An inhibitory role of NEK6 in TGFβ/Smad signaling pathway

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INTRODUCTION

The NEK6 (NIMA-related kinases 6) is reported to play potential 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 TGFβ 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 tumorigenesis. [BMB Reports 2015; 48(8): 473-478]

RESULTS AND DISCUSSION

NEK6 interacts with Smad4 and suppresses TGFβ-mediated transcription

To study the role of NEK6 in tumorigenesis, we searched the potential NEK6 interacting proteins from BioGRID 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 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.
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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 immunoprecipitation 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 immunoblotting with anti-His mAb (upper panel), respectively. (D) NEK6 represses transcription of TGFβ-Smad responsive reporter induced by TGFβ1. (E) NEK6 represses transcription of TGFβ/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: immunoprecipitation.

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 cdc25A. 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% (N1 and N38 respectively). Cell cycle analysis revealed that over-expression of NEK6 reversed the increase in cell numbers in G1 phase 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-
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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 suppresses cell 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 expression in stable cell lines. 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 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.

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 NEK6 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...
**NEK6 enhances the expression of Smad7, an antagonist of the TGFβ/Smad signaling pathway.**

**Fig. 4.** Expression of NEK6 is regulated by TGFβ and hypoxia. (A) NEK6 transcription is down-regulated by TGFβ treatment. NEK6 transcription in SMMC-7721 cells (upper panel) and Hep3B cells (lower panel) with the indicated treatments was analyzed with real-time PCR. Data were normalized to non-treated control (0 h). (B) NEK6 protein expression is regulated by TGFβ treatment. NEK6 protein expression in SMMC-7721 cells and Hep3B cells with indicated treatments was analyzed by Western blotting. Expression of c-myc protein was used as a positive control of TGFβ treatment. Data were quantified in (C). (D) NEK6 transcription is induced by hypoxia. Transcription of NEK6 in Hep3B cells with indicated hypoxia and normoxia treatments was examined by real-time PCR. (E) NEK6 transcription in SMMC-7721 cells (left panel) and Hep3B (right 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 c-myc 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.

**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 TFDB 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 TGFβ 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 HIFα [19]. Both NEK6 mRNA and protein were significantly up-regulated following the treatment. Induction of HIFα 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% CO2.

**Immunoprecipitation assay**

Hep3B cells were transfected with indicated plasmids using Lipofectamine (Invitrogen) according to the manufacturer’s protocols. Forty-eight hours later, 106 cells were lysed using 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 lysates 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-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-luciferase reporter plasmid and (CAGA)₉ -MLP-Luc. Cells were lysed 36 hours following transfection, and luciferase activities were determined by the Dual Luciferase reporter system (Promega) on a Lumat LB 9507 luminometer (Berthold). Values were normalized to the renilla luciferase activity expressed from pRL-SV40 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.

**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'-TTCCAACAA-CCTCTGCCACACC-3', NEK6-R: 5'-CAGACTCTGGCTCCTGCGC-TTG-3', β2-MG-F: 5'-ATGAGTATGCTCTGCCGTTGAG-3', β2-MG-R: 5'-TGTGGAGCAACCTGGCTCAGATAC-3', VEGF-F: 5'-TGACCCCATGGCAAGAAGGAG-3', VEGF-R: 5'-GGTCTGGCCTTGTGAGGATGG-3', DAPK1-F: 5'-AATGGAGTTGGCGATTTCACCCTG-3', DAPK1-R: 5'-AAGGGACTTCAGGAAACTGAGCCA-3', p27-F: 5'-AACCGACGATTCTTCTACTC-3', p27-R: 5'-TGAATATCTCTTTGCTICATC-3', cdc25A-F: 5'-AGGGTTATCGTCTTTCATACAGTTGC-3', cdc25A-R: 5'-ACAGCTTGTCGATCATGAGC-3', c-myc-F: 5'-ACGACTCTGAGGAAGGAACGAGAGCCA-3', c-myc-R: 5'-ACTCTGACCTTTGCGGAGGAAAC-3', c-myc-F: 5'-ACGACTCTGAGGAAGGAACGAGAGCCA-3', c-myc-R: 5'-ACGACTCTGAGGAAGGAACGAGAGCCA-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 1.0×10⁴ 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-2-yl)-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 by FACS Calibur (BD Biosciences). Cell cycle distribution was analyzed with ModFit.

**Immunofluorescence microscopy**

Cells were serum starved for 8 hours and then treated with TGFβ1 (5 ng/ml or 10 ng/ml) for additional 5 hours before harvest. Immunofluorescence microscopy was performed as 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.
rutes and permeabilized with 0.2% Triton X-100 for 5 minutes at room temperature. Samples were washed thrice with TBS (50 mM Tris-HCl pH 7.4, 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 polyclonal 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 MgCl2, 0.05% Nonidet P-40, 1 mM DTT, 8 ng/ml aprotinin, 2 ng/ml leupeptin, and 1 mM PMSF) for 10 minutes. Then, cytoplasmatic 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 MgCl2, 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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REFERENCES
1. O’Connell MJ, Krien MJ and Hunter T (2003) Never say never. The NIMA-related protein kinases in mitotic control. Trends Cell Biol 13, 221-228
2. Belham C, Roig J, Caldwell JA et al (2003) A mitotic cascade of NIMA family kinases. Niecc1/Nek9 activates the Nek6 and Nek7 kinases. J Biol Chem 278, 34897-34909
3. Lee MY, Kim HJ, Kim MA et al (2008) Nek6 is involved in G2/M phase cell cycle arrest through DNA damage-induced phosphorylation. Cell Cycle 7, 2705-2709
4. Jee HJ, Kim AJ, Song N et al (2010) Nek6 overexpression antagonizes p53-induced senescence in human cancer cells. Cell Cycle 9, 4703-4710
5. Jee HJ, Kim HJ, Kim AJ, Song N, Kim M and Yun J (2011) Nek6 suppresses the premature senescence of human cancer cells induced by camptothecin and doxorubicin treatment. Biochem Biophys Res Commun 408, 669-673
6. Takeno A, Takemasa I, Doki Y et al (2008) Integrative approach for differentially overexpressed genes in gastric cancer by combining large-scale gene expression profiling and network analysis. Br J Cancer 99, 1307-1315
7. Cao X, Xia Y, Yang J et al (2012) Clinical and biological significance of never in mitosis gene A-related kinase 6 (NEK6) expression in hepatic cell cancer. Pathol Oncol Res 18, 201-207
8. Nassirpour R, Shao L, Flanagan P et al (2010) Nek6 mediates human cancer cell transformation and is a potential cancer therapeutic target. Mol Cancer Res 8, 717-728
9. Jeon YJ, Lee KY and Cho YY (2010) Role of Nek6 in tumor promoter-induced transformation in JB6 C141 mouse skin epithelial cells. J Biol Chem 285, 28126-28133
10. Massagué J (1996) TGFbeta signaling: receptors, transducers, and Mad proteins. Cell 85, 947-950
11. Inman GJ (2011) Switching TGFbeta from a tumor suppressor to a tumor promoter. Curr Opin Genet Dev 21, 93-99
12. O’Regan L and Fry AM (2009) The Nek6 and Nek7 protein kinases are required for robust mitotic spindle formation and cytokinesis. Mol Cell Biol 29, 3975-3990
13. Barrios-Rodiles M, Brown KR, Oztacar B et al (2005) High-throughput mapping of a dynamic signaling network in mammalian cells. Science 307, 1621-1625
14. Dennyler S, Itoh S, Vivien D, ten Dijke P, Huet S and Gauthier JM (1998) Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor type 1 gene. EMBO J 17, 3091-3100
15. Lee KY and Bae SC (2002) TGF-beta-dependent cell growth arrest and apoptosis. J Biochem Mol Biol 35, 47-53
16. Zhang W, Ou J, Inagaki Y, Greenwel P and Ramirez F (2000) Synergistic cooperation between Sp1 and Smad3/Smad4 mediates transforming growth factor beta1 stimulation of alpha 2(I)-collagen (COL1A2) transcription. J Biol Chem 275, 39237-39245
17. Nakao A, Afarkhte M, Morén A et al (1997) Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. Nature 389, 631-635
18. Höckel M and Vaupel P (2001) Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 93, 266-276
19. Chen J, Li L, Zhang Y et al (2006) Interaction of Pin1 with Nek6 and characterization of their expression correlation in Chinese hepatocellular carcinoma patients. Biochem Biophys Res Commun 341, 1059-1065