Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 344 (2006) 727–734
BBRC
www.elsevier.com/locate/ybbrc

Ubc9 interacts with SOX4 and represses its transcriptional activity ☆

Xin Pan [1], Huiyan Li [1], Peijing Zhang, Baofeng Jin, Jianghong Man, Lifeng Tian, Guiying Su, Jie Zhao, Weihua Li, Hui Liu, Weili Gong, Tao Zhou, Xuemin Zhang *

National Center of Biomedical Analysis, Beijing 100850, PR China

Received 23 March 2006
Available online 17 April 2006

Abstract

SOX4 is a member of SOX transcriptional factor family that is crucial for many cellular processes. In this study, a yeast two-hybrid screening of human mammary cDNA library identified human ubiquitin-conjugating enzyme 9 (hUbc9) that interacted with SOX4. This interaction was confirmed by GST pull-down in vitro and co-immunoprecipitation assays in vivo. Deletion mapping demonstrated that HMG-box domain of SOX4 is required to mediate the interaction with Ubc9 in yeast. Furthermore, confocal microscopy showed that Ubc9 co-localized with SOX4 in the nucleus. Luciferase assays found that Ubc9 specifically repressed SOX4 transcriptional activity in 293T cells. We further demonstrated that Ubc9 could functionally repress the transcriptional activity of endogenous SOX4 induced by progesterone in T47D cells. The C93S mutant of Ubc9, which abrogates SUMO-1 conjugation activity, did not abolish the ability to repress SOX4 activity. It shows that Ubc9 interacts with SOX4 and represses its transcriptional activity independent of its SUMO-1-conjugating activity.

Keywords: SOX4; Ubc9; Yeast two-hybrid; Protein interaction; Transcriptional activity; SUMO-1

The SOX (SRY-related HMG-box) genes define a family of transcriptional factor which play key roles in the regulation of a variety of developmental processes, such as sex determination, neural development, cardiac development, chondrogenesis development, and lung and lens development [1–5]. All SOX proteins contain a highly conserved HMG-box DNA-binding domain. According to the sequence similarity of the HMG-box, the SOX family can be further subdivided into groups A–H. SOX4, a member of the C subgroup of SOX family and first identified as the Sry-related gene a4 [6], was expressed in a wide range of tissues such as ovary, testis, and thymus of adult mice and in mouse T and pre-B lymphocytic cell lines [7]. It has been shown to be involved in many developmental processes such as embryonic cardiac development [1], nervous system development [8], thymocyte development [9], as well as the differentiation and development of B- and T-lymphocyte [10]. Serine-rich C terminus of murine SOX4 functions as its transactivation domain [7].

In addition to its pivotal roles in regulation of development, SOX4 has also been implicated in other physiological and pathological processes. In normal breast and breast cancer cells, the expression and transcription activity of SOX4 is under progesterone control, which suggests changes in SOX4 gene expression may play a role in commitment to the differentiated phenotype in the normal and malignant mammary gland [11]. Furthermore, there are increasing evidences that SOX4 is highly expressed in several tumors including breast cancer, colon cancer, lung cancer, meduloblastoma, and hepatocellular carcinoma, suggesting that SOX4 may be involved in tumorigenesis [12–19]. Up-regulated SOX4 expression during prostaglandin-induced apoptosis in hepatocellular carcinoma cells has

☆ *Abbreviations:* SOX, SRY-related HMG-box; HMG, high-mobility group; Ubc9, ubiquitin-conjugating enzyme 9; Δ, deleted allele; GFP, green fluorescence protein; RFP, red fluorescence protein; DBD, DNA-binding domain; AD, activation domain; SUMO, small ubiquitin-related modifier.

* Corresponding author. Fax: +86 10 68246161.
E-mail address: xmzhang@nic.bmi.ac.cn (X. Zhang).

[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.03.194

also been reported to play roles in regulating apoptosis through its central domain [19–21]. Although the evidences are accumulating on the important roles of SOX4 in such a variety of cellular processes, the mechanism by which SOX4 regulates transcription remains to be elucidated.

In this study, a yeast two-hybrid screening of human mammary cDNA library identified ubiquitin-conjugating enzyme 9 (hUbc9) that interacted with SOX4. The interaction was confirmed both in vitro and in vivo. Further studies demonstrated that Ubc9 repressed SOX4 transcriptional activity through physical association and independent of its SUMO-1-conjugating activity.

Materials and methods

Yeast two-hybrid screening. The bait plasmid was created by inserting a PCR-amplified fragment encoding the human SOX4 sequence (amino acid 1–133) into the *Eco*RI–*Bam*HI restriction sites of pGBKT7, resulting in an in-frame fusion with the GAL4 DNA-binding domain. The resultant plasmid and a human mammary cDNA library were simultaneously transformed into AH109 yeast strains according to the manufacturer's instructions (Clontech Laboratories, Inc.). Transformants were plated on synthetic medium without tryptophan, leucine, histidine, and adenine, followed by X-α-galactosidase assays. Approximately 2.16 million clones were screened, and then we restreaked candidate clones to allow loss of some of the AD/library plasmids and reintroduced them back to the same yeast strain to confirm the specific interaction between the candidate clones and the SOX4 bait. The cDNA inserts were sequenced and rescued in *Escherichia coli* DH5α.

Cell culture, transfection, and antibodies. COS-7 cells were maintained in RPMI1640 supplemented with 10% fetal calf serum (FCS). T47D cells derived from human breast cancer and 293T cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, at 37 °C in a humidified atmosphere of 5% CO_2. Transient transfections were performed using Lipofectamine 2000 reagent according to the manufacturer's instructions. HA and Myc antibodies were obtained from Santa Cruz.

Expression plasmids and reporters. Full-length human SOX4 cDNA was a gift from Dr. Farr C.J. (University of Cambridge). Full-length human SOX11 cDNA was a gift from Dr. Phillipe Jay. SOX4 and its truncation mutants were prepared by PCR specific primers and cloned in-frame into the pGBKT7 vector (Clontech), and the human Ubc9 cDNA was isolated from a human mammary cDNA library and cloned into the GAL4 activation domain vector pGADT7 (Clontech). All constructs were verified by sequencing. Myc and HA epitope tags were introduced to the N-terminus of SOX4 and Ubc9 by cloning into the pXJ40-myc or the pXJ40-HA mammalian expressing vectors, respectively. Constructs encoding GST fusion proteins were prepared by amplification of each sequence by standard PCR methods, and the fragments were cloned in-frame into pGEX-KG (Amersham Pharmacia Biotech). The Ubc9C93S with cysteine 93 substitution by serine was generated by PCR-mediated site-directed mutagenesis. For co-localization assays, the SOX4 cDNA and Ubc9 (Ubc9C93S) were amplified and ligated into the pEGFP-N1 and pDsRed-N1 vectors (Clontech), respectively.

GST pull-down assay. GST and GST-Ubc9 fusion were expressed and purified as previously described [22]. The fusion proteins were adsorbed to glutathione–Sepharose 4B beads. The expression vector for SOX4 was in vitro translated using the TNT Reticulocyte Lysate System (Promega). The ^{35}S-labeled SOX4 was then incubated with GST or GST fusion proteins in the binding buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.3 mM dithiothreitol, 0.1% Nonidet P-40, and protease inhibitor tablets from Roche Diagnostics). The binding reaction was rocked at 4 °C for 2 h, and the beads were subsequently washed four times with the washing buffer (the same as the binding buffer). The bound proteins were then boiled in 10 μl of sample buffer and separated by SDS–PAGE and autoradiography.

Co-immunoprecipitation and immunoblotting. For co-immunoprecipitation experiments, 293T cells were transfected using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM EDTA) containing a mixture of protease inhibitors and whole cell extracts were centrifuged at 12,000 rpm for 20 min to remove cell debris. Immunoprecipitations were performed by incubating whole cell extracts with anti-Myc monoclonal antibody, while rocking at 4 °C for 4 h. Immunoprecipitates were washed three times with the same buffer, resuspended in 40 μl of 1× SDS sample buffer, and then resolved by SDS–PAGE. For Western blot analysis, cells were lysed in lysis buffer supplemented with a mixture of protease inhibitors. Whole lysates were separated by SDS–PAGE and subsequently transferred onto nitrocellulose (Amersham Biosciences) and probed with the primary antibody after blocking. Membranes were developed using the enhanced chemiluminescence method (ECL, Amersham).

Fluorescence microscopy analysis. COS-7 cells transiently transfected with expression vectors of pEGFP-SOX4 and pDsRED-Ubc9 or pDsRED-Ubc9C93S. Twenty-four hours after the transfection, confocal imaging for GFP and RFP was performed by using a Bio-Rad Radiance 2100™ confocal laser system connected to a Nikon TE300 microscope. The green fluorescence of GFP-labeled SOX4 was excited with an argon laser (488-nm excitation line with 515-nm long pass barrier filter). RFP-Ubc9 or RFP-Ubc9C93S was simultaneously excited with a He–Ne laser (543-nm excitation line with 570-nm long pass barrier filter). All images were taken with an oil immersion objective and processed as TIFF (tagged image file format) on Photoshop 7.0 using standard image processing techniques.

Luciferase assays. Luciferase assays were performed as described [23]. Human 293T cells seeded onto 12-well dishes were transfected with SOX4 reporter plasmid (from Dr. Coffer P.) or GAL-DBD-Luc plasmid (from Dr. Ye Qinong) and with combinations of SOX4 and wild-type Ubc9 or Ubc9C93S mutant expression plasmids, together with internal control *Renilla* luciferase vector pRL-TK (Promega). Twenty-four hours after transfection, luciferase activity was measured by using a Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was determined as ratio of Firefly/*Renilla* luciferase activities, and data are expressed as means ± SD of triplicate values obtained from a representative experiment that was independently repeated at least three times.

Results

Identification of Ubc9 as a SOX4-interacting protein by yeast two-hybrid

In the attempt to identify SOX4-interacting proteins that could clarify the role of SOX4 in cells, we performed a yeast two-hybrid screen using the N-terminal of SOX4 as bait to screen human mammary cDNA library. From 2.16×10^6 independent transformants, we obtained four cDNA clones that specifically interacted with the bait. Subsequent sequence analysis identified the inserts from four positive clones were Ubc9 (Fig. 1A). Full-length cDNA for Ubc9 was then obtained from a mammary library, encoding 147 amino acid residues. We then tested in yeast whether Ubc9 could interact with SOX11, which shows a high similarity with SOX4 in protein sequence. The result showed that SOX11 could not interact with Ubc9 (data not shown). With different truncated deletions of SOX4 being created (Fig. 1B), the HMG-box domain was further determined to be essential for the interaction of SOX4 with Ubc9 in yeast