Transcriptional Activation of the SALL1 by the Human SIX1 Homeodomain during Kidney Development*

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SALL1 is a member of the SAL gene family that encodes a group of putative developmental transcription factors. SALL1 plays a critical role during kidney development as mutations of the human SALL1 gene cause Townes-Brocks syndrome, which is associated with kidney malformation. Deletion of the mouse Sall1 gene results in renal agenesis or severe dysgenesis. To date, little is known about the molecular mechanisms controlling the regulation of SALL1 expression. This report describes the cloning and characterization of the human SALL1 gene promoter. Consensus binding sites were identified for several transcription factors, with multiple sites for WT1 and SIX1. In transient transfection assays, SALL1 promoter activity was higher in HEK-293 human kidney cells and COS-7 monkey kidney cells than in NIH-3T3 fibroblasts, consistent with its role in kidney development. Transcription from the SALL1 promoter was strikingly activated by the SIX1 protein. Utilizing a luciferase reporter gene assay, endogenous or exogenously added SIX1 activated the SALL1 promoter. Overexpression of SIX1 induced a significant increase in the endogenous SIX1 protein. In addition, co-expression of SIX1 and Eya1 resulted in a significant increase in the SALL1 promoter activity when compared with either SIX1 or Eya1 alone. Finally, we demonstrate that SIX1 was able to bind to the SALL1 promoter by retardation assays and that deletion of the putative element of SIX1 significantly diminishes the SALL1 promoter activity response to SIX1 stimulation. Our findings, when taken together, indicate that SALL1 is a likely target gene for SIX1 during kidney development.

Townes-Brocks syndrome (Online Mendelian Inheritance in Man (OMIM) 107480) is an autosomal-dominant disorder characterized predominantly by renal, urogenital, anal, limb, and ear anomalies (1, 2). Kidney malformations occur in ~60% of Townes-Brocks syndrome patients, including unilateral or bilateral hypoplastic or dysplastic kidneys, renal agenesis, and multicystic kidneys. The human homologue of the Drosophila SALL1 gene, SALL1, maps to chromosome 16q12.1, and alterations of this locus were found in Townes-Brocks syndrome families (3, 4). All mutations identified so far are found in the 5′-region of the triple zinc finger motif and result in premature termination of translation (5, 6). Genetic targeting of the mouse Sall1 results in severe renal dysplasia or completeagenesis, which indicates that SALL1 plays an essential role in early kidney development (7).

In Drosophila melanogaster, Sal is a region-specific homeotic gene that acts as an important downstream target of the HH (hedgehog) and the DPP (decapentaplegic) signaling pathways (8–10). DPP signals in Drosophila are analogous to those of the TGFβ gene family in humans (11–13). Drosophila Sal is required for specification of the posterior head and anterior tail segments, as well as for the larval tracheal system (14) and adult wings (15). SAL-related genes have been isolated from Caenorhabditis elegans nematodes (16), fish (10), Xenopus frogs (17), mice (18), and humans (3). Each homologue is expressed during embryonic development, as well as in specific adult tissues. Drosophila SAL mutation leads to the incomplete transformation of head and tail segments into trunk-like characters and the establishment of larval legs and discs (19, 20).

Renal ontogeny begins with the interaction of the ureteric bud and the metanephric mesenchyme (21, 22). The ureteric bud induces the metanephric blastema to condense and aggregate, with subsequent formation of the nephron, the functional unit of the kidney. Conversion of metanephric mesenchyme into differentiated renal epithelium is a process that must be accompanied by activation or induction of specific factors that coordinate morphological changes in the developing kidney (22, 23). The human SIX1, a member of the SIX classes of homeodomain genes, is one such transcription factor functioning as a master regulatory protein and activating multiple sets of genes during kidney development. During normal mouse kidney development, Six1 is expressed in uninduced metanephric mesenchyme and in the induced mesenchyme around the ureteric bud. In Six1 null mutants, the ureter fails to invade the mesenchyme, which consequently undergoes apoptosis. Six1 is considered to be required for the earliest phase of kidney development. Therefore, downstream direct targets of SIX1 need to be identified for better understanding of kidney development.

We previously showed that the SALL1 gene was expressed in the metanephric mesenchyme within and around the ureteric bud and in early or differentiated epithelial structures (24). SALL1 is not expressed in glomeruli (24). Similar to Six1, Nishi-
nakamura et al. (7) showed that homozygous deletion of the Sall1 gene resulted in failure of ureteric bud outgrowth and apoptosis of the mesenchyme. All together, properly timed activation and suppression of SALL1 are essential for normal kidney development, and SALL1 is tightly controlled during kidney development. Little is known, however, about the mechanism of SALL1 gene regulation. We have investigated transcriptional regulation of the SALL1 promoter. Our data suggest that SALL1 is a target of SIX1 transcription during the course of normal kidney development.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK-293 human kidney cells, COS-7 monkey kidney cells, NIH-3T3 fibroblasts, and PC3 prostate cancer cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained at 37 °C in a humidified environment with 5% carbon dioxide and 10% fetal serum.

Plasmid Constructs—SIX1FL, full-length human SIX1 cDNA, ΔNterm, deletion construct lacking the SIX1 N terminus (from the start codon to nucleotide 689, which is in the first helix of the encoded domain), ΔCterm, deletion construct lacking the SIX1 C terminus (beginning from nucleotide 822, just after the homeodomain, and terminating at the STOP codon), and ΔHD, deletion construct lacking the N-terminal and C-terminal portion of the SIX1 (see Fig. 5A), were kindly provided by Dr. Heide L. Ford (25). Other SIX member constructs and Eya plasmids were described elsewhere (26, 27).

Rapid Amplification of cDNA Ends—The 5’-end of the SALL1 cDNA was determined with the 5’-RACE4-PCR method. Human kidney Marathon-Ready cDNA (Clontech) was amplified with the PCR primer adaptor AP1 and the cDNA-specific primer R1 of SALL1 according to the manufacturer’s instructions.

Isolation of BAC Clones for SALL1—A two-step PCR was used to screen a human BAC library for a BAC clone containing the SALL1 gene. Positive BAC clones were purified and verified by direct nucleotide sequencing.

Chromosomal Localization—Chromosomal localization was determined by fluorescence in situ hybridization. DNA isolated from a SALL1 BAC clone was labeled with digoxigenin and hybridized to human peripheral blood lymphocytes in metaphase. Signals were detected via fluorescence-conjugated avidin. Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride.

SALL1 Promoter Constructs and Site-directed Mutagenesis—The 5′-flanking region of SALL1 was amplified with primers (5′-primer, 5′-GGCCATGGCTCGATCATCCCGGACGC-CAGTACAGCTCCGA-3′, and 3′-primer, 5′-CGCCATGG-AAGCGTTGGCTAAACATCAGCTGGGGCAAT-3′) to generate a fragment from 1 to 2002 bp upstream of the first ATG, with HindIII and XhoI sites at each end, respectively. The SALL1 BAC DNA, instead of human genomic DNA, was used as a template to avoid amplification of a SALL1 pseudogene. After HindIII and XhoI digestion, this fragment was cloned into

4 The abbreviations used are: RACE, rapid amplification of cDNA ends; BAC, bacterial artificial chromosome; siRNA, small interfering RNA; WT, wild type; Luc, luciferase.

FIGURE 1. Genomic organization and chromosomal localization of SALL1 and 5′-RACE of human SALL1. A, genomic organization of SALL1. Boxes represent exons, and boxes highlighted by shading indicate zinc finger motifs. The lines indicate introns. B, chromosomal localization by fluorescent in situ hybridization to metaphase chromosomes of human peripheral lymphocytes. Inset, magnified view of labeled chromosome. SALL1 is located on chromosome 16q12.1. C, 5′-RACE of human SALL1. The diagram shows the positions of the primers used for 5′-RACE. Human fetal kidney Marathon-Ready cDNA was used as a template for PCR amplification with an adaptor, AP1 and a gene-specific primer, R1, for SALL1. D, results of agarose gel electrophoresis and ethidium bromide staining of amplification products are shown. The major size of the PCR product was identified, subcloned, and sequenced. Lane 1, 5′-RACE PCR product; lane 2, DNA ladder.

the promoterless pGL3-Basic luciferase reporter plasmid (Promega) at the HindIII and XhoI sites to generate −2002Luc. The fragments of −1500-Luc, −1000-Luc, −500-Luc, and −21-Luc were created in the same manner as −2002-Luc. A deletion mutant of a −2002-Luc SALL1 promoter construct that lacks the consensus site for SIX1 was generated using a QuickChange II mutagenesis kit (Stratagene) according to the manufacturer’s recommendations.

Transfection—Transfection was performed with FuGENE 6 transfection reagent (Roche Applied Science) according to the
manufacturer's instructions. Cells were plated in 24-well plates at a density of \( \sim 1 \times 10^5 \) cells/well. Cells were harvested 24 h after transfection. Plasmid DNA for transient transfection was prepared with the Qiagen plasmid mini kit (Qiagen). To analyze the SALL1 promoter constructs, 0.8 \( \mu g \) of the SALL1 promoter reporter plasmid and 0.2 \( \mu g \) of the internal control \( \beta \)-galactosidase plasmid, CMV-\( \beta \)-galactosidase (Invitrogen), were used. For co-transfection experiments with SIX1, a total of 1.2 \( \mu g \) of plasmid DNA was used. The reaction contained 0.5 \( \mu g \) of the SALL1 promoter reporter plasmid and either 0.5 \( \mu g \) of empty vector DNA or 0.5 \( \mu g \) of the SIX1 expression plasmid plus 0.2 \( \mu g \) of internal control CMV-\( \beta \)-galactosidase plasmid.

\( \beta \)-Galactosidase and Luciferase Assays—Cells were extracted with the use of 100 \( \mu l \) of luciferase cell culture lysis reagent (Promega) 24 h after transfection. The \( \beta \)-galactosidase assay, performed with 10 \( \mu l \) of cell extract, used the \( \beta \)-galactosidase enzyme assay system (Promega) and the standard assay protocol provided by the manufacturer (except that 1 M Tris base was used as stopping buffer instead of sodium carbonate). For the luciferase assay, 5 \( \mu l \) of extract was used in accordance with the manufacturer's instructions. After subtraction of the background, luciferase activity (arbitrary units) was normalized to \( \beta \)-galactosidase activity (arbitrary units) for each sample.

SiRNA—HEK-293 cells were seeded at a density of \( 5 \times 10^5 \) cells/ml into 24-well dishes and transfected with \( EYA1 \) small interference RNA (siRNA) (Santa Cruz Biotechnology) using Lipofectin 2000 (Invitrogen) according to the manufacturer's instructions. Renilla plasmid (Promega) was used as an internal control. \( EYA1 \) siRNA was a pool of three separate strands targeting different \( EYA1 \) mRNA locations. The strand information is as follows: Strand A, 5' - CCAAUGAGCAGCAGUGAAA-3'; Strand B, 5' - GCAACAAGCUACAGCCCUAU-3'; Strand C, 5' - GCAACGCUCAGUGUUA-3'. An siRNA that consists of a scrambled sequence was also used as a control.

Western Immunoblotting—Cell lines were harvested with ice-cold phosphate-buffered saline containing 5 mM EDTA and lysed in a buffer composed of 150 mM NaCl, 20 mM Hepes, pH 7.5, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 \( \mu g/ml \) aprotinin, 1 mM leupeptin, 1 mM pyrophosphate, 1 mM vanadate, and 1 mM dithiothreitol. Protein content of the lysates was determined using the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard. Samples were subjected to 10% SDS-PAGE followed by electrophoretic transfer of the proteins to nitrocellulose membranes. Membranes were blocked with 3% bovine serum albumin in T-TBS (20 mM Tris-HCl, pH 7.5, 135 mM NaCl, and 0.1% Tween 20) and then incubated either with an anti-SIX1 antibody (Santa Cruz Biotechnology) or with an anti-human \( \beta \)-actin antibody (0.1 \( \mu g/ml \); Santa Cruz Biotechnology). Membranes were then washed extensively with T-TBS and incu-
bated with horseradish peroxidase-conjugated secondary antibody. Bands corresponding to SIX1, SALL1, and β-actin proteins were detected using the SuperSignal West Pico® chemiluminescent substrate (Pierce).

**Antibody Generation**—The N-terminal peptide (DGDD-TEKQPSRPTKSDK) and the C-terminal peptide (EKMASSEN-GTNRFTR) of SALL1 were chosen for their potential antigenicity and used for immunization of chickens in collaboration with Lampire Biological Laboratories (Pipersville, PA). SALL1 antibodies IgY were purified from chicken eggs.

**Electrophoresis Mobility Shift Assays**—Double-stranded oligonucleotides containing 5’-protruding ends were labeled by [α-32P]dCTP with Klenow fragment and used for gel mobility shift assays as probes. Nuclear extracts were prepared from the HEK-293 cells as described previously (28). An antiserum to SIX1 was mixed with the nuclear extracts before the addition of the labeled probes. The gel mobility shift assay was performed using the method described previously in Ref. 28. Wild type and mutant probe, WT (5’-primer, 5’-TGAAC-CCATCAGGTTCACACGT-3’, 3’-primer, 5’-ACGTTTGGGAAC-GTATGGTTCA-3’), mutant type probe, MT (5’-primer: 5’-TGATCGGACTAGTTGGCCAAAAG-GT-3’, and 3’-primer, 5’-ACGTTTG-GAGGACACTAGTCCGATCA-3’) were used for electrophoretic mobility shift assays.

**RESULTS**

**Cloning and Sequencing of the Human SALL1 5’-flanking Region**—To identify the promoter region(s) upstream of SALL1, we screened a human BAC genomic library. One BAC clone was isolated, and direct nucleotide sequencing and restriction mapping showed that it contained the full-length SALL1 gene. An intronless pseudogene, SALL1P, has been reported and bears identical sequences, except for several mutations in the coding region. This pseudogene has been mapped to chromosome Xp11.2 (29). To determine whether the isolated BAC clone was specific for SALL1 gene, DNA from the SALL1 BAC clone was labeled with digoxigenin-11-dUTP by nick translation and hybridized in situ to normal human chromosomes derived from peripheral blood lymphocytes. Fluorescence in situ hybridization revealed that the BAC clone containing SALL1 localized to the proximal long arm of chromosome 16 (q12.1) (Fig. 1, A and B), consistent with a previous report of the SALL1 locus (3). This study demonstrated that the isolated BAC clone indeed contained the SALL1 gene but not the SALL1P gene.

**Cloning of the 5’-End of SALL1 cDNA**—The 5’-end of SALL1 cDNA was determined by 5’-RACE-PCR using human fetal kidney Marathon-Ready cDNA pool as templates. The PCR product obtained by this method, using primers AP1 and R1, appeared as a major band on agarose gel electrophoresis (Fig. 1, C and D). The sequence of this major PCR product subcloned into the pBluescript (Stratagene) vector revealed the major 5’-end as −69 (the translation start site was set at +1).

**Analysis of SALL1 Promoter Sequence**—Sequencing of the 5’-flanking region of SALL1 did not reveal a classic TATA or CAAT box. This region, however, was GC-rich and contained several GC boxes. A search of Genomatrix, the Transcription Factor Database, revealed several putative binding sites for regulatory transcription factors scattered within 2.0 kb upstream of the ATG translation site, including 11 sites for WTI and one
SALL1 Promoter and SIX1 in Kidney Development

FIGURE 4. Effect of SIX and Eya on SALL1 promoter activity. Transient transfections of HEK-293 cells with each of the SIX and Eya plasmids were performed. Fold differences, i.e. relative luciferase activity, were calculated as arbitrary luciferase activity of SALL1-Luc (−2002-Luc construct) over that of the vector, pGL3 basic (Promega) without promoter. Transfection efficiencies were normalized by β-galactosidase activity derived from CMV-β-galactosidase control plasmid.

To define the minimal DNA sequence required for the SALL1 promoter activity and to analyze DNA sequences potentially important for regulation of SALL1 transcription, transient transfections were performed with a series of deleted DNA fragments surrounding the transcription start sites in a luciferase reporter gene assay. Each resulting recombinant plasmid DNA construct was then transiently transfected into HEK-293 cells. The series of deleted promoter fragments used in the transfection containing −2002, −1502, −1002, −502, and −21 bp upstream sequences are shown in Fig. 3C. All five constructs exhibited functional promoter activity in HEK-293 cells. Among the five constructs, the −1002 bp upstream sequence showed the highest relative luciferase activity (Fig. 3C). Luciferase activity decreased progressively to ~35% of that of −1002 as the sequence length increased, which suggested the presence of upstream suppressor activity between −2002 and −1002. Removal of the upstream region (−1002/−21) led to basic
levels of luciferase activity similar to those of pGL3-Basic, indicating the presence of strong promoter activity in this region.

Transcriptional Activation of Human SALL1 Promoter by SIX1—Sequence analysis of the SALL1 promoter revealed a potential SIX binding site. Given the known importance of the SIX and Eya networks in the earliest kidney development, we assessed whether these transcription factors could alter transcription from the SALL1 promoter. HEK-293 cells were transfected with each of the SIX and Eya members. The SALL1 promoter was activated by different SIX members that we examined, except for SIX5 (Fig. 4), although the degree of SALL1 promoter activation varies among the SIX1 members. However, little or no increase in a luciferase activity in HEK-293 cells was observed by co-transfection of each of the Eya members with the SALL1 promoter construct. This could be due to the fact that Eya alone bears no apparent DNA binding activity. It serves as a co-activator for SIX1 in gene transcription regulation function (30). Despite the presence of endogenous SIX1 in HEK-293 cells, its expression level is still significantly low when compared with that of SIX1 co-transfected with Eya; thus only low SALL1 promoter activities were observed.

Since SIX1 is the best characterized gene for studying kidney development and the mutant phenotype of Six1 in mice clearly resembles that of Sall1 heterozygous in renal development (7, 30), we next looked at the effects of the cellular context of endogenous SIX1 on SALL1 promoter in different cell lines. We analyzed the effects of SIX1 on SALL1 promoter activity with transient transfection assays. Human SALL1 promoter reporter gene expressions were examined in the HEK-293, COS-7, PC3, and NIH-3T3 cells. SIX1 was able to activate the SALL1 promoter by 3-fold in HEK-293 cells, 7-fold in COS-7 cells, 28-fold in PC3 cells, and 20-fold in the NIH-3T3 cells when compared with the pCDNA3 control (Fig. 5A). PC3 and NIH-3T3 expressed weak or no endogenous SALL1 protein (Fig. 3B), and high levels of exogenous SIX1 greatly increased SALL1 promoter activity in these cell lines.

To determine dose-dependent activation of the SALL1 promoter, the SIX1 responsiveness of the SALL1 promoter was evaluated by co-transfection of 1 μg of the SALL1 promoter construct and 0.2 μg of β-galactosidase plasmid, together with increasing amounts of the SIX1 expression construct (0–3 μg) (Fig. 5B). SALL1 promoter activity was increased more than 4-fold by co-transfection with an ~1-fold excess of the SIX1 construct. When this experiment was repeated with the use of a 3:1 molar excess of the SIX1 construct, the promoter was further greatly activated, showing a greater than 11-fold increase (Fig. 5B).

To determine the essential region of SIX1 that exerts these activation effects, different deletion constructs were used to

FIGURE 6. Overexpression of SIX1 induces expression of the endogenous SALL1 protein and SIX1 is able to bind to SALL1 promoter. A, characterization of SALL1 peptide antibodies by immunoblotting. Lysates from HEK-293 cells transiently expressing control vector (lane 1 in each panel) and His-SALL1 (lane 2 in each panel) were resolved by 10% SDS-PAGE gel, transferred onto a nitrocellulose, and probed with the N-terminal SALL1 peptide antibody (N Antibody, left panel), C-terminal SALL1 peptide antibody (C Antibody, middle panel) and anti-polyhistidine (His6) antibody (His Antibody, right panel). B, whole-cell extracts were prepared from HEK-293 cells transiently transfected with either pcDNA3 control (lane 1) or an increased amount of SIX1 construct and subjected to Western blot analysis using anti-SIX1, SALL1, or β-actin. C, binding of SIX1 transcription factor to the SALL1 promoter. Nuclear extracts from HEK-293 cells were incubated with radiolabeled 21-bp oligonucleotide, which contained the consensus site for SIX1 (WT) or mutated consensus sequence for SIX1 (Mutant). Anti-SIX1 antibody was mixed with reaction mixture and incubated for 1 h at 4 °C. The specific DNA-SIX1 complex and the supershifted band are indicated by arrows. D, SIX1 stimulation of −2000-Luc SALL1 promoter or −2000-Luc SALL1 promoter lacking the consensus site for SIX1 and SALL1m were analyzed by luciferase reporter assays. SALL1m or −2000-Luc SALL1 construct was co-transfected into HEK-293 cells with either pcDNA3 vector or plasmid encoding SIX1 using Lipofectin 2000. Luciferase assays were performed 24 h after transfection. Each bar represents mean ± standard deviation of at least three independent experiments.

SALL1 Promoter and SIX1 in Kidney Development
SALL1 Promoter and SIX1 in Kidney Development

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**FIGURE 7. Cooperation of SIX1 and Eya1 in activation of SALL1 promoter and proposed model of SIX1-Eya1 genetic network in regulation of SALL1 expression.** A. −2000-Luc of SALL1 promoter construct was co-transfected with SIX1 or Eya1 or pcDNA3 or both the SIX1 and the Eya1 together in the HEK-293 cells. Luciferase activity in the cell lysate was normalized with β-galactosidase activity of pCMV-β-galactosidase as an internal control. B. diminishment of SIX1 in activation of the SALL1 promoter by knock-down of the endogenous EYA1. Upper panel, siRNA-mediated reduction of the endogenous EYA1 mRNA expression in HEK-293 cells was determined by reverse transcription-PCR. HEK-293 cells were treated with EYA1 siRNA, and the control group treated with non-targeting siRNA. EYA1 mRNA expression was examined 24 h after transfection. To show relative amounts of mRNA in each sample, β-actin was used as a constitutively expressed marker. Bottom panel, SALL1 promoter and SIX1 construct were co-transfected into HEK-293 cells, either with EYA1 siRNA or with control siRNA. 24 h after transfection, luciferase reporter assays were performed as described above. Experiments were repeated at least three times. C and D, model of SIX1-Eya1 regulating the target gene, SALL1. In the absence of Eya1, SIX1 produces only a marginal effect on SALL1 promoter activity. SIX1 recruits Eya1 by binding to and translocating it from the cytoplasm to the nucleus in where synergistic effect of SIX1 and Eya1 on the SALL1 promoter occurs (30). Eya1 is located in the cytoplasm, and Eya1 alone bears on effect on the SALL1 promoter activity.
To test the effect of the mutation of the identified SIX1 binding site on the SALL1 promoter activity, the consensus SIX1 binding site (−948 to −942) was deleted and introduced into the SALL1 promoter (SALL1m). The luciferase activities of the resulting construct (SALL1m) were then compared with the activity of the wild type or co-transfection with SIX1 in HEK-293 cells. There is no difference in luciferase activities between the SALL1 promoter mutant (SALL1m) and the wild type promoter construct in HEK-293 cells. However, deletion of 7 bp (−948 to −942) of the putative binding site of SIX1 resulted in 3-fold reduction in the luciferase activity (Fig. 6D) when compared with that of the wild type. This study indicates that the SIX1 consensus binding site is a functional site present on the SALL1 promoter.

Cooperation of SIX1 and Eya1 in Activation of SALL1 Promoter—The SIX and Eya families are co-expressed in multiple organs. Eya, which has no apparent DNA binding activity, serves as co-activator (30). Previous studies have shown that SIX1 and Eya1 are able to synergistically activate reporter gene transcription. We next investigated the SALL1 reporter gene activity by further transient co-transfection assays. Transfection of SIX1 resulted in an ~1.5-fold increase in luciferase activity, and the activation level was increased to ~2.5-fold by the co-transfection of Eya1. However, Eya1 alone had a minimal effect on the luciferase activity (Fig. 7A).

To further examine the role of Eya1 in the SIX1/Eya1/SALL1 network, we disrupted Eya1 expression in the HEK-293 cells by an siRNA approach. Three different siRNA sequences targeting Eya1 and a scrambled control siRNA comprised of a non-targeting sequence without significant similarity to any known human gene sequences were used. Eya1 mRNA was highly expressed in HEK-293 cells (Fig. 7B). Completely knocking down Eya1 expression significantly diminished the SALL1 promoter activity response to SIX1 stimulation (Fig. 7B).

**DISCUSSION**

Kidney development requires SALL1 expression, and the expression of SALL1 in the kidney is associated with proliferating cells of the ureteric bud and the differentiating nephrogenic mesenchyme. How SALL1 expression is induced in this tissue is unknown. What we detail here is the first characterization of the human SALL1 gene promoter and its activation by SIX1.

The SALL1 promoter has no TATA or CCAAT boxes in the vicinity of the transcription start sites, and this is similar to the situation with SALL2, another member of SALL gene family. The TATA-less promoters, which are usually associated with multiple transcription start sites, are observed primarily in housekeeping genes and many genes in the hematopoietic system. Deletion analysis of the promoter region revealed a negative element, which may reside between −2000 and −500 bp upstream of the ATG initiation codon. Significant homology exists between murine and human SALL1 promoter sequences of the 5′-flanking region, suggesting a conserved function of regulation of SALL1 gene expression.

Putative binding sites were identified in the promoter region. The most interesting finding was the presence of consensus sites for WT1 and SIX. The WT1 and SIX binding sites were highly conserved between human and mouse sequences. The functional importance of WT1 and SIX with respect to modulation of the SALL1 expression needs to be examined. Previously, we have shown that the WT1 was able to suppress expression of SALL2, the other member of SALL family (31). The presence of multiple potential binding sites in the SALL1 promoter region indicates that WT1 may be a transcriptional regulator for SALL1 as well. Using a reporter gene assay, transcription from the SALL1 promoter was strikingly suppressed by the WT1 tumor suppressor protein in a dose dependent manner (data not shown).

We discovered a positive regulatory element between −948 and −942 of SALL1, which contained a consensus binding site for SIX1. To determine whether SIX1 modulates SALL1 expression in kidney, we examined the promoter regulatory regions, utilizing a functional luciferase assay with co-transfection of the SIX1 plasmid in HEK-293 cells. The SALL1 promoter was found to be significantly activated by the SIX1. In addition, we observed dose-dependent and cell type-specific activation of the SALL1 promoter by co-transfection of SIX1 in several different cell lines. The exogenous SIX1 could up-regulate the transcription of the SALL1 reporter gene construct in vitro. These studies are consistent with previous findings in animals that deletion of SIX1 in mice results in the loss of SALL1 expression (32).

Mutations of transcription factors SIX1 and SALL1 result in incomplete ureteric bud outgrowth and mesenchymal apoptosis, leading to a failure of tubule formation (7, 33). Kidney phenotypes of SALL1 in deficient mice, similar to those of SIX1, raise the possibility that both SIX1 and SALL1 act in the same pathway(s) in kidney development. To further explore this model, we extend our study to a co-activator of SIX1, Eya1. Mutation in human Eya1 is associated with branchio-oto-renal syndrome with abnormalities including renal and otic organs similar to a human disease caused by a SALL1 mutation. These findings suggest that there is a complex gene network involving SIX1/Eya1/SALL1. Eya1 bears no apparent DNA binding activity and can be translocated to the nucleus by the SIX1 protein (30). Previous studies have shown that SIX1 and Eya1 are able to synergistically activate the target genes. We examined the effect of the Eya1 on the transcriptional induction of SALL1 in a reporter gene assay. Co-expression of SIX1 and Eya1 resulted in a significant increase in the SALL1 promoter activity when compared with either SIX1 or Eya1 alone. Endogenous Eya1 mRNA knockouts by siRNA sequences targeting Eya1 significantly diminish SALL1 promoter activity. This further indicates that Eya1 can serve as a co-activator of SIX1 in the regulation of the SALL1 expression. A proposal of the SIX1-Eya1 genetic network in the regulation of the SALL1 expression is shown in Fig. 7, C and D.

In conclusion, our studies provide a starting point for a more detailed examination of human SALL gene regulation. Our studies strongly suggest that SIX1 likely plays a role in the induction of SALL1 expression during normal kidney development. Further studies are needed to elucidate the biological function of the SIX1-mediated transcript of SALL1 in kidney development.

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