NEK9-dependent proliferation of cancer cells lacking functional p53

Daisuke Kurioka1, Fumitaka Takeshita2, Koji Tsuta3, Hiromi Sakamoto3, Shun-ichi Watanabe6, Kenji Matsumoto7, Masatoshi Watanabe8, Hitoshi Nakagama4, Takahiro Ochiya2, Jun Yokota1,9, Takashi Kohno1 & Naoto Tsuchiya1

1Division of Genome Biology, National Cancer Center Research Institute, 2Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 3Division of Genetics, National Cancer Center Research Institute, 4Division of Cancer Development System, National Cancer Center Research Institute, 5Division of Pathology and Clinical Laboratories, National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo-ku Tokyo 104-0045, 6Division of Thoracic Surgery, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku Tokyo 104-0045, 7Division of Allergy & Immunology, National Research Institute for Child Health and Development, 2-10-1 Okura Setagaya-ku Tokyo, 157-8535, 8Laboratory for Medical Engineering, Division of Materials and Chemical Engineering, Graduate School of Engineering, Yokohama National University, 79-1 Tokiwadai, Hodogaya-ku Yokohama 240-8501, Japan, 9Institute of Predictive and Personalized Medicine of Cancer (IMPPC), Barcelona, Spain.

Dysfunction of the p53 network is a major cause of cancer development, and selective elimination of p53-inactivated cancer cells therefore represents an ideal therapeutic strategy. In this study, we performed a microRNA target screen that identified NEK9 (NIMA-related kinase 9) as a crucial regulator of cell-cycle progression in p53-inactivated cancer cells. NEK9 depletion selectively inhibited proliferation in p53-deficient cancer cells both in vitro and in vivo. The resultant cell-cycle arrest occurred predominantly in G1 phase, and exhibited senescence-like features. Furthermore, NEK9 repression affected expression of a broad range of genes encoding cell-cycle regulators and factors involved in mRNA processing, suggesting a novel role for NEK9 in p53-deficient cells. Lung adenocarcinoma patients with positive staining for NEK9 and mutant p53 proteins exhibited significantly poorer prognoses, suggesting that expression of both proteins promotes tumor growth. Our findings demonstrate that a novel NEK9 network regulates the growth of cancer cells lacking functional p53.

The p53 tumor-suppressor pathway is the most important cellular network involved in preventing transformation of normal cells following exposure to various oncogenic insults. In response to oncogenic stress, p53 activation leads to cell-cycle arrest, allowing for repair of damage and survival, or apoptosis, allowing for elimination of damaged cells, through stimulus-dependent transactivation of its target genes. Cancer genome sequencing studies have shown that TP53 is one of the genes most frequently mutated in human cancers. Thus, dysfunction of the p53 signaling pathway(s) is a major cause of tumor onset and/or progression in almost all human cancers. Furthermore, the molecular network(s) specifically activated in p53-deficient contexts may promote proliferation of cancer cells. Several lines of evidence strongly suggest that TP53 mutations contribute to maintenance of the malignant gain-of-function phenotypes of cancer cells, including cell-cycle progression and activation of cell migration, as well as loss of wild-type tumor-suppressor functions. In light of this novel concept, mutant p53 is an attractive target for therapeutics directed against a wide range of cancers.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level. Dysfunction of miRNAs is deeply involved in cancer development. Many miRNAs are oncogenic and/or tumor-suppressive factors; in particular, several miRNAs serve as intrinsic mediators that coordinate the p53 tumor-suppressor network in response to oncogenic stresses. Each miRNA represses expression of a distinct set of target genes, determined in part by cellular characteristics influenced by p53 mutation status; consequently, these miRNAs induce different phenotypes in different cancer cells. Thus, by exploiting their ability to affect the regulation of specific oncogenic or tumor-suppressive networks, individual miRNAs can be used as screening tools to identify therapeutic molecular targets in cancer cells.

Previously, we showed that miR-22, which acts on the p53 network, induces cell death in cancer cells with wild-type p53. On the other hand, it induces cell-cycle arrest in p53-mutant cancer cells that express CDK6, CDK3, SIRT1, and HDAC4, which are critical factors involved in cell-cycle progression and potential targets of miR-22. This finding led us to hypothesize that miR-22 has a unique set of target genes that determine the fate of cancer.
quantitated using TaqMan qRT-PCR assays. Red letters indicate potential in silico genes by Table 1). Finally, we searched for MUT and KO cell lines than in WT cells (Fig. 1A, Supplementary were down-regulated by miR-22. Comparison of these five miRNAs identified by miRWalk analysis. A 70 kDa protein that stained with amido black was used as a loading control. The original full scan images are available in Supplementary Figure 7. (D) Cell proliferation assay. Cells were counted 72 h after transfection with specified siRNAs, indicated below the graph. Two siRNAs, designated 1 and 2, were used to knock down each gene. Error bars indicate standard deviation (SD, n = 3).

Results
Identification of NEK9 by miRNA target screen. To identify targets selectively repressed in p53 mutant (MUT) and knockout (KO) cells, we compared changes in mRNA levels resulting from miR-22 expression between cancer cell lines with p53 KO, MUT, or wild-type (WT). As depicted in Supplementary Figure 1, we first chose the genes down-regulated in both p53 MUT and KO cells by miR-22, and compared their expression levels in p53 WT cells, and then selected genes exhibiting a differential response to miR-22. Furthermore, we narrowed-down the candidate genes classified as encoding cell-cycle regulators in the Gene Ontology database. The resultant 14 genes were down-regulated by miR-22 expression to greater extents in p53 KO cells expressing mutant p53, but not in those expressing WT p53, and expression of the NEK9 open reading frame (ORF) in NEK9-knockdown (KD) cells restored cell proliferation. Furthermore, NEK9 KD in cancer cells induced senescence-like changes ascribed to prolonged G1 arrest or slowdown of cell-cycle progression. In addition, human lung adenocarcinoma patients with expression of both NEK9 and mutant p53 proteins exhibited significantly poorer prognoses, suggesting that NEK9 expression confers growth advantages on p53 mutant tumors. Taken together, our findings strongly suggest that the NEK9 network, which is selectively activated in p53-deficient contexts, is a crucial cellular pathway responsible for the growth capacity of cancer cells lacking functional p53.

NEK9 expression is required for proliferation of p53-inactivated cancer cells. To confirm that NEK9 depletion specifically suppresses growth of p53-mutant cells, we knocked down this protein in a set of cancer cell lines with different p53 mutation status (Fig. 2A, Supplementary Figure 3A). NEK9 KD significantly suppressed the growth of three cancer cell lines with p53 mutations, but did not affect the growth of three cancer cell lines with WT p53. In four subsequent experiments, we verified that the effect of NEK9 KD depended on p53 status. First, we stably knocked down p53 in HCT116 cells, which normally express WT p53, via expression of a specific shRNA. NEK9 KD significantly decreased growth in p53 KD cells, but not in cells expressing a control shRNA (Fig. 2B, Supplementary Figure 3B). Second, we established H1299 cell lines (a p53-null lung cancer cell line) that exogenously expressed either WT p53 or one of four p53 mutants (Supplementary Figure 3C). Three of the mutants, R175H, R273H, and R249S, are frequently observed in a variety of human cancers (IARC p53 database, http://p53.iarc.fr/); the fourth, R280K, is less common. In H1299 cells expressing mutant p53, but not in those expressing WT p53, siRNA-mediated NEK9 KD significantly suppressed growth (Fig. 2C).

Quantitative RT-PCR analysis confirmed that these five genes were significantly down-regulated by miR-22 in both p53 KO and WT cells, relative to WT cells (Fig. 1B). MiR-22 expression significantly decreased the level of NEK9 and SHC1 proteins in both p53 KO and MUT cells, but did not affect the levels in WT cells (Fig. 1C). Furthermore, siRNA-mediated knockdown (KD) of the five predicted miR-22 targets revealed that only NEK9 depletion significantly suppressed proliferation in MUT cells; however, NEK9 KD did not affect proliferation in WT cells (Fig. 1D). Using a reporter-gene assay, we verified that NEK9 mRNA is a direct target of miR-22 (Supplementary Figure 2). Based on these results, we concluded that NEK9 is an miR-22 target gene whose repression specifically suppresses growth of p53-mutant cancer cells.
and anchorage-independent growth of MUT cells (Fig. 2E). Third, we asked whether NEK9 protein expression would restore cell proliferation in p53-mutant cells. Endogenous NEK9 was knocked down by siRNA targeting the 3′ UTR of NEK9 mRNA, followed by expression of a cDNA encoding an siRNA-resistant NEK9 ORF (Fig. 2F), and then the cells were counted. As shown in Fig. 2G, NEK9 re-expression restored cell growth after KD of endogenous NEK9. Expression of an amino-acid-substituted form of NEK9, T210A, in which an autophosphorylation site required for kinase activity of NEK9 is altered, also complemented the inhibition of cell proliferation after NEK9 KD, suggesting the possibility that kinase activity is not required for the growth-promoting function (Fig. 2F and G). Fourth, we evaluated the effect of a NEK9 siRNA/drug-delivery system (DDS) carrier complex on cell proliferation in p53-mutant xenograft tumors consisting of SW480 cells, derived from a p53-mutant human colon cancer, in mice. As indicated in Fig. 3A, the siRNA/DDS carrier complex was administered into transplanted tumors five times at 2-day intervals, and tumor size was measured at every 2 days. In these animals, NEK9 KD markedly inhibited in vivo growth of p53 mutant cancer cells, but not of p53 WT cells (Fig. 3B, C, and D), providing further evidence that NEK9 contributes to cell proliferation in p53-mutant cancer cells. Taken together, these results clearly indicate that p53 deficiency makes cells dependent on NEK9 for proliferation, both in vitro and in vivo.

Inhibition of G1-S progression and induction of senescence-like features by NEK9 KD. Because NEK9 regulates proper positioning of bipolar spindles, we hypothesized that NEK9 KD prevents G2/M progression. In flow-cytometry analysis, however, NEK9 KD had
no significant effect on the fraction of cells in G2/M phase; rather, in the two p53-mutant cell lines SW480 and PANC1, NEK9 KD significantly increased the G1-phase population while significantly decreasing the S-phase population (Fig. 4A). We obtained similar results in H1299 transfectants expressing p53 mutants, as well as in p53 KD HCT116 cells (Supplementary Figure 4). These data indicated that NEK9 is necessary for the G1–S transition in p53-deficient cancer cells.

The growth arrest resulting from NEK9 KD was associated with flattened and enlarged cellular morphologies typical of cellular senescence. Consistent with this, the cells stained positively for senescence-associated β-galactosidase (SA-β-gal) (Fig. 4B). Flow-
cytometry analysis confirmed that SA-β-gal staining increased upon NEK9 depletion to a much greater extent in p53-mutant cells than in p53 WT cells (Fig. 4C). These results strongly suggest that depletion of NEK9 causes senescence-like phenotypic changes in p53-deficient cancer cells, because such cells require NEK9 function to progress from G1 to S phase.

Induction of broad changes in the gene expression profile upon NEK9 KD. To further elucidate the molecular connection between NEK9 depletion and cell-cycle arrest/senescence, we analyzed changes in mRNA expression profiles following NEK9 KD in p53-MUT cells. NEK9 KD caused broad changes in gene expression in p53-deficient cells (Fig. 4D): about 25% of genes up-regulated by NEK9 KD were expressed at significantly lower levels in p53 MUT and KO than in WT, suggesting that these genes were down-regulated due to loss of functional p53 (Fig. 4E). On the other hand, about 20% of genes down-regulated by NEK9 KD were expressed at significantly higher levels in MUT and KO than in WT, suggesting that their expression was increased by loss of functional p53 (Fig. 4E). Thus, NEK9 appears to be involved in regulation of a wide range of signaling pathways required for proliferation in p53-deficient cells.

Next, we performed a gene network analysis, using sets of genes affected in opposite directions by NEK9 KD and p53 deficiency.
NEK9 expression in human lung cancer specimens expressing mutant p53. To elucidate whether NEK9 dependency occurs in human tumors, we next analyzed the expression of NEK9 and p53 proteins in cancerous and non-cancerous cells in vivo by an immunohistochemical staining of tissue microarray. In this method, positive staining of p53 indicates the expression of mutant p53 protein. NEK9 signal was absent or faint in non-cancerous lung epithelial cells (Supplementary Figure 5A and B). By contrast, NEK9 was frequently expressed in several types of lung cancer: 33/57 (57.9%) of adenocarcinomas (ADC), 54/56 (96.4%) of squamous cell carcinomas (SQC), 23/23 (100%) of large cell neuroendocrine carcinomas (LCNEC), and 22/22 (100%) of small cell carcinomas (SCLC) (Fig. 5A). NEK9 protein was detectable in cancer cells that stained either positively or negatively with an anti-p53 antibody, i.e., again cells harboring either mutant or WT TP53, respectively. In the ADC and SQC samples, NEK9 staining did not correlate with either histological differentiation or p53 status. Notably, however, half of ADCs (13/26), the vast majority of SQCs (28/30), and all LCNECs (20/20) and SCLCs (14/14) that were positive for p53 staining also stained positively for NEK9 (Fig. 5B), indicating that NEK9 dependency occurs in lung SQCs, LCNECs, and SCLCs with p53 mutations. In addition, Kaplan–Meier analysis demonstrated that NEK9/p53 double-positive cases had significantly poorer prognoses than other cases (Fig. 5C and Supplementary Figure 5C), suggesting that NEK9 confers a growth advantage on ADCs harboring p53 mutations, resulting in aggressive tumors with poor clinical outcomes. To extend and validate these results, we used microarrays to measure NEK9 mRNA levels in a different set of 76 lung ADC samples, in which TP53 mutations were defined by exome sequencing (Fig. 5D, Supplementary Table 4). In addition, we used The Cancer Genome Atlas (TCGA)28 data to analyze NEK9 expression levels in various cancers. As indicated in Fig. 5D and Supplementary Figure 5D, NEK9 mRNA was expressed at similar levels in ADCs and SQCs of the lung, colon, stomach, and serous ovarian cancers, regardless of TP53 status. Judging from these results, NEK9 dependency makes a considerable contribution to growth and/or progression of tumors harboring TP53 mutations.

Discussion

Through miR-22 target screening, we identified NEK9 as a crucial regulator of cell-cycle progression in cancer cells lacking functional p53. The miRNA target screen is based on unique features of miRNAs, which target multiple mRNAs and induce phenotypes in a cell type–dependent manner. In a previous study, we showed that miR-22 induces apoptosis in p53 WT cancer cells, but causes cell-cycle arrest in p53 MUT and KO cells that express CDKs and HDACs, critical regulators of the cell cycle that are potential targets of miR-2210. Therefore, we hypothesized that specific factor(s) repressed by miR-22 are required for cell-cycle progression in cancer cells with p53 deficiencies. Consistent with this idea, our screen successfully identified NEK9 as a crucial factor involved in cell-cycle progression in p53-deficient cancer cells. NEK9 regulates mitotic entry by phosphorylating two other NEK family proteins, NEK6 and 717–20,29,30, and it is also activated sequentially by PLK1 and CDK1 to regulate formation of the mitotic spindle18–20. PLK1 was previously identified as a factor required for survival of isogenic p53 KO cell lines12. The results described here demonstrate that NEK9 inhibition does not affect the proportion of cells in G2/M phase, indicating that NEK9 is involved in molecular mechanism(s) distinct from those dependent on PLK1, at least in p53-deficient cancer cells. In fact, proliferation in p53 mutant cells was not specifically inhibited following knockdown of NEK6 or 7 (Supplementary Figure 6A and B).

NEK9 interacts with the FACT (facilitates chromosome transcription) complex in HeLa cells21, in which functional p53 is disrupted by a viral protein. Knockdown of FACT components inhibits progression from G1 to S phase22. These findings are consistent with our own observation that NEK9 KD caused cell-cycle arrest at G1 phase and modulates the global gene expression profile in p53-deficient cells. Furthermore, phosphorylation of NEK9 at T210, which is required for activation of kinase activity23, is important for the interaction between NEK9 and FACT24. Our findings, however, demonstrated that expression of NEK9 T210A restored proliferation in p53 mutant cells, suggesting that the role of NEK9 in regulating G1–S progression is kinase-independent (Fig. 2G). Consistent with this, a previous report showed that phosphorylation at T210 and NEK9 kinase activity are only detectable during G2/M phase, and are required for G2 phase progression25. However, the possibility that an uncharacterized phosphorylation site(s) and/or an interaction of protein partner(s) is required for the kinase activity of NEK9 to regulate the progression of the cell cycle from G1 to S phase in the p53-inactivated cells cannot be excluded. Further studies are needed to clarify this important issue.

A recent study demonstrated that p53 mutations exert tumor-promoting functions that affect cell-cycle progression and metastatic activity in cancer cells26. Therefore, we hypothesized that NEK9 promotes these oncogenic functions of mutant p53. However, we did not detect an interaction between NEK9 and mutant p53 proteins (data not shown). Furthermore, NEK9 KD inhibited the proliferation of p53 KD cells (Fig. 2B). These observations suggest that NEK9 is associated with proliferation and cell-cycle progression in a manner that does not involve a direct interaction with p53. Currently, the relationships between NEK9, mutant p53, and the downstream targets of NEK9 remain unclear due to the unique functions of NEK9, including its kinase independent activity. Therefore, a more detailed analysis of NEK9 function will be required to identify the network(s) controlling the proliferation of p53-inactivated human cancer cells.

Mutant p53 proteins have attracted a great deal of attention as potential targets for cancer therapy. Based on this idea, several approaches have been attempted, including allele-specific activation of WT p53 and restoration of WT function to mutant p53 using small molecules17,19. Inhibition of NEK9 scaffolding (or NEK9 complex formation) and kinase functions may provide a means for selectively targeting cancer cells that lack functional p53; thus, our findings might lead to the development of novel cancer therapies.

Methods

Cell lines. Cell lines used for this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HCT116 p53–/– cells were kindly provided by Dr. Bert Vogelstein (The Johns Hopkins University, Baltimore, MD, USA). Colon and pancreatic cancer cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), and lung cancer cell lines were maintained in RPMI-1640 (Gibco) supplemented with 10% FBS.

Microarray analyses. Microarray analysis was carried out as described previously14. In brief, SW480 or HCT116 p53– cells, seeded at 5.0 × 10^6 cells/ml, were transfected for 48 h with either miR-22 or miR-negative control (Ambion) at a final concentration of 5 nM using HiPerFect Reagent (Qiagen). Total RNA was prepared using RNAeasy columns (Qiagen) and labeled with Cy3 using the Low Input Quick Amp labeling kit (Agilent). Microarray analysis was performed using Whole Human
Figure 5 | Expression of NEK9 protein in lung cancer. (A) Representative images of NEK9 and p53 double-positive lung adenocarcinoma (ADC, upper left pair of images), squamous cell carcinoma (SQC, upper right pair), large cell neuroendocrine carcinoma (LCNEC, bottom left pair), and small cell lung carcinoma (SCLC, bottom right pair). The tables below each image pair show the number of samples of each type of cancer that contained positively stained cells. (B) NEK9- and p53-positive cases of ADC and SQC, with histological differentiation type indicated for each case. (C) Kaplan–Meier survival curves comparing NEK9 and p53 double-positive ADCs versus other types of ADCs. Vertical axis indicates overall survival (OS); horizontal axis indicates duration (months). Statistical analysis was carried out by Wilcoxon test. (D) Levels of NEK9 mRNA in human lung ADCs were determined by microarray analysis. TP53 mutation status was determined by whole-exome sequencing. Boxes represent the upper and lower quartiles and the median, and whiskers show the maximum and minimum values.
Genome_Oligo_Microarrays (4 × 44K.v2, Agilent), and the data were analyzed using the GenoGene GX1.15 software (Agilent).

For the generation of mRNA expression profiles in NEK9 KD cells, SW480 cells were transfected with NEK9 siRNAs, total RNAs were prepared, and microarray analysis was carried out as described above. Network analysis was performed using WikiPathways (http://wiki.pathways.org/).

Human tumor xenograft model. SW480 cells (derived from a p53–mutant human colon cancer) were inoculated at 5.0 × 10^6 cells per site on the backs of 6-week-old female nude mice (CLEA Japan, Inc.) in vivo–jetPEI (Polyplus Transfection)/siRNA complexes were prepared in a volume of 100 μl (1 μmol/site) according to the manufacturer’s instructions, and then injected intratumorally into each animal five times at 2-day intervals. Tumor size was monitored every 2 days by measuring the length (L) and width (W) with calipers, and volume (V) was calculated according to the formula V = (L × W^2) × 0.5. Tumor volume is expressed as percentage of the volume on the day of the first siRNA injection. The animal study protocol was approved by the Ethics Committee in Animal Experimentation of the National Cancer Center (NCC) Research Institute, and the experiments were carried out according to the NCC guidelines.

Analysis of human lung cancer samples. Surgical specimens of primary human lung cancer were obtained from patients treated at the National Cancer Center (NCC) Hospital, with documented informed consent obtained in each case. Whole-exome sequencing analysis was carried out using the Agilent SureSelect Human All Exon 50-kb kit, and sequencing was performed on the Illumina HiSeq 2000 platform. TP53 mutations were evaluated using sequencing data. Gene expression profiles were generated using Agilent Whole Human Genome DNA Microarrays. NEK9 expression levels in lung cancer were then determined.

Immunohistochemical analysis was conducted on tissue microarray sections derived from anonymized samples from patients with ADC, SQC, LCNEC, or SCLC (2007). NEK9 protein expression was used to assess the clinical utility of NEK9 in lung cancer in a prospective study of patients with ADC, SQC, LCNEC, and SCLC.

Supplementary information. Supplementary information includes extended Methods, eight figures and 5 tables.

Acknowledgments

This work was supported by the Advanced Research for Medical Products Mining Program of the National Institute of Biomedical Innovation (NIBIO, 08-02, 12-01), and by Grants-in-Aid for the Ministry of Health, Labor, and Welfare of Japan; the Ministry of Education, Culture, Sports, Technology of Japan; and the National Cancer Center (NCC) Research and Development Fund (to N.T.). Whole-exome sequencing and expression analysis of clinical samples were supported by NIBIO (10-41, 10-43). We also thank the core facility of the NCCRI for microarray analysis and animal experiments, which were supported by the NCC Research and Development Fund (23-A-7).

Author contributions

D.K. and N.T. designed all research, performed experiments, analyzed data, and wrote the paper. F.T. and T.O. carried out some experiments. M.W. and H.N. analyzed some data. H.S., K.M., Y. and T.K. performed clinical and pathological analyses. K.T. and S.W. performed clinical and pathological analyses.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports/

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Kuriksa, D. et al. NEK9-dependent proliferation of cancer cells lacking functional p53. Sci. Rep. 4, 6111; DOI:10.1038/srep06111 (2014).

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated by a license in the caption lines: if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/4.0/