALL1, the product of the *TBS* gene, has recently been deduced and contains several novel zinc finger motifs, it likely serves as a transcription factor. However, our immunohistochemical studies have shown that the role for SALL1 in development and subcellular localization seems to vary among different tissues. Cytoplasmic localization of SALL1 was seen in all endocrine cells that we examined, while the spermatogonia showed nuclear localization. In addition, renal collecting tubules and the blastemal component of Wilms' tumors also exhibited nuclear staining (Ma *et al.* 2001). These studies raise the possibility that SALL1 activity, like that of the *Exd* gene (homeodomain protein) (Mann & Abu-Shaar 1996), and *rel* transcription factors NF κ B and Dorsal, may be controlled by their subcellular localization (Rushlow & Warrior 1992). This is also true for many signal transduction pathways in which nuclear entry of specific proteins is regulated in response to signal transmission. Examples include transcription factor Smad7, where nuclear localization relies on the presence or absence of the TGF β signal (Itoh *et al.* 1998). Therefore, subcellular localization of SALL1 might vary according to physiological condition or cell type.

In summary, the intriguing profiles of SALL1 expression combined with our previous studies point to an important role for SALL1 at multiple levels of the reproductive axis.

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