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SAMD4B, a novel SAM-containing protein, inhibits AP-1-, p53- and p21-mediated transcriptional activity

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The sterile alpha motif (SAM) is a putative protein interaction domain involved in a wide variety of biological processes. Here we report the identification and characterization of a novel gene, *SAMD4B*, which encodes a putative protein of 694 amino acids with a SAM domain. Northern blot and RT-PCR analysis showed that *SAMD4B* is widely expressed in human embryonic and adult tissues. Transcriptional activity assays show *SAMD4B* suppresses transcriptional activity of L8G5-luciferase. Over-expression of *SAMD4B* in mammalian cells inhibited the transcriptional activities of activator protein-1 (AP-1), p53 and p21, and the inhibitory effects can be relieved by siRNA. Deletion analysis indicates that the SAM domain is the main region for transcriptional suppression. The results suggest that *SAMD4B* is a widely expressed gene involved in AP-1-, p53- and p21-mediated transcriptional signaling activity. [BMB reports 2010; 43(5): 355-361]

INTRODUCTION

The p53 pathway is composed of hundreds of genes and their products that respond to a wide variety of stress signals. These responses to stress include apoptosis, cellular senescence or cell cycle arrest (1). The p53 protein is employed as a transcriptional activator of p53-regulated genes. In addition, the p53-regulated genes produce proteins that communicate with adjacent cells, prevent and repair damaged DNA and set up positive and negative feedback loops that enhance or attenuate the functions of the p53 protein and communicate with other signal transduction pathways (2, 3). In the p53 pathway, one of the most important upstream mediators of the p53 response is AP-1, which is composed of dimers of Fos and Jun proteins and has been linked to a startling breadth of cellular events including cell transformation, proliferation, differentiation and apoptosis (4, 5). The downstream components in the p53 pathway are widely believed including the cancer suppressor $p21^{WAF1/CIP1}$, which plays an important role in the control of the cell cycle by inhibiting all the cyclin-dependent kinases (CDK) (6, 7). Many transcription factors have also been identified as components of the p53 pathway, including the family of sterile alpha motif (SAM)-containing proteins.

SAM is an evolutionary conserved protein binding domain that is involved in the regulation of numerous developmental processes among diverse eukaryotes (8). SAM domains have been shown to form homo- and hetero-oligomers, forming multiple self-association structures that also bind to various non-SAM domain-containing proteins although with a low affinity (9). SAM domains are protein-protein interaction domains that are able to bind other SAM domains as well as to SH2 domains via phosphorylation of a conserved tyrosine in SH2 domains. They occur in proteins involved in both signaling and developmental regulation (10), and recent studies indicate that three SAM-containing proteins, Sqp53, p63 and p73, are likely to function in the p53 pathway (11, 12). Whether any other SAM domain proteins are involved in the p53 pathway is unknown.

In this study, we isolated a novel human gene named *SAMD4B*, encoding a SAM domain-containing protein, from a human heart cDNA library. Northern blot and RT-PCR analysis indicates that *SAMD4B* is expressed in most of embryonic and adult tissues tested. The highest expression of mRNA was detected in adult testis and the lowest in adult liver. Reporter gene assays showed that *SAMD4B* was a transcriptional repressor. Overexpression of pCMV-Tag2B-*SAMD4B* in HEK-293 cells inhibited AP-1, p53 and p21-mediated transcriptional activities, and the inhibitory effects were relieved by siRNA. The results suggest that *SAMD4B* might play a role in p53 pathway, which is known to affect cell cycle and apoptosis.

RESULTS AND DISCUSSION

Molecular characterization and evolutionary conservation analysis of the human *SAMD4B*

Many SAM domain containing proteins, such as Pmk1, DPYK1,

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p67, p73 and Sqp53, have been identified as playing important roles in regulating embryonic development (6). To specifically study the function of the SAM containing proteins, we screened the human EST database with the conserved SAM motif. A series of ESTs, CR983898, BF768721, DN996769, CN394606 and CB852248, with high sequence similarity, were obtained. Homology comparison of those ESTs against human EST databases was carried out. A 5.1 kb fulllength cDNA sequence of a predicted novel human gene was obtained from these ESTs by splicing on an EST annotation machine at IFOM (http://bio.ifom-firc.it/EST_MACHINE/index.html). An ORF sequence containing these ESTs was obtained using Genscan software. Results of a comparison against the nr database showed that the sequence represented a novel gene, termed *SAMD4B* (GenBank accession number EF601121) as approved by HUGO Nomenclature Committee. To confirm the cDNA sequences identified from the database, the PCR was performed using specific primers pORF1/pORF2 (Supplement material Table 1) and the PCR product (2192 bp) was sequenced. The result showed that it was identical to the predicted sequence. The full length of the cDNA is 5.1 kb with an open-reading frame (ORF) of 2085 bp extending from nucleotide 1032 to 3116, encoding a SAM domain-containing protein of 694 amino acids. Human genomic BLAST showed that *SAMD4B* maps to chromosome 19q13.2.

Phylogenetic tree analysis of amino acid sequences deduced from *SAMD4B* DNA sequence was performed using the MegAlign program of DNASTAR. GenBank accession numbers of previously known members of the SAM domain-containing protein and the novel SAM gene sequences were used for this analysis and the results show that *SAMD4B* is one of the conserved proteins during evolution (Supplement material Fig. 1). The most closely related protein to *SAMD4B* is XP_001136382 (Pan troglodytes).

Expression of *SAMD4B* in human adult and embryonic tissues

In order to understand the expression pattern of the *SAMD4B* gene in various human tissues, the expression of *SAMD4B* in multiple human embryonic tissues was examined by RT-PCR. The results showed that the target bands were detected in all the embryonic tissues tested (Fig. 1A). The expression profiles of the *SAMD4B* gene was also examined in adult tissues using northern blot analysis. The results indicated that a single expressed mRNA band of 5.1 kb was observed in various adult tissues, with a higher level in adult testis and lower in adult liver (Fig. 1B). The results indicate that *SAMD4B* is expressed in multiple human embryonic and adult tissues.

Subcellular localization of the *SAMD4B* protein

To examine the subcellular distribution of *SAMD4B*, the pEGFP-N2-*SAMD4B* recombinant plasmid was constructed and transiently introduced into COS-7 cells by liposome transfection. Under fluorescence microscope, the green fluorescence produced by pEGFP-N2-*SAMD4B* was detected in the nucleus and cytoplasm of COS-7 cells labeled with DAPI for the nucleus 48 h post-transfection. As shown in (Fig. 1C-E), this result suggested that *SAMD4B* protein distributes evenly in both the nucleus and the cytoplasm.

SAMD4B has transcriptional repressor activity

Although *SAMD4B* protein belongs to SAM domain-containing protein, the potential role of *SAMD4B* is not clear. To examine the transcriptional activity of *SAMD4B*, we constructed a fusion protein of *SAMD4B* with the DNA-binding domain (BD) of yeast transcription factor GAL-4 driving by a CMV promoter (pCMV-BD-*SAMD4B*). The luciferase reporter exhibited a basal level of transcriptional activity when transfected with the control plasmid pCMV-BD encoding the GAL4-DBD alone. LexA-VP16, which can directly activate the expression of the lucifer-

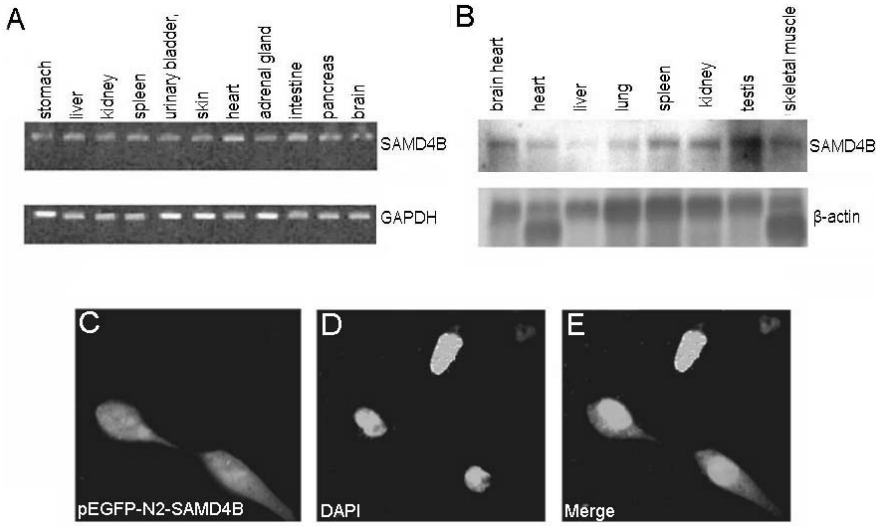

Fig. 1. Expression of *SAMD4B* in human tissues and subcellular localization of *SAMD4B* protein in COS-7 cells. (A) Expression of *SAMD4B* in a human embryo at 6 months gestation analyzed by RT-PCR. (B) Expression of *SAMD4B* in human adult tissues analyzed by Northern blot. The RNA filters were hybridized with the *SAMD4B* cDNA probe labeled with Digoxin (DDLK-010). Hybridization with β-actin was used to normalize loading amounts. A band at 2.0 kilobases (kb) was detected. (C) EGFP-*SAMD4B* transfected into the COS-7 cells is localized in both the nucleus and the cytoplasm. (D) Nucleus of COS-7 cells was stained with DAPI. (E) The combined image of (C) and (D), showing subcellular localization of *SAMD4B* when overexpressed in COS-7 cells. EGFP-*SAMD4B* is localized in both the nucleus and the cytoplasm.

ase reporter gene, was used as a positive control (13). Co-transfecting with pL8G5-luc plasmid, the GAL4-*SAMD4B* fusion protein inhibited luciferase activity by approximately two-fold (Fig. 2); while co-transfecting with pLexA-VP16, the GAL4-*SAMD4B* fusion protein inhibited the VP16 activated luciferase activity by approximately six-fold (Fig. 2), suggesting that *SAMD4B* is a potential transcription repressor.

SAMD4B suppresses the transcriptional activities of AP-1, p53 and p21, but has no effect on c-Jun

SAM domain is a putative protein-protein interaction module present in a wide variety of proteins involved in many biological processes (8, 14). To investigate the role of full-length *SAMD4B* and identify the key regulatory motifs in *SAMD4B* protein, Pathway-specific reporter gene assays were performed to measure the effect of *SAMD4B* on different pathways (such as AP-1, c-Jun, p53 and p21) in the HEK-293 cells. We found *SAMD4B* suppressed AP-1, p53 and p21 pathway more obviously than others. As shown in Fig. 3A, using pAP-1-Luc designed for monitoring induction of AP-1, full-length *SAMD4B* suppresses the AP-1-luciferase activity by ~82%. The 5′DOMA, SAM and 3′DOMA motif of *SAMD4B* inhibited the luciferase activity by ~28%, ~71% and ~48%, respectively (Fig. 3A). Using p53-luc, which contains a luciferase gene under the

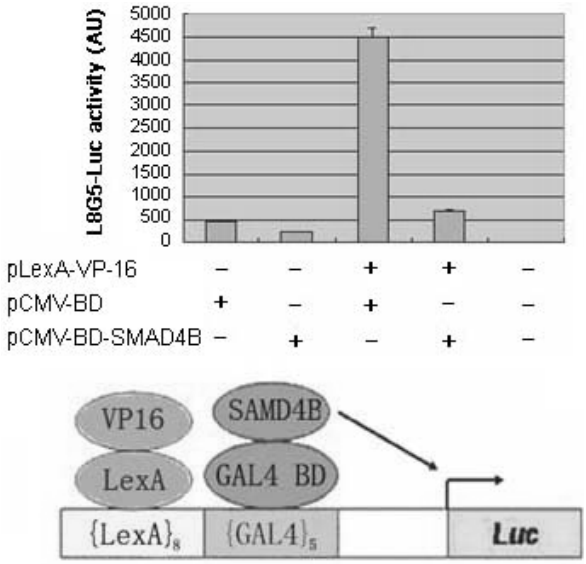

Fig. 2. *SAMD4B* is a transcription repressor. pCMV-BD-*SAMD4B* or pCMV-BD is transiently co-transfected into COS-7 cells along with the pL8G5-Luc reporter and pLexA-VP16 as indicated in the figure. Forty eight hours later, the luciferase activity assay was performed. The data are means of three repeats in a single experiment after normalization for β-galactosidase activity. Each experiment was repeated at least three times.

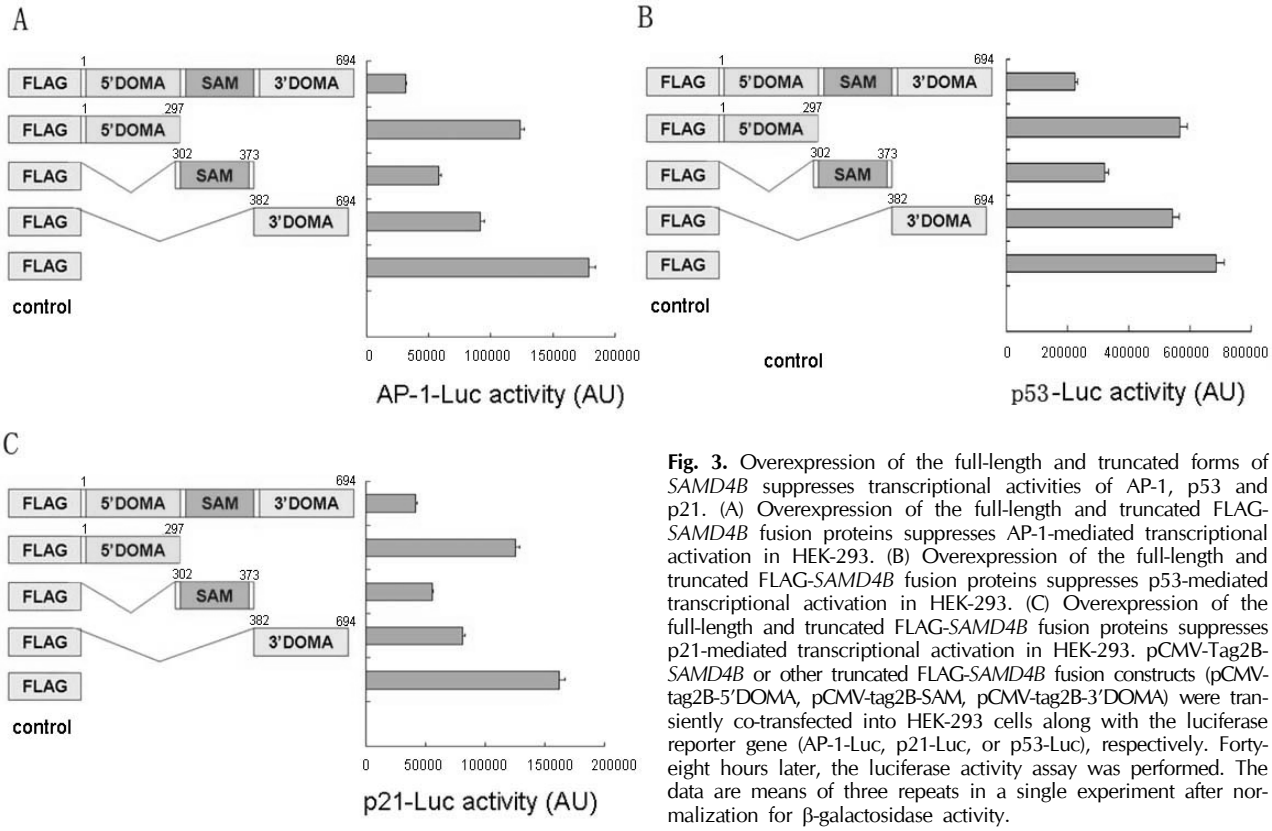

Fig. 3. Overexpression of the full-length and truncated forms of *SAMD4B* suppresses transcriptional activities of AP-1, p53 and p21. (A) Overexpression of the full-length and truncated FLAG-*SAMD4B* fusion proteins suppresses AP-1-mediated transcriptional activation in HEK-293. (B) Overexpression of the full-length and truncated FLAG-*SAMD4B* fusion proteins suppresses p53-mediated transcriptional activation in HEK-293. (C) Overexpression of the full-length and truncated FLAG-*SAMD4B* fusion proteins suppresses p21-mediated transcriptional activation in HEK-293. pCMV-Tag2B-*SAMD4B* or other truncated FLAG-*SAMD4B* fusion constructs (pCMV-tag2B-5′DOMA, pCMV-tag2B-SAM, pCMV-tag2B-3′DOMA) were transiently co-transfected into HEK-293 cells along with the luciferase reporter gene (AP-1-Luc, p21-Luc, or p53-Luc), respectively. Forty-eight hours later, the luciferase activity assay was performed. The data are means of three repeats in a single experiment after normalization for β-galactosidase activity.

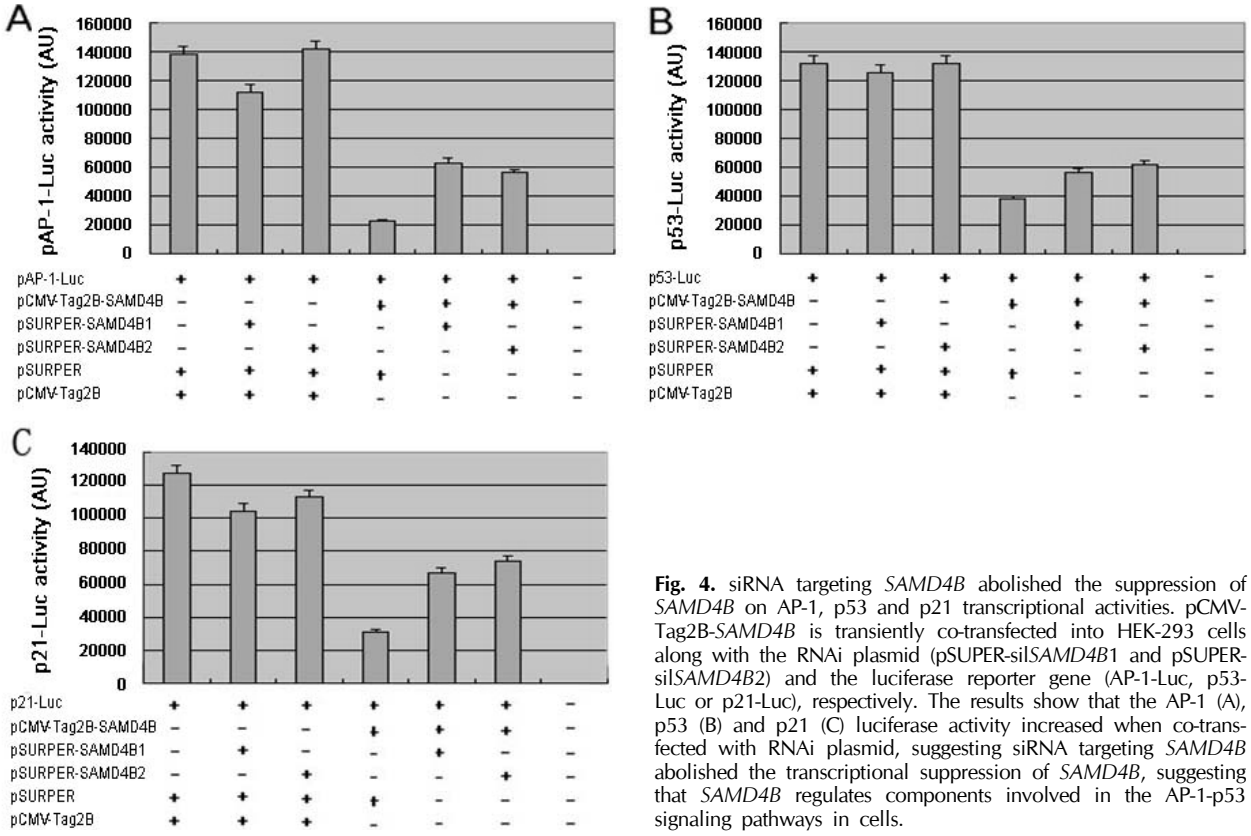

Fig. 4. siRNA targeting *SAMD4B* abolished the suppression of *SAMD4B* on AP-1, p53 and p21 transcriptional activities. pCMV-Tag2B-*SAMD4B* is transiently co-transfected into HEK-293 cells along with the RNAi plasmid (pSUPER-si*SAMD4B*1 and pSUPER-si*SAMD4B*2) and the luciferase reporter gene (AP-1-Luc, p53-Luc or p21-Luc), respectively. The results show that the AP-1 (A), p53 (B) and p21 (C) luciferase activity increased when co-transfected with RNAi plasmid, suggesting siRNA targeting *SAMD4B* abolished the transcriptional suppression of *SAMD4B*, suggesting that *SAMD4B* regulates components involved in the AP-1-p53 signaling pathways in cells.

control of p53 response elements, the full-length and three truncated *SAMD4B* protein inhibited the luciferase activity by ~66%, ~20%, ~54% and ~23%, respectively (Fig. 3B). Furthermore, using p21-luc, which encodes for luciferase controlled by p21, the full-length *SAMD4B* protein inhibited the luciferase activity by ~73%, the three truncated *SAMD4B* protein inhibited the luciferase activity by ~24%, ~67% and ~47%, respectively (Fig. 3C). In addition, overexpressing full-length *SAMD4B* does not affect the c-Jun luciferase activity (Supplement Fig. 2). These results indicate that *SAMD4B* may potentially participates in the transcriptional repression involved in the AP-1, p53 and p21 signaling pathway in cells.

To further demonstrate the role of *SAMD4B* in cell signaling transduction, we used RNA interference to block the translation of *SAMD4B*. HEK-293 cells were transfected with one of two different pSUPER-si*SAMD4B* constructs (pSUPER-si*SAMD4B*1 and pSUPER-si*SAMD4B*2), together with pAP-1-Luc (p53-Luc or p21-Luc) and pCMVTag2B-*SAMD4B*, respectively. Both of the si*SAMD4B* sequences could relieve *SAMD4B*'s repression of AP-1, p53 and p21 transcriptional activities (Fig. 4).

Recently, studies focusing on the SAM domain containing proteins have suggested their extensive involvement in development, gene regulation, diseases and embryogenesis, such as p63 and p73, homologue of the tumour suppressor p53, are positively regulated in c-deficient tumors in response to oncogene over expression, and their expression are increased in several tumor types (11). Both proteins have a SAM domain near their C-terminus, and deletion of the SAM domain of two proteins will work as tumour suppressor as p53, but complete protein of p63 and p73 can repress p53 pathway, so the SAM domain is thought to be responsible for regulating p53-like functions as a repressor (11, 12). Our results indicated that the three domains (5'DOMA, SAM and 3'DOMA) contribute to the transcriptional repression by *SAMD4B*; in contrast, the effect by the SAM domain of *SAMD4B* seems to be more obviously than the other two DOMA domains.

AP-1 is a dimeric transcription factor composed of Jun, Fos or ATF (activating transcription factor) subunits and has been linked to modulation of p53 pathways to explain the role of AP-1 in cell survival. It is thought that AP-1(c-fos) is stimulated by p53 (17); p53 can activate the c-Jun NH2-terminal kinase (JNK) pathway to drive apoptosis (18). We have shown that *SAMD4B* inhibits the transcription of AP-1 but does not affect transcriptional of c-Jun, suggesting that the repression of the AP-1(c-fos) may be indirectly caused through suppressing p53 by *SAMD4B*. It has been reported that p21 is one of the downstream genes of p53 and p53 that strongly induces p21 expression resulting in cell-cycle arrest (6); We examined the

transcriptional effect of *SAMD4B* on p21 to confirm an effect of *SAMD4B* in the p53 pathway and the results suggest that *SAMD4B* suppresses p21 transcriptional activity, so we suppose that the similar repression of the p21 may be caused through suppressing p53 by *SAMD4B*. Our results suggested that *SAMD4B* may function as a negative transcriptional regulator in AP-1-p53 signaling pathways.

CONCLUSION

In summary, we have cloned a novel human SAM domain containing gene, *SAMD4B*. The deduced protein is composed of 694 amino acids. Phylogenetic tree analysis indicates that *SAMD4B* is a highly conserved protein. *SAMD4B* is expressed in multiple human tissues. *SAMD4B* protein is localized evenly in both the nucleus and the cytoplasm and acts as a transcriptional repressor. Overexpression of *SAMD4B* inhibits the transcriptional activities of AP-1, p53 and p21. Studies with truncated *SAMD4B* protein show that the SAM domain of *SAMD4B* is the main region for transcriptional suppression. Our studies suggested that *SAMD4B* might function as a negative transcriptional regulator in AP-1-p53 signaling pathways, which is known to affect cell cycle and apoptosis.

MATERIALS AND METHODS

Construction of cDNA library of human embryo heart

The total RNA from 20-week human embryo heart was extracted using standard methods. Briefly, 5 μg mRNA was purified from 500 μg total RNA using Rapid mRNA purification Kit (AMRESCO). Reverse transcription reactions were performed with the purified embryonic heart mRNA and oligo(dT)-RA primer according to cDNA Synthesis kit protocol. After Cassette Adaptor Ligation reactions using cDNA PCR Library Kit, cDNA amplification reactions were performed with RA primer, CA primer, and TaKaRa Ex Taq (19).

Molecular cloning of *SAMD4B* and bioinformatics analysis

A search of the human EST database with the conserved SAM motifs was performed through a combined BLAST search as previously described (15, 16). The obtained sequence was subjected to human homology searching against expressed sequence tag (EST) database using Blastn (http://www.ncbi.nlm.nih.gov/blast). We searched with the *SAMD4B* consensus sequence and identified five overlapping human expressed sequence tags (ESTs) (CR983898, BF768721, DN996769, CN394606 and CB852248) corresponding to a novel gene *SAMD4B*. The sequence of *SAMD4B* open-reading frame (ORF) was confirmed by PCR amplification with the gene-specific primer pair pORF1/pORF2 (Supplement material Table 1) based on the sequences of two contigs from ESTs, BU855513 and BF970676. The PCR products were cloned into pMD18-T-vector (Takara) and sequenced with 3770 DNA Sequencer (ABI PRISM). Sequence analysis was performed using the DNASTAR program and BLAST program from NCBI. Both the sequence alignment and phylogenetic tree analysis were performed with MegAlign program (DNASTAR).

Northern blot hybridization

The *SAMD4B* cDNA was used as a probe which was labeled with Digoxin (DDLK-010) using a random prime labeling kit (TaKaRa). An adult human Multiple Tissue Northern blot (Yinuojin of China) was hybridized to the labeled *SAMD4B* cDNA probe and β-actin cDNA probe (Clontech). Hybridization was carried out with 5×SSC, 5×Denhardt's, 10% dextran sulfate, and denatured human DNA, at 65°C overnight. After hybridization, the blots were washed three times at 65°C in 2×SSC containing 0.1% SDS for 5 min, twice in 0.1×SSC, and 0.1% SDS at 65°C for 15 min, and then subjected to autoradiography at 80°C. The blots were stripped by incubating for 10 min in 0.1×SSC and 0.5% SDS at 95°C. The membranes were reprobed with radio labeled β-actin cDNA as an indicator of mRNA loading (20, 21).

RNA isolation and RT-PCR

Human tissues from therapeutically aborted fetuses were obtained under the approval of Health Center of Changsha Women and Children Hospital, People's Republic of China, with the consent of the patients and under the regulation of university policy. Total RNA from various tissues (stomach, liver, kidney, spleen, urinary bladder, skin, heart, adrenal gland, intestine, pancreas and brain) was extracted using an RNA isolation kit (Invitrogen, Carlsbad, USA). cDNA was synthesized according to the instructions (Promega, Madison, USA) and was used as a template in the PCR amplification with the primer pair pRT1/pRT2 (Supplement material Table 1) and TaKaRa Ex Taq, GAPDH was amplified with primers Gs and Ga (Supplement material Table 1) as the control.

Plasmid construction

The L8G5-Luc, p53-Luc, p21-Luc and pAP-1-Luc constructs used were generated previously in the lab (20, 23). The *SAMD4B* ORF was amplified by PCR with primers pE1 and pE2 (Supplement material Table 1) and was then subcloned into the *Eco*RI and *Bam*HI sites of pEGFP-N2 vector with the GAG codon instead of the TAG stop codon. To generate a fusion protein of *SAMD4B* with GAL4 or FLAG tag, the *SAMD4B* ORF was amplified by PCR with primers pA1 and pA2 (Supplement Table 2) and then subcloned in-frame into the BamHI and *Eco*RI site of the pCMV-BD or pCMV-Tag2B, respectively. To generate a fusion protein of 5'DOMA (amino acids 1-297), SAM (amino acids 302-373) and 3'DOMA (amino acids 382-694) fragments with FLAG tag, the three fragments were amplified by PCR with primers pA1/pB1, pC1/pC2, pD1/pA2 (Supplement Table 1), respectively, and then subcloned to the BamHI and *Eco*RI sites of the pCMV-Tag2B.

Cell culture and subcellular localization analysis

COS-7 cells and HEK-293 cells used in all studies were maintained and passaged according to standard methods described previously (24). Cells were transfected with pEGFP-N2-*SAMD4B* plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Forty-eight hours after the transfection, Subcellular localization of the fusion protein (EGFP-*SAMD4B*) was detected by fluorescence microscopy (Nikon, E400) after labeling with DAPI for nuclei. The COS-7 cells transfected with pEGFP-N2 vector were used as a control.

Transient expression reporter gene assay

To investigate the effect of *SAMD4B* on transcriptional activity, pCMV-BD-*SAMD4B* was transiently co-transfected into COS-7 cells along with the pL8G5-Luc reporter and pLexA-VP-16 as described above. The luciferase activity assay was performed 48 h later according to the methods described previously (23).

To investigate the effect of *SAMD4B* on the transcriptional activity of the pathway-specific reporter gene (such as AP-1-Luc, p21-Luc, p53-Luc and c-Jun-Luc), pCMV-Tag2B-*SAMD4B* or other truncated FLAG-*SAMD4B* fusion constructs were transiently co-transfected into HEK-293 cells along with the luciferase reporter gene (AP-1-Luc, p21-Luc, p53-Luc or c-Jun-Luc), respectively. Luciferase activity was assayed as described previously, and each assay was repeated at least three times.

RNAi analysis

A pSUPER.retro.puro vector-based system was used to deliver siRNA into HEK-293 cells (25). Pairs of oligonucleotides were designed by the RNAi program (http://www.openbiosystems.com/RNAi) and two nucleotide (nt) sequences specific for *SAMD4B* (si*SAMD4B*-1: CCAGACACAATCTCTATGAGT; si*SAMD4B*-2: GCAGAAGAAACGGCTGCTA) are selected. Each pair of the forward and reverse oligos (si*SAMD4B*-s1/si*SAMD4B*-anti-s1 and si*SAMD4B*-s2/si*SAMD4B*-anti-s2, Supplement material Table 1) was annealed and inserted into the pSUPER.retro.puro vector between the *Bgl*II and *Xho*I enzyme sites, respectively. The Luciferase activity assay for the reporter gene (AP-1-Luc, p21-Luc or p53-Luc), pCMV-Tag2B-*SAMD4B*, and pSUPER si*SAMD4B*, or pSUPER.retro.puro vector was performed according to the protocols described above.

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