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Interaction between Tbx1 and HoxD10 and connection with TGFβ-BMP signal pathway during kidney development

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ABSTRACT

Renal malformations are commonly found among patients carrying a 22q11 deletion which renders loss of *Tbx1* gene, an important transcriptional factor implicated in a number of developmental processes. Smad1 is known to interact with Tbx1, but the exact mechanism remains unknown. In this study, we have measured the expression of *Tbx1* in both murine and human tissues using RT-PCR, and analyzed its protein product and protein-protein interactions with Western blotting and immunoprecipitation assays. Precipitated proteins were verified with mass spectrometry. As discovered, Tbx1 binds with Hoxd10. *Tbx1* and *Hoxd10* genes also have similar expression profiles during murine kidney development. Based on homology between mouse and human, we hypothesized that such interaction also exists in human. Through a RNA interference experiment using a human embryonic kidney HEK293 cell line, we demonstrated that TBX1 can alter TGF-β/BMP, an important signaling pathway, through interacting with HOXD10. Above findings may shed light on the mechanism of *TBX1* mutations leading to renal malformations found in patients carrying a 22q11 deletion.

1. Introduction

TBX1 is a member of T-box family, a group of DNA-binding transcriptional factors which play important roles in the regulation of developmental processes (Papaioannou and Silver, 1998; Smith, 1999). In mice, mutations in *Tbx1* gene can cause a DiGeorge syndrome-like phenotype, where development of aortic arch, thymus, parathyroid gland, skull and face, ear, kidneys and teeth can be affected (Baldini, 2005). 22q11 deletion is the most common cause of DiGeorge syndrome (OMIM 188400) in humans. A high prevalence of genitourinary malformations has been discovered among individuals carrying a 22q11 deletion (Kobrynski and Sullivan, 2007; Kujat et al., 2006; Wilson and Conlon, 2002; Wu et al., 2002; Zhang et al., 2006), which suggested that particular gene(s) from the deleted region is involved in the development of genitourinary system. Mutations of such gene(s) may result in defects such as renal malformations, metanephric duct/urinary bladder and hypospadia.

Human *TBX1* gene has been mapped to the critical region of 22q11 deletion. A number of genes including *SMADs* and *WNTs* are known to be regulated by *TBX1* (Fulcoli et al., 2009; Huh and Ornitz, 2010; Ivins et al., 2005; Liao et al., 2008; Mitsiadis et al., 2008). During early development, *TBX1* also functions in a dose-dependent manner in major signaling systems including sonic hedgehog (SHH), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) (Garg et al., 2001; Merscher et al., 2001; Yu et al., 2002). Animal experiment also suggested that *Tbx1* knockout or haploinsufficiency can cause phenotypes similar to human patients carrying a 22q11 deletion.

The present study was set to explore proteins which interact with TBX1, the mechanism of such regulation and its connections with known molecular pathways involved in kidney development.

2. Materials and methods

2.1. Immunoprecipitation and mass spectrometry

Protein was extracted from 0.5 d mice kidneys. 400–800 μg nucleoprotein was transferred to two 1.5 ml Eppendorf tubes. One was used as sample, and the other was used as negative control. Isolated protein was incubated with 2–4 μg of antibodies (anti-Tbx1,

Abbreviations: A, adenosine; BMP, bone morphogenetic protein; bp, base pair; BSA, bovine serum albumin; C, cytidine; cDNA, DNA complementary to RNA; cpm, counts per minute; DMEM, Dulbecco's modified eagle medium; dNTP, deoxyribonucleoside triphosphate; G, guanosine; IP, immunoprecipitation; kb, kilobase or 1000 bp; kDa, kilodalton(s); MALDI-TOF-MS, matrix-assisted laser desorption/Ionization time of flight mass spectrometry; N, any nucleotide; siRNA, small interfering RNA; T, thymidine; *Tbx1*, T-box 1 gene; TBST, Tris-buffered saline (TBS) plus 0.1% Tween; TGF-β, transforming growth factor β.

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Zymed Laboratories, USA; anti-Hoxd10, Santa Cruz, CA, USA) or equal amount of mouse IgG (Santa Cruz, CA, USA). The solution was topped with a lysis buffer (Beyotime, China) to 1 ml. 100 μl resuspended protein G PLUS-agarose (Santa Cruz, CA, USA) was added, and the mixture was incubated at 4 °C overnight. Immunoprecipitates was centrifuged at 3,500 rpm for 5 min. at 4 °C and washed 4 times with 1 ml of lysis buffer. After the final wash, immunoprecipitates was resuspended with 20 μl of 1× electrophoresis buffer and boiled for 2–3 min. before further analysis with 12% SDS-PAGE. Unused samples were stored at −20 °C.

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) was accomplished by Tianjin Biochip Corporation (Tianjin, China). The results were searched against MS-Fit at http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msfitstandard, Database: NCBInr. 2008.11.25, SwissProt.2008.06.10 and UniProtKB.2008.06.10.

2.2. Western blotting

Proteins were extracted from 0.5 d murine kidney and human embryonic kidney HEK293 cells. Protein concentrations were determined through a modified Lowry procedure with bovine serum albumin (BSA) as the standard. Equal amounts of proteins were separated by electrophoresis on a 12% SDS-polyacrylamide gel, and transferred onto a polyvinylidene difluoride (PVDF) membrane (Lillipore, Bedford, MA, USA). The membranes were blocked with Tris-buffered saline (TBS) plus 0.1% Tween (TBST) supplemented with 5% nonfat milk for at least 3 h, and incubated with primary antibodies (mouse Tbx1 antibody, 1:1000 dilution, Invitrogen, CA, USA; human TBX1 antibody, 1:1000 dilution, Abcam, MA, USA; mouse Hoxd10 antibody, 1:1000 dilution, Santa Cruz, CA, USA; human HOXD10 antibody, 1:1000 dilution, Abcam, MA, USA; human β-ACTIN antibody, 1:1000 dilution, Santa Cruz, CA, USA) overnight at 4 °C. After extensive washing with TBST, the membranes were incubated for 1 h with IgG-HRP (goat anti-rabbit IgG for human TBX1, human HOXD10, mouse Tbx1 and human β-actin, rabbit anti-goat IgG for mouse Hoxd10, 1:1000; Zhongshan, China). After washing for 3 times, the blots were visualized using an ECL system (Thermo, USA) following autoradiography.

2.3. Cell culture and transfection

Human embryonic kidney cell line HEK293 was maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, laevomycetin and streptomycin (100 U/ml). Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

For small interfering RNA (siRNA) experiment, the cells were plated into 6-well plates 24 h prior to transfection, and then transfected with human TBX1 siRNA oligo and a negative control siRNA oligo synthesized by GenePharma (Shanghai, China). (Table 1) Transfected cells were respectively labeled as HEK293/*TBX1*-siRNA and HEK293/NC-siRNA. Transient transfection was carried out using a lipofectamine 2000 reagent (Invitrogen, CA, USA) according to manufacturer's protocol. Medium was replaced 4–6 h after the transfection. For all experiments, a siRNA control was set at equivalent concentrations. The cells were harvested at 48 h after transfection.

Table 1
Sequence of siRNA.

siRNA	Sequence (5′ to 3′)	
TBX1-588	Sense	CCGACUAUAUGCUGCUCAUTT
	Anti-sense	AUGAGCAGCAUAUAGUCGGTT
TBX1-872	Sense	CGCAAAGAUACGCAGAAAUTT
	Anti-sense	AUUUCUCGCUAUCUUUGCGTT
TBX1-976	Sense	GCUCAAGAUUGCCAGCAAUTT
	Anti-sense	AUUGCUGGCAAUCUUGAGCTT
Negative control	Sense	UUCUCCGAACGUGUCACGUTT
	Anti-sense	ACGUGACACGUUCGGAGAATT

2.4. RNA extraction and reverse transcription PCR

Total RNA was isolated using a TRIZOL reagent (Invitrogen, CA, USA) from HEK293/*TBX1*-588-siRNA, HEK293/*TBX1*-872-siRNA, HEK293/*TBX1*-976-siRNA and HEK293/NC-siRNA cells as well as kidney tissue of embryo (E 17.5 d), newborn (0.5 d) and adult mice.

Reverse transcription was carried out with extracted total RNA with a Reverse Transcription System (Promega, Madison, WI, USA). The reaction mixture contained 4 μl of 25 mM $MgCl_2$, 2 μl of 10× reverse transcription buffer, 2 μl of 10 mM dNTP mixture, 0.5 μl of recombinant RNasin ribonuclease inhibitor, 15 U of AMV reverse transcriptase, 0.5 μg of Oligo $(dT)_{15}$ primer or random primers, and 1 μg of total RNA in a total volume of 20 μl. RNAs were incubated for 15 min. at 42 °C, followed by 5 min. at 95 °C to inactivate reverse transcriptase and 5 min. at 0–5 °C.

Derived cDNA was amplified with PCR. Specific primers for human *TBX1*, *β-actin* and mouse *Tbx1*, *Hoxd10* and *β-actin* were designed with Primer3 software (http://www.basic.nwu.edu/biotools/Primer3). Aliquots of PCR product were verified by electrophoresis on a 2% agarose gel, with the fragments visualized by ethidium bromide staining (Table 2). Gene sequences were verified with DNA sequencing and comparison with data from GenBank using a BLAST algorithm. Target cDNA was quantified by relative gray-scale value, which was normalized by comparing the gray-scale values of *Tbx1* and *Hoxd10* with that of *β-actin*, and analyzed with a UVP Gelworks ID software (advanced version 2.5) (Li et al., 2009). Each experiment was independently performed for three times.

2.5. Semi-quantitative real-time PCR

Semi-quantitative real-time PCR was conducted on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. All reactions had consisted of 10 μM of forward primer, 10 μM of reverse primer, 12.5 μl of 2× SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA) and 1 μl of cDNA in a final reaction volume of 25 μl. Gene expression was quantified using a comparative *Ct* method, which normalizes the *Ct* values to an internal housekeeping gene (*GAPDH*) and calculates the relative expression value (Fink et al., 1998). Primer sequences for *TBX1*, *HOXD10*, *SMAD1* and *GAPDH* are listed in Table 3. The cycling conditions were 50 °C for 2 min., with an initial denaturation step at 95 °C for 10 min., followed by 40 cycles with denaturation at 95 °C for 15 s and annealing of the primers at 60 °C for 1 min. All experiments were repeated for at least three times to ensure the accuracy of results.

2.6. Statistical analysis

Gene expression was determined with a software from the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster

Table 2
Primer sequence for reverse transcription PCR.

Gene	Sequence (5′ to 3′)	
TBX1	Sense	ATGCTGCTCATGGACTTCGTGC
	Anti-sense	CTCGCTATCTTTGCGTGGGTC
β-actin (homo)	Sense	CTCTTCCAGCCTTCCTTCCT
	Anti-sense	CACCTTCACCGTTCCAGTTT
Tbx1	Sense	CTGTGGGACGAGTTCAATCAG
	Anti-sense	TTGTCATCTACGGGCACAAAG
Hoxd10	Sense	TGAGGTTTCCGTGTCCAGTCCCGA
	Anti-sense	ATTCCAGCGTTTGGTGCTTGGTGTA
β-actin (mus)	Sense	GAGACCTTCAACACCCCAGC
	Anti-sense	CCACAGGATTCCATACCCAA