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An inhibitory role of NEK6 in TGFβ/Smad signaling pathway

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The NEK6 (NIMA-related kinases 6) is reported to play po-tential roles in tumorigenesis. Although it is suggested to function in several cellular pathways, the underlying mechanism in tumorigenesis is still largely unknown. In the present study, we discovered interaction of NEK6 with Smad4, a key member of transforming growth factor beta (TGFβ) pathway. Over-expression of NEK6 in hepatocellular carcinoma (HCC) cell lines suppresses TGFβ-mediated transcription activity in a kinase activity-dependent manner. In addition, NEK6 suppresses the cell growth arrest induced by TGFβ. Mechanically, NEK6 blocks nuclear translocation of Smad4, which is essential for TGF β function. Moreover, we identified that NEK6 could be regulated by TGFβ and hypoxia. Our study sheds new light on the roles of NEK6 in canonical TGFβ/Smad pathway and tum-origenesis. [BMB Reports 2015; 48(8): 473-478]

INTRODUCTION

Human NIMA-related kinases is a family of 11 members, which are implicated in mitotic control (1). During the mitotic process, NEK6 is phosphorylated and activated by NEK9, forming a mitotic cascade required for normal spindle organization and mitotic progression (2). NEK6 inhibits the premature senescence induced by p53 or anticancer drug, and is also identified as a target of cell cycle checkpoint following DNA damage (3-5).

In cancers, the expression and kinase activity of NEK6 are up-regulated and are strongly associated with the advanced stage in gastric and liver cancers (6, 7). Deregulation of NEK6 expression affects cell growth, apoptosis, transformation and tumorigenesis (8, 9). However, the underlying mechanism is not fully understood.

Canonical TGFβ signaling pathway is activated upon binding of TGFβ family members to type II TGFβ receptor (TβRII). Subsequently, TβRII heterodimerizes and phosphorylates type I receptor (TβRI), which phosphorylates the downstream R-Smads (Smad2 and Smad3). Phosphorylated R-Smads, together with the Co-Smad (Smad4), translocates into nucleus, binds to the "CAGA" consensus sequence, and regulates the transcription of target genes (2). TGFβ can function as both tumor suppressor and promoter. In benign epithelia and many early-stage tumors, TGFβ is a potent inducer of growth arrest. However, TGFβ promotes cell motility, invasion, and metastasis in advanced tumors (3).

In the current study, we identified inhibitory role of NEK6 on TGFβ pathway by interfering with nuclear translocation of Smad4. NEK6 itself is negatively regulated by TGFβ pathway, and is positively regulated by hypoxia. Therefore, our results suggest that deregulated NEK6 facilitate tumorigenesis via inhibition of TGFβ pathway and NEK6 may serve as a novel target for cancer therapy.

RESULTS AND DISCUSSION

NEK6 interacts with Smad4 and suppresses TGFβ-mediated transcription

To study the role of NKE6 in tumorigenesis, we searched the potential NEK6 interacting proteins from BioGRID[beta] database (http://www.thebiogrid.org), a protein-protein interaction networks in mammalian cells (13). We found that several key members in TGFβ pathway, including Smad4, TGFBR1 and Smurf2, were indicated to interact with Nek6 in *Mus Musculus*.

We hypothesized that human NEK6 may function in TGFβ pathway through its interaction with human Smad4, TGFBR1 and Smurf2. To confirm this hypothesis, interaction between NEK6 and these proteins was examined by co-immunoprecipitation assays. We showed that Smad4 was co-precipitated with NEK6 both *in vitro* and *in vivo* (Fig. 1A and B), but not TGFBRI or Smurf2 (data not shown). This interaction was also confirmed by GST pull-down assay (Fig. 1C). Thus, these results suggest interaction of Smad4 with NEK6.

We have considered other members in Smad family, including Smad2 and Smad3 in TGFβ pathway, and Smad1 and Smad5 in BMP pathway, since Smad family members have two conservative Mad homologous, which were also reported to be indispensable in interaction with other proteins. However, we were not able to find additional interaction between these family members with human NEK6.

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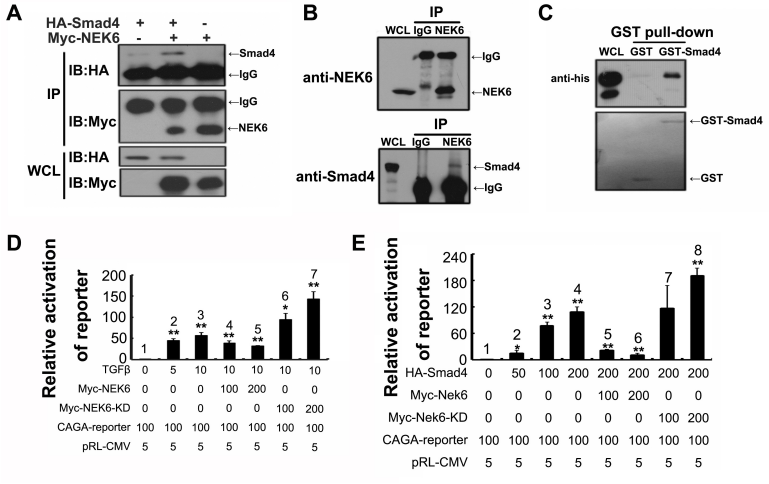

Fig. 1. Interaction of NEK6 with Smad4 and its suppressive effect on TGFβ-mediated reporter activation (A) NEK6 interacts with Smad4 *in vitro*. Cell lysates from Hep3B cells transfected with indicated plasmids were subjected to immunoprecipitation assay with anti-myc mAb. Samples were then analyzed with Western blot with indicated antibodies. (B) NEK6 interacts with Smad4 in vivo. Cell lysates from Hep3B cells were subjected to imunoprecipitation assay with anti-NEK6 antibody and IgG. Samples were then analyzed with Western blot with indicated antibodies. (C) NEK6 interacts with Smad4 directly. Cell lysates from Hep3B cells expressing His-tagged NEK6 were subjected to GST pull-down assay. Precipitated GST-Smad4 and NEK6 were detected by Coomassie brilliant blue G250 staining (lower panel) and immuoblotting with anti-His mAb (upper panel), respectively. (D) NEK6 represses transcription of TGFβ/Smad responsive reporter induced by TGFβ1. (E) NEK6 represses transcription of TGF β/Smad responsive reporter induced by Smad4. Luciferase assay was performed in Hep3B cells as described in Materials and Methods. Results (mean±SD) are shown as fold induction after normalization to non-treated cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. IB: immunoblotting, WCL: whole cell lysate, IP: imunoprecipitation.

Subsequently, we investigated the role of NEK6 in TGFβ pathway by luciferase assay in HCC cell line Hep3B. We found that transcription of $(CAGA)_9$ MLP-Luc, an artificial TGFβ/-Smad-responsive reporter, could be significantly induced by either TGFβ1 stimulation or transfecting Smad4 in a dose-dependent manner. However, in the presence of NEK6, transcription of reporter gene was significantly inhibited (Fig. 1D, E). Interestingly, the kinase dead mutation of NEK6 not only abolished its suppressive role, but also behaved as an activator. The possible reason for such a behavior could be due to its dominant negative role that might have interfered with the endogenous NEK6.

NEK6 inhibits TGFβ-induced cell growth arrest by targeting downstream proliferation-associated genes

Furthermore, we examined the role of NEK6 in regulating the transcription of TGFβ/Smad downstream target genes. As shown in Fig. 2A, the up-regulation of *DAPK1*, *p27*, *p21*, and *Smad7*, and the down-regulation of *c-myc* and cdc25A induced by TGFβ1 were both inhibited by NEK6 in a kinase activity-dependent manner.

Two classes of anti-proliferative genes are known to be induced by TGFβ and account for their cell growth arrest function. The first class is the Cdk-inhibitory responses that include the up-regulation of *p15*, *p21*, and *p27*, and the down-regulation of *cdc*25A. The second class is the down-regulation of c-myc observed in most cell types (4). Since NEK6 inhibited the transcriptional regulation of these genes, we hypothesized that NEK6 may play a suppressive role in TGFβ-induced cell growth arrest. Thus, we established control (C1 and C2) and Z cell line SMMC-7721. Expression of NEK6 was confirmed by Western blotting with the indicated antibodies (Fig. 2B). Interestingly, we were unable to get NEK6 kinase dead stable cell lines, probably due to its indispensable role in cell proliferation (8). Stable cell lines were cultured in medium with or without TGFβ1 (10 ng/ml) for 5 days and the cell growth was detected with cell proliferation assay. As shown in Fig. 2C, control cells were inhibited by 40.23% ± 2.35% and 41.31% ± 4.75 (C1 and C2 respectively) when compared to the non-treated cells,. While in the case of NEK6 overexpressed cells, the inhibition was attenuated by 61.17% ± 5.41% and 63.11% ± 2.75% (N11 and N38 respectively). Cell cycle analysis revealed that over-expression of NEK6 reversed the increase in cell numbers in G1 phage and decrease in cell numbers in S phase induced by TGFβ1 (Fig. 2D).

NEK6 blocks TGFβ-induced Smad4 nuclear translocation

Further research is needed to explore the mechanism accounting for the antagonistic role of NEK6 in TGFβ pathway. One of the possibilities is that NEK6 may interfere with the nuclear translocation of Smad4. As shown in fig. 3A, Smad4 was translocated into nuclear in cell line C1 following TGFβ1 treat-

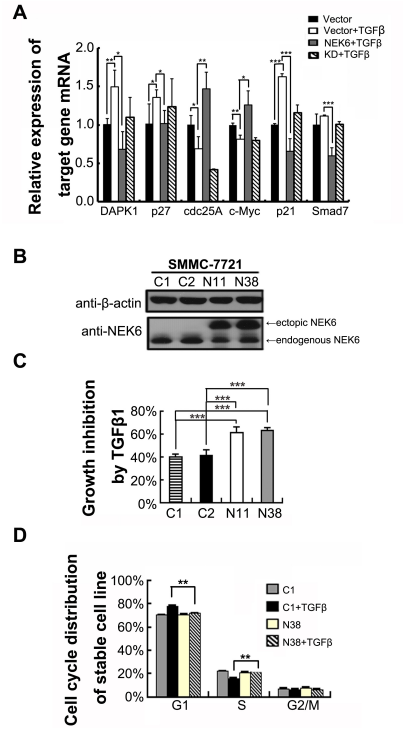

Fig. 2. NEK6 suppresses TGFβ/Smad-mediated cell growth arrest by targeting downstream genes (A) NEK6 inhibits TGFβ1-mediated target gene transcription. Hep3B cells transfected with indicated plasmid were treated with or without 10 ng/ml TGFβ1. Expression of target genes was examined by real time PCR. (B) NEK6 protein expression was analyzed in stable cell lines. (C) NEK6 inhibits growth arrest induced by TGFβ1. Following cultured cells in medium with 10 ng/ml TGFβ1 for 5 days, cell growth of stable cell lines was analyzed by cell proliferation assay. (D) NEK6 suppresses cell growth arrest induced by TGFβ1. Cell cycle analysis was performed as described in Materials and Methods. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

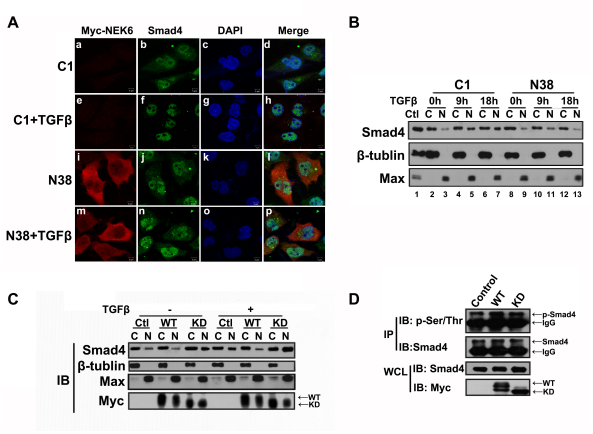

Fig. 3. NEK6 blocks TGFβ-induced nuclear translocation of Smad4(A) NEK6 blocks nuclear translocation of Smad4 induced by TGFβ1. Fluorescent micrographs of ectopic NEK6 (red) and endogenous Smad4 (green) in stable cell line C1 and N38 were taken following indicated treatments. Bar, 5 μm. (B) NEK6 blocks nuclear translocation of Smad4 induced by TGFβ1 in stable cell line. Subcellular distribution of Smad4 in C1 and N38 cells was analyzed by Western blotting with the indicated antibodies. β-tubulin and Max were used as positive control for cytoplasmic and nuclear extracts, respectively. (C) NEK6 blocks nuclear translocation of Smad4 induced by TGFβ1 in a kinase activity dependent manner. Subcellular distribution of Smad4 in control cells and cells expression wild type NEK6 or kinase dead NEK6 was analyzed by Western blotting with the indicated antibodies. (D) NEK6 phosphorylates endogenous Smad4. Control cells and cells expressing wild type NEK6 or kinase dead NEK6 were subjected to immunoprecipitation by anti-Smad4 antibody. Phosphorylation status of Smad4 was determined employing anti-phosphoserine/threonine antibody.

ment, while its translocation was blocked in cell line N38. In addition, we performed the nuclear and cytosolic fractionation assay from TGFβ1 treated C1 and N38 cells. Consistently, nuclear accumulation of Smad4 upon TGFβ1 treatment was attenuated in N38 cell line, when compared with the control C1 cell line (Fig. 3B). In order to determine kinase activity of NEK6 for its role in nuclear translocation of Smad4, we transiently transfected the control vector, vector expressing wild type or kinase dead mutant of NEK6 into Hep3B cells, and performed the cell fractionation assay. As shown in fig. 3C, the kinase dead mutant of NEK6 abolished the nuclear translocation of Smad4 after TGFβ stimulation. It was intriguing to observe that kinase dead mutant of NEK6 had a higher ratio of Smad4 in nuclear to cytoplasm than cells expressing control vector even in non-treated cells. This result displays dominant negative role of kinase dead NEK6, which was consistent with our data from luciferase assay (Fig. 1D and E). These results suggest that NEK6 suppresses TGFβ signaling pathway by blocking nuclear translocation of Smad4 in a kinase activity dependent manner. However, the underlying mechanism is unknown. Since NEK6 is a serine/threonine kinase, we examined whether Smad4 would be phosphorylated at its serine/threonine sites. We transfected Hep3B cells with wild type or kinase dead NEK6 and enriched endogenous Smad4 by immunoprecipitation. Anti-phosphoserine/threonine antibody was used to evaluate the phosphorylation status of Smad4. Interestingly, when wild type NEK6 caused an obvious elevation of Smad4 phosphorylation, kinase dead construct also caused a slightly elevation (Fig. 3D). This could be a result of balance between direct and indirect role of NKE6 kinase activity. As Smad4 has 40 serine sites and 29 threonine sites, NEK6 may phosphorylate only some of them, and kinase dead construct could abolish basal phosphorylation at these sites due to its dominant negative role. Hence, it is possible that other kinases could have led to phosphorylation of remaining sites in Smad4. When these kinase activities are affected by NEK6 kinase, for example inhibition by NEK6, a reduction in phosphorylation level of Smad4 could be observed. On the contrary, kinase dead construct may release inhibition of such kinases and then promote

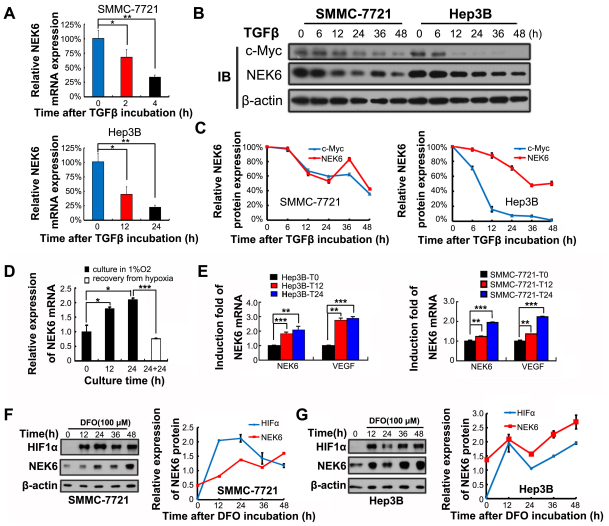

Fig. 4. Expression of NEK6 is regulated by TGFβ stimulation and hypoxia (A) NEK6 transcription is down-regulated by TGFβ1 treatment. NEK6 transcription in SMMC-7721 cells (upper panel) and Hep3B cells (lower panel) with the indicated treatment was analyzed with real time PCR. Data were normalized to non-treated control (0 h). (B) NEK6 protein expression is regulated by TGF β 1 treatment. NEK6 protein expression in SMMC-7721 cells and Hep3B cells with indicated treatment was analyzed by Western blotting. Expression of c-myc protein was used as a positive control of TGFβ1 treatment. Data were quantified in (C). (D) Transcription of NEK6 is induced by hypoxia. Transcription of NEK6 in Hep3B cells with indicated hypoxia and normoxia treatments was examined by real-time PCR. (E) Transcription of NEK6 is induced by DFO. Transcription of NEK6 in SMMC-7721 cells (right panel) and Hep3B (left panel) with indicated treatments was examined by real-time PCR. Transcription of VEGF was used as a positive marker for successful DFO treatment. Protein expression of NEK6 in SMMC-7721 (F) cells and Hep3B cells (G) following indicated treatments was analyzed by Western blotting. The expression of HIFα was used as a positive marker for successful DFO treatment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

further phosphorylation of Smad4. Further studies as well as prediction based on database are needed to confirm this hypothesis. Also, these results could not rule out the notation that NEK6 may activate other partners in NEK6-Smad4 complex, which results in retention of SMAD4 in cytoplasm.

NEK6 is down-regulated by TGFβ and up-regulated by hypoxia

Based on the potential function of NEK6 in regulating the TGFβ pathway in cancers, we further investigated the factors that may regulate the expression of NEK6. We retrieved 2500bp of NEK6 promoter (Chr9 127018040-127020539) and searched for transcription factor binding sites through TFD database (http://www.ifti.org/). We found one potential TGFβ responsive binding site (from -1754 to -1762bp), which contained the characteristic CAGA box (16). As shown in Fig. 4A-C, both mRNA and protein of NEK6 were down-regulated in SMMC-7721 and Hep3B cells upon TGFβ1 treatment. Protein expression of c-myc was used as a positive control of TGFβ1 treatment. These data suggest a mutual regulation between TGFβ pathway and NEK6, which is similar to Smad7, an antagonist and target gene of TGFβ/Smad pathway (17).

In addition to the TGFβ responsive binding site, we found two potential hypoxia inducible factor (HIFα) binding sites, named as hypoxia responsive elements (HRE). As shown in Fig. 4D, NEK6 transcription was induced significantly by hypoxia (1% O2), and was reduced to normal after normoxia culture (21% O2) for additional 24 hours. In addition, we treated Hep3B and SMMC-7721 cells with desferrioxamine (DFO), which mimicked hypoxia induction of HIF α [19]. Both NEK6 mRNA and protein were significantly up-regulated following the treatment. Induction of HIF α and VEGF were used as positive controls of hypoxia treatment (Fig. 4E-G). As a common feature of tumor microenvironments, hypoxia promotes malignant progression or transformation of tumors. Even when the solid tumors are in their early stages, they would contain acute and chronic hypoxia (5). In these stages, NEK6 could be up-regulated by hypoxia, attenuate cell growth arrest induced by TGFβ through regulation of related target genes, and create a favorable growth condition for tumor cells. Suppressed TGFβ signaling further enhances the expression of NEK6, which will strengthen its tumor-promoting role. We found that NEK6 was significantly up-regulated in HCC tumors with portal vein tumor thrombus and HCC cell lines with strong metastasis capability (Data not shown). Consequently, it is proposed that NEK6 could be a potential target for cancer therapy in the early stages of tumor development. Function of NEK6 in the advanced stages of tumors needs further investigation, since at this stage; TGFβ promotes cell motility, invasion, and metastasis and acts as a tumor promoter.

MATERIALS AND METHODS

Cell cultures

Hep3B and SMMC-7721 cells were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (PAA) at 37°C in a humidified incubator containing 5% CO_2.

Immunoprecipitaion assay

Hep3B cells were transfected with indicated plasmids using Lipofectamine (Invitrogen) according to the manufacturer's protocols. Forty-eight hours later, 10^6 cells were lysed with cold lysis buffer [5 mM EDTA, 0.5% NP-40, 0.1 mM phenylmethane sulphonyl-flouride (PMSF), 10 μM pepstatin A, 10 μM leupeptin and 25 μg/ml aprotinin]. Cell lysates were then collected and pre-cleared by 20 μl protein G Plus/protein A agarose beads (Amersham) at 4°C for 1 hour with rotation. Then, pre-cleared cell lysates were incubated with 40 μl protein G Plus/protein A agarose beads and 1 μg anti-myc mono-clonal antibody (mAb)

at 4°C for 6 hours with rotation. Agarose beads were collected and washed five times with lysis buffer, and then the samples were subjected to SDS-PAGE and Western blot assay. For in vivo immunoprecipitation, 5 μg anti-NEK6 mAb (Sigma) and mouse IgG (Sigma) were used. Whole cell lystes and precipitated complex were immunoblotted by anti-NEK6 mAb and anti-Smad4 antibody (Epitomics).

GST pull-down assay

Glutathione S-transferase (GST) fused Smad4 protein was kindly provided by Dr Jian An (Fudan University, China). Cell lysates from Hep3B cells expressing His-tagged NEK6 were incubated with 10 μg GST-Smad4 or 25 μg GST proteins, together with 40 μl glutathione-S-Sepharose beads (Amersham) in lysis buffer at 4°C for 6 hours. Sepharose beads were then collected and washed with lysis buffer for three times. Samples were then analyzed by SDS-PAGE and Western blot and precipitated GST-protein was determined by Coomassie brilliant blue G250 staining.

Luciferase assay

Hep3B cells were transfected with plasmid combination including $(CAGA)_9$ -MLP-Luc. Cells were lysed 36 hours following transfection, and luciferase activities were determined by using the Dual Luciferase reporter system (Promega) on a Lumat LB 9507 luminometer (Berthold). Values were normalized with the renilla luciferase activity expressed from pRL-CMV. In TGFβ1 stimulation assay, cells were serum starved for 8 hours, then treated with TGFβ1 (5 ng/ml or 10 ng/ml) for additional 5 hours before harvest.

Plasmids and cytokines

Preparation of pCMV-Myc-NEK6 and pcDNA3.1-NEK6 has been described previously (19). The kinase dead mutant NEK6-K74MK75M was generated by point mutagenesis. Human Smad4 plasmid and $(CAGA)_9$ MLP-luciferase reporter plasmid were kindly gifted by Dr Jian An (Fudan University, China). Human recombinant TGFβ1 was purchased from Peprotech.

Western blot

Protein samples separated by SDS-PAGE were electro-transferred onto nitrocellulose membranes (Amersham). Then membranes were blocked with 5% non-fat milk blocking solution and incubated with specific primary antibody at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-labeled secondary antibody (Santa Cruz). Signals were visualized by enhanced chemiluminescence reagents (Santa Cruz). Anti-β-actin, anti-myc, anti-HA, anti-his, anti-β-tubulin, anti-HIFα, anti-c-myc, and anti-NEK6 antibodies were purchased from Sigma. Anti-Smad4 and anti-Max antibodies were purchased from Epitomics. Anti-phosphoserine/threonine antibody was purchased from Abcam.

Quantitative real-time PCR

Total RNA was extracted from cells with Trizol reagent (Invitrogen) and the first-strand cDNA was synthesized using a reverse transcription kit (Invitrogen) following the manufacturer's instructions. Real-time PCR was performed by using SYBR Green PCR master mix (TOYOBO) on a Light Cycler 480II detection System (Roche). Gene expression was normalized to that of the housekeeping gene β2-microglobulin (β2-MG). Primers used are listed as below. NEK6-F: 5′-TTCCAACAACCTCTGCCACACC-3′, NEK6-R: 5′-CACAGTCCTGCCTCGCCTTG-3′, β2-MG-F: 5′-ATGAGTATGCCTGCCGTGTGAAC-3′, β2-MG-R: 5′-TGTGGAGCAACCTGCTCAGATAC-3′, VEGF-F: 5′--TGCACCCATGGCAGAAGGAG-3′, VEGF-R: 5′-TGTGCTGGCCTTGGTGAGGT-3′, DAPK1-F: 5′-AATGGAGTTGGCGATTTCAGCGTG-3′, DAPK1-R: 5′-AAGGGACTTCAGGAAACTGAGCCA-3′, p27-F: 5′-AACCGACGATTCTTCTACTC-3′, p27-R: 5′-TGTATATCTTCCTTGCTTCATC-3′, cdc25A-F: 5′-AGGGTTATCTCTTTCATACAGTTGC-3′, cdc25A-R: 5′-ACACGCTTGCCATCAGTAGG-3′, c-myc-F: 5′-AGCGACTCTGAGGAGGAACA-3′, c-myc-R: 5′-ACTCTGACCTTTTGCCAGGA -3′, p21-F: 5′-GGACAGCAGAGGAAGACC-3′, p21-R: 5′-CTAAGGCAGAAGATGTAGAGC-3′, Smad7-F: 5′-CCTTAGCCGACTCTGCGAACTA-3′, Smad7-R: 5′-CCAGATAATTCGTTCCCCCTGT-3′.

Selection of stable cell lines

SMMC-7721 cells were transfected with pcDNA3.1-NEK6 or control vector. Twenty-four hours later, cells were collected and seeded into 100-mm dishes. Subsequently, cells were selected in the presence of 800 μg/ml G418 for two weeks. Cell colonies were isolated and subjected to Western blot for NEK6 expression.

Cell Proliferation assay

Cells were seeded in 96-well plates at the density of 1.0×10^3 cells per well; and cultured in medium with or without 10 ng/ml TGFβ1. Fresh medium was changed every 2 days. Five days later, cells were subjected to 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H -tetrazolium (MTS) assay (Promega) according to the manufacturer's instructions. Spectrophotometric absorbance at 490 nm was read by a microtiter reader (HITACHI).

Flow cytometry analysis

Cells were harvested, washed with ice-cold phosphate-buffered saline (PBS) and then incubated with staining buffer (PBS with 0.03% TritonX-100, 50 μg/ml propidium iodide, 100 μg/ml RNase) for 10 minutes at room temperature. DNA content of cells was analyzed with FACS Calibur (BD Biosciences). Cell cycle distribution was analyzed with ModFit.

Immunofluorescence microscopy

Cells were serum starved for 8 hours and then treated with normal medium with 10 ng/ml TGFβ1 for additional 5 hours. Then cells were fixed with 4% paraformaldehyde for 10 mi-

nutes and permeabilized with 0.2% Triton X-100 for 5 minutes at room temperature. Samples were washed thrice with TBS (50 mM Tris-HCl pH7.4, 150 mM NaCl) and blocked with blocking buffer (PBS with 1% horse serum, 1% BSA, 0.02% NaN3) for 1 hour. Next, cells were incubated with the combination of 1:200 (v/v) diluted anti-myc mAb and anti-Smad4 poly-clonal antibody at 4°C overnight. Cells were then washed three times with TBS and incubated with 1:5000 (v/v) diluted Cy3-conjugated rat anti-mouse IgG and FITC-conjugated goat anti-Rabbit IgG for 45 minutes. Cells were counterstained with DAPI for 20 minutes and mounted on slides for confocal microscopy analysis by LSM700 laser scanning microscope (Carl Zeiss).

Preparation of cytoplasmic and nuclear extracts

Ten million cells were suspended in 0.5 ml ice-cold lysis buffer A (20 mM HEPES, pH 7.9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM DTT, 8 ng/ml aprotinin, 2 ng/ml leupeptin, and 1 mM PMSF) for 10 minutes. Then, cytoplasmic fraction was collected by centrifugation at 6500 rpm at 4°C for 10 minutes, and the nuclear pellet was washed three times with lysis buffer A and re-suspended in 0.3 ml ice-cold buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 1 mM DTT, 8 ng/ml aprotinin, 2 ng/ml leupeptin, and 1 mM PMSF) for 30 minutes. Subsequently, the samples were centrifuged at 12,000 rpm at 4°C for 30 minutes, and the supernatant was collected as nuclear extract.

Statistical analysis

Statistical significance was examined with two-tailed Students t-test. Results with P values less than 0.05 were considered significant.

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