Abstract

The tumor suppressor gene p53 and its family members p63/p73 are critical determinants of tumorigenesis. DAp63 is a splice variant of p63, which lacks the N-terminal transactivation domain. It is thought to antagonize p53- , p63- , and p73-dependent translation, thus blocking their tumor suppressor activity. In our studies of the pediatric solid tumors neuroblastoma and osteosarcoma, we find overexpression of DAp63; however, there is no correlation of DAp63 expression with p53 mutation status. Our data suggest that DAp63 itself endows cells with a gain-of-function that leads to malignant transformation, a function independent of any p53 antagonism. Here, we demonstrate that DAp63 overexpression, independent of p53, increases secretion of interleukin (IL)-6 and IL-8, leading to elevated phosphorylation of STAT3 (Tyr-705). We show that elevated phosphorylation of STAT3 leads to stabilization of hypoxia-inducible factor 1 (HIF-1α) protein, resulting in VEGF secretion. We also show human clinical data, which suggest a mechanistic role for DAp63 in osteosarcoma metastasis. In summary, our studies reveal the mechanism by which DAp63, as a master transcription factor, modulates tumor angiogenesis.

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Introduction

p63 exhibits high sequence and structural homology to the tumor suppressor protein p53 (Trp53), and to another member of this family, p73 (Trp73; refs. 1, 2). Studies using genetically engineered mice have elucidated a central role for p63 in skin development and the aging of epithelial tissues (3–7). Through the use of alternative promoters, the p63 gene generates transcripts encoding two major classes of protein isoforms, TAp63 and Np63. TAp63 contains an N-terminal transactivation domain, whereas Np63 lacks this domain (2). Additional diversity arises through alternative splicing, which generates proteins with unique C-Termini, designated α, β, and γ. Together, these give rise to six unique p63 transcripts. All of these maintain a DNA-binding domain with significant homology to p53, but with somewhat altered target specificity (8, 9).

Several reports both from in vitro and in vivo experiments have suggested that the TAp63 isoforms act as tumor suppressor genes. For instance, TAp63 isoforms suppress metastasis through induction of senescence (10) and transcriptional activation of Dicer1 and mir-130b (11). TAp63+/− mice develop metastatic mammary and lung adenocarcinoma, as well as squamous cell carcinoma (SCC) with metastases to the lung, liver, and brain (11).

The roles of the DAp63 isoforms seem to be more complex. Early studies showed that the DAp63 isoforms oppose p53-, TAp63-, and TAp73-mediated transcription (and therefore apoptosis and cell-cycle arrest), suggesting an oncogenic role for DAp63 isoforms (2, 9, 12–14). Other studies have demonstrated effects that are independent of any dominant negative inhibitory activity, such as targeting of the chromatin remodeler Lsh by DAp63, which results in stem cell proliferation and tumorigenesis (15). Some reports show that DAp63 isoforms retain transcriptional activity and can transactivate genes involved in epidermal morphogenesis (16,17) and DNA repair (18). DAp63 is overexpressed in some types of adult human cancer (19), particularly SCC (20), in which it promotes oncogenesis by suppressing TAp73 (21). In other tumor types, such as adenocarcinoma of the breast and prostate, DAp63 expression is lost during the tumorigenic process (22). Although mice that exhibit knocked down (17) or total loss of expression (23) of DAp63 have been described, their cancer-associated phenotypes have not yet been reported.

Taken together, the role that p63 isoforms play in cancer merits further investigation. Here, we show that two childhood malignancies, neuroblastoma and osteosarcoma, overexpress DAp63. In these tumors we find no correlation between p53 mutation and DAp63 overexpression. We hypothesize that DAp63 is a key modulator of tumorigenesis in these childhood cancers, independent of p53 status. We demonstrate that...
ΔNp63α exhibits gain-of-function activity, which leads to the expression of crucial angiogenic factors and promotes tumor development. Finally, we show that there is a selection for cells expressing high levels of ΔNp63 in osteosarcoma metastasis. Together, these data suggest a central role for ΔNp63 in the progression and dissemination of these childhood cancers.

Materials and Methods

Animal studies

All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee of the Research Institute at Nationwide Children’s Hospital (Columbus, OH). The approved protocols were designed to minimize the numbers of mice used and to minimize any pain or distress. For analysis of tumorigenicity, lentiviral transduced cells (1.5 × 10⁶ cells/mouse) were suspended in 100 μL of 1 × PBS and injected subcutaneously into the flank of 6-week-old CB17SC; scid-/- female mice (Taconic Farms). All mice were maintained under barrier conditions. Tumor volumes were measured once per week as previously described (24).

Cell culture

The neuroblastoma cell line SKNSH was maintained in RPMI supplemented with 10% FBS. SKNDZ was maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS plus 0.1 mmol/L non-essential amino acids. OHS osteosarcoma cells were obtained from Dr. Oystein Fodstad (Radium Hospital Oslo, Oslo, Norway). OS-17 and OHS were cultured in RPMI supplemented with 10% FBS. Hek-293T cells were cultured in DMEM supplemented with 10% FBS. Normal human dermal fibroblasts (NHDF) were obtained from American Type Cell Collection (ATCC) and cultured in fibroblast basal medium (ATCC; PCS-201-030) supplemented with the Fibroblast Growth Kit Low serum (ATCC; PCS-201-041) and penicillin-streptomycin (Life Technologies). JHU-011 cells were a generous gift of Dr. David Sidransky, (Johns Hopkins University, Baltimore, MD) and maintained in RPMI plus 10% FBS. Control and STAT3-/-/- mouse embryonic fibroblasts (MEF) kindly provided by Dr. Valeria Poli (University of Dundee, Dundee, UK) were cultured in DMEM supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVEC) were obtained from ATCC.

Immunoprecipitation and chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer’s instructions (EZ ChIP Kit; Millipore). Protein-DNA complexes were precipitated using a p63 antibody (4A4; Santa Cruz Biotechnology) and PCR was carried out using following specific primers: inter-leukin (IL)-6, forward, 5'-TATAATGTTCTCAATCGCC-3'; reverse, 5'-CTCCAGTCTATATTTATGGG-3'; IL-8 forward, 5'-CATCAATGCAAATCGTGGA-3'; reverse, 5'-GTGTGTGCCC-TATGAGTG-3'.

Immunoblot analysis, immunohistochemistry, and cell-cycle analysis

Cells were lysed on ice with lysis buffer (Cell Signaling Technology) supplemented with protease and phosphatase inhibitors (Thermo Scientific) and 1 mmol/L PMSF (Sigma). Immunoblots were probed with the following antibodies: Antibodies for the detection of β-actin and p63 (4A4) were purchased from Santa Cruz Biotechnology. STAT3, pSTAT3 (Tyr-705), PARP, hypoxia-inducible factor 1α (HIF-1α), and VHL were detected using Cell Signaling Technology antibodies. Total proteins of control tissues were purchased from BioChain. Immunohistochemistry (IHC) assay was performed as previously described (25). For the cell-cycle analysis, osteosarcoma and neuroblastoma cells (60% confluent) were transfected with scrambled or targeting ΔNp63 siRNAs, and cell-cycle distribution was determined by fluorescence-activated cell sorting analysis staining the DNA with propidium iodide (BD Biosciences) according to the manufacturer’s protocol.

VEGF-luciferase assays, quantitative ELISA, and human cytokine array

Luciferase assays were performed according to the manufacturer’s instructions (Promega) by using following plasmids: The VEGF-Luc reporter construct was kindly provided by Dr. P. Amore (Harvard Medical School, Boston, MA) and hypoxia response element (HRE)-Luc reporter plasmid obtained from Addgene (Plasmid: 26731). pcDNA3.1 is from Invitrogen and pcDNA3.1-ΔNp63 is from Addgene (Plasmid: 26979). VEGF secretion assays were performed by using the Human VEGF Quantikine ELISA Kit (Cat. No. DVE00) according to the manufacturer’s instructions (R&D Systems). For human cytokine array, proteome profiler antibody array (R&D Systems) was used according to the manufacturer’s instructions to detect the relative levels of expression of 36 different cytokine-related proteins in NHDF electroporated with pcDNA3 or pcDNA3-ΔNp63α.

Quantitative migration, wound healing, and endothelial cell tube formation assay

Transwell migration assays were performed using previously published methods (25). Migration was quantified using the ratio of the migrated cells to the total cells (migrated plus remaining cells) to determine the fraction of migrated cells in each individual experiment. Each experiment was performed in duplicate. Wound healing and endothelial cell tube formation assay were performed as previously described (25).

RNA isolation, cDNA synthesis, and quantitative real-time PCR

RNA isolation and reverse transcription were performed using the RNeasy Mini Kit (Qiagen) and Omniscript Reverse Transcriptase (Qiagen) according to the manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) was performed on an ABI Prism 7900HD Sequence Detection System (Applied Biosystems) using the TaqMan Universal Mastermix (Applied Biosystems). Tp63 and ΔNp63 gene expressions were quantified by TaqMan Gene Expression Assay (Applied Biosystems) and normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Applied Biosystems). Total RNA from control tissues used as a negative control in qRT-PCR assays were purchased from BioChain.
Quantitative real-time PCR analysis of formalin-fixed paraffin-embedded primary osteosarcomas and lung metastases

Tissue from formalin-fixed paraffin-embedded (FFPE) primary osteosarcomas and corresponding lung metastases was identified through our local pathology department after approval by our local Institutional Review Board. Eleven 5-μm sections were cut from each tumor and dried on glass slides. The central section was stained with hematoxylin and eosin (H&E) and scanned to a virtual slide. Nontumor portions of each unstained slide were scraped away using microdissecting blades. The remaining tissue was then deparaffinized in xylene and fresh 100% ethanol. The remaining tissue was then scraped into a microcentrifuge tube. This was then processed using a Qiagen FFPE RNeasy Kit according to the protocol recommended by the manufacturer. cDNA was generated using 0.5 μg of the isolated RNA using a High Capacity Reverse Transcriptase Kit from Applied Biosciences, utilizing the gene-specific primers as noted below (pooled reverse primers). These resulting cDNAs were used as template to perform qRT-PCR with RT2 SYBR Green qRT-PCR Master Mix from Qiagen on an Applied Biosciences 7900HT machine. Dissociation curves were generated for each reaction and the specificity of each reaction was verified by single peak formation. Samples were normalized to the geometric mean of the expression of β-actin and RPL13A. Primers used were ΔNp63 forward 5'-ACCTGGAACACATGC-CCAGA-3’, reverse 5’-ACGAGGAGCGTCTCTGAATC-3’, 99 bp product ACTB forward 5’-ACAGAGCCTCGCTTTTG-3’, reverse 5’-CGCCGCGATATCATCATCA-3’, 76 bp product RPL13A forward 5’-GGCCCCAGTGACTCTGAAT-3’, reverse 5’-AGATGGCGAGGTGACAG-3’, 93 bp product.

Lentiviral production and siRNAs

The short hairpin RNA (shRNA) lentiviral constructs and high-titer lentiviral stocks were generated as described in the Addgene’s pLKO.1 protocol. siRNAs targeting IL-6 and IL-8 were purchased from Dharmaco and transfected with Lipofectamin 2000 (Invitrogen) into the neuroblastoma and osteosarcoma cell lines.

Results

Childhood neuroblastoma and osteosarcoma express high levels of ΔNp63α

To examine the expression of ΔNp63 in childhood cancers, we surveyed the solid tumor models available through the Pediatric Preclinical Testing Program (Supplementary Fig. S1). Using qRT-PCR with primers designed to distinguish transactivation from ΔN isoforms of p63, we found that the ΔNp63 isoform is highly expressed in more than 50% of neuroblastoma and osteosarcoma tumor xenografts, ranging in abundance from 3 to 4.5 log of expression in contrast with tissues of origin (Fig. 1A). Interestingly, transactivation isoforms of p63 were not detectable by qRT-PCR in any of the analyzed samples. We also examined p63 protein expression in these xenografts. As

Figure 1. Neuroblastoma and osteosarcoma childhood tumors overexpress ΔNp63. A, qRT-PCR was used to assay ΔNp63 mRNA levels in neuroblastoma and osteosarcoma xenografts relative to control tissues. Total RNA isolated from xenografts and control tissues (AG, adrenal gland; BM, bone marrow) was reverse-transcribed and subjected to qRT-PCR with probes specific for ΔNp63. Results were normalized to GAPDH. B, immunoblot analysis of p63 protein expression in neuroblastoma and osteosarcoma xenografts. The predominant band detected corresponds to ΔNp63α and levels of ΔNp63 mRNA correlate with those of the ΔNp63α protein. C, p63 protein expression analysis in neuroblastoma and osteosarcoma cell lines. Cell extracts were analyzed by Western blot analysis with antibodies as shown.
shown in Fig. 1B, the expression of the ΔNp63 protein in these xenografts correlated with the expression of the ΔNp63 mRNA transcript (Fig. 1A). In addition to xenografts, we also examined p63 protein expression in neuroblastoma and osteosarcoma cell lines. We found high-level ΔNp63 protein expression in more than 50% of the analyzed neuroblastoma cell lines and two analyzed osteosarcoma cell lines (Fig. 1C). Consistent with prior studies showing ΔNp63α to be the major isoform (26, 27), we detected p63 protein migration at 68 kDa, which is consistent with the mobility for the ΔNp63α isoform.

**Oncogenic effects of ΔNp63α in neuroblastoma and osteosarcoma**

ΔNp63α has been hypothesized to contribute to tumorigenesis based on its ability to inhibit p53-dependent transactivation (2, 28). By this argument, overexpression of ΔNp63α might simply inactivate p53, abrogating the requirement for its loss during tumorigenesis. Were that hypothesis true, one would expect to find either ΔNp63 overexpression or p53 mutation, but not both. We found no consistent correlation between p53 mutation and ΔNp63α overexpression in neuroblastoma and osteosarcoma (Supplementary Table S1), which makes this hypothesis difficult to substantiate, at least in these tumors. We therefore hypothesized that ΔNp63α might endow cells with a gain-of-function, rather than simply blocking transcriptional activity of p53, TAp63, and TAp73.

To explore the precise function and biochemical mechanisms of ΔNp63α activity, we chose two representative cell lines from each tumor type that differ in their p53 status. We sought to understand the biologic consequence of high-level ΔNp63α expression in neuroblastoma and osteosarcoma by using a lentiviral-mediated shRNA approach. We designed and tested three constructs expressing ΔNp63α-targeted shRNA in pLKO.1 vectors (Supplementary Fig. S2A). None of these shRNA constructs inhibited expression of the TAp63 isoform (Supplementary Fig. S2B). Importantly, we were able to optimize lentiviral production and infection conditions to ensure essentially 100% infection of targeted cells, as assessed using viruses coexpressing the eGFP protein (Supplementary Fig. S2C). Under these conditions, we observed approximately 80% knockdown of endogenous ΔNp63α protein and mRNA in JHU-011 cells. These same constructs were used to infect two different neuroblastoma (SKNSH express wild-type p53 and SKNDZ express mutated p53) and osteosarcoma (OS-17 express wild-type p53 and OHS express mutated p53) cell lines (Supplementary Fig. S2D).

These transduced cell lines were used to study the effects of ΔNp63α expression on anchorage-independent growth and cell proliferation. As shown in Supplementary Fig. S3A and S3B, we found that inhibition of ΔNp63α significantly reduced both cell proliferation and colony formation in a soft agar assay. There was no difference in proliferation or colony formation that could be attributed to p53 status. The reduction in cell proliferation and colony formation following knockdown of endogenous ΔNp63α was not associated with apoptosis (Supplementary Fig. S3C).

We next investigated whether high ΔNp63α expression contributes to the tumorigenic phenotype in neuroblastoma and osteosarcoma. As shown in Fig. 2, all neuroblastoma and osteosarcoma cell lines transduced with a nonsilencing control shRNA formed tumors in nude mice. In contrast, neuroblastoma cell lines with silenced ΔNp63α expression formed tumors in only 8 of 40 mice (Fig. 2A), whereas osteosarcoma cell lines formed tumors in only 4 of 40 mice (Fig. 2B). Similar to our in vitro experiment, decrease in tumorigenicity of endogenous ΔNp63α expression did not correlate with the p53 status of the injected cells.

We assessed these tumors for expression of Ki-67, a well-described marker of proliferative activity. Depletion of

![Figure 2. Knockdown of endogenous ΔNp63α reduces tumor growth. A, neuroblastoma cell lines, SKNSH and SKNDZ, transduced with a nonsilencing control shRNA or two different shRNAs targeting ΔNp63 isoforms were injected subcutaneously into 4- to 6-week-old CB17SC scid−/− mice and observed for up to 7 weeks (n = 10 for each treatment). Tumor volumes were measured every week. B, osteosarcoma cell lines, OS-17 and OHS, transduced with a nonsilencing control shRNA or two different shRNAs targeting ΔNp63 isoforms were injected subcutaneously into 4- to 6-week-old CB17SC scid−/− mice and observed for up to 7 weeks (n = 10 for each treatment). Tumor volumes were measured every week.](cancerres.aacjrournals.org)
endogenous ΔNp63α significantly reduced Ki-67 reactivity in tumors (Fig. 3A–D). Given the essential role that angiogenesis plays in tumor formation, we sought to evaluate angiogenic activity in these same tumors. Inhibition of endogenous ΔNp63α resulted in significant decrease of CD34 immunostaining, a well-established marker for tumor angiogenesis (Fig. 3A–D). These data suggested that the contribution of ΔNp63 to progression in these tumors lies in a p53-independent, gain-of-function mechanism that involves both growth and angiogenic pathways.

**ΔNp63α regulates VEGF activity and promotes migration**

We sought to understand the mechanisms by which ΔNp63α modulates angiogenesis in neuroblastoma and osteosarcoma. One quick assessment of angiogenesis in vitro is the measurement of the ability of HUVECs to form three-dimensional structures (tube formation). To test whether overexpression of ΔNp63α in HEK-293T could stimulate tube formation in endothelial cells, we examined endothelial tube formation using a Transwell assay system, in which soluble factors from HEK-293T cells stimulate endothelial tube formation (Fig. 4A). We found that overexpression of ΔNp63α in HEK-293T cells resulted in stimulation of tubular structures in HUVEC cells (Fig. 4B and C). Moreover, we found that overexpression of ΔNp63α increased wound-healing motility in HEK-293T and in primary NHDF (Supplementary Fig. S4A and S4B). Depletion of the ΔNp63 isoform in neuroblastoma (SKNDZ) and osteosarcoma (OS-17) cell lines reduced wound-healing motility, an effect not seen in control shRNA–treated cells (Supplementary Fig. S4C).

To determine whether the ΔNp63α isoform regulates VEGF secretion, we used ELISA to assess secreted VEGF levels in HEK-293T and NHDF cells transfected with either empty vector or a ΔNp63α expression construct. In contrast with control cells, ΔNp63α-overexpressing cells secreted approximately 4-fold more VEGF into the medium (Fig. 4D). Moreover, the number of cells staining positive or vessel numbers were counted by a blinded observer in five random × 40 fields. Treated (sh1 ΔNp63) and controls (sh Ctrl) were compared using a Student t test and are quantified in histograms (right). *, P < 0.05.
ΔNp63 knockdown markedly decreased VEGF secretion in SKNDZ and OS-17 cell lines (Fig. 4E), suggesting a key role for ΔNp63α in VEGF secretion in these childhood tumors.

Given that p63 isoforms are proteins with sequence-specific DNA-binding properties, we hypothesized that ΔNp63α might alter the expression of genes critical to angiogenesis. Therefore, we examined VEGF expression in the presence of ΔNp63α in HEK-293T and NHDF cells. As shown in Fig. 4F, expression of ΔNp63α increased luciferase activity in HEK-293T and NHDF cells that were cotransfected with a VEGF promoter–luciferase construct.

ΔNp63α induces STAT3 phosphorylation

NHDF and HEK-293T cells do not express detectable amounts of endogenous p63 or p73, and HEK-293T cells express SV40 large T antigen that inhibit endogenous p53. Thus, transcriptional activation of the VEGF-Luc reporter by the ΔNp63α isoform was not likely due to any dominant negative effect on endogenous p53, p73, or p63. It is more probable that ΔN isoforms mediate a gain-of-function, which modulates cellular factors that are important in the regulation of VEGF promoter activity.

HIF-1 is a heterodimeric transcription factor that regulates transcription of several genes associated with angiogenesis, including VEGF (29, 30). It has been shown that ΔNp63 promotes HIF-1α stabilization in the H1299 cell line by an unknown mechanism (31). Consistent with this report, we determined that overexpression of ΔNp63α isoforms induced HIF-1α stabilization in primary NHDF and HEK-293T cells under normoxic conditions (Fig. 5A), leading to strong HRE reporter activity (Fig. 5B).

We then focused on the underlying mechanism of HIF-1 stabilization in our models. Under normoxic conditions, Von Hippel–Lindau (VHL) protein directs the ubiquitination and subsequent proteosomal degradation of the HIF-1α protein; we hypothesized that high-level ΔNp63 expression might reduce VHL protein expression, leading to increased HIF-1 protein levels. To test this hypothesis, we examined the VHL protein expression in the same cell lysates. As shown in Fig. 5C, the expression of VHL protein level did not change with ΔNp63α overexpression in either cell line, suggesting a mechanism of stabilization that is independent of VHL protein expression.

Other studies have shown that STAT3 can modulate the stability and activity of HIF-1α, leading to VEGF expression (32). It has also been shown that transcriptional activity of the ΔNp63 promoter can be regulated by STAT3 and that expression of ΔNp63 cells induces STAT3 phosphorylation (Tyr-705) in Hep3B cells, generating a positive feedback loop between STAT3 and ΔNp63 (33). To investigate whether a similar process might be occurring in our NHDF and HEK-293T cells, we probed cell lysates for activated STAT3. We found that high-level ΔNp63α-overexpressing cells showed increased STAT3 phosphorylation at Tyr-705 (Fig. 5D). In addition, ΔNp63

Role of ΔNp63 Expression in Neuroblastoma and Osteosarcoma

Figure 4. Effect of ΔNp63α on endothelial tube formation, VEGF secretion, and VEGF promoter activity. A, a schematic illustration of the Transwell assay. HUVECs were incubated on Matrigel in the bottom chamber, and transfected HEK-293T cells were grown in the top chamber separated from the HUVECs. Only secreted mediators can diffuse into the bottom chamber to stimulate HUVEC tube formation. B, tube formations in HUVECs were determined for transfected HEK-293T cells grown in the top chamber separated from the HUVECs.

Figure 4A-4D. A, schematic illustration of the Transwell assay. B, tube formations in HUVECs were determined for transfected HEK-293T cells grown in the top chamber separated from the HUVECs. C, to quantify tube formation, branching points of HUVEC cells were counted under a fluorescence microscope. D, ectopic expression of ΔNp63α in HEK-293T and NHDF induces VEGF secretion. E, knockdown of endogenous ΔNp63 in SKNDZ and OS-17 cell lines results in decreased secretion of VEGF. F, ectopic expression of ΔNp63α induces VEGF promoter activity in HEK-293T and NHDF cells.
knockdown in SKNDZ and OS-17 cell lines markedly decreased STAT3 phosphorylation at Tyr-705 (Fig. 5E). To show whether induction of HIF-1 reporter were abrogated in the absence of Np63, we compared the expression on STAT3 and osteosarcoma cell lines. We also examined the necessity of B family can interact together to affect transcription of NF-κB/Rel target genes, including IL-6 and IL-8 promoters. Canonical IL-6 and IL-8 signaling occurs through Janus-activated kinases, which in turn phosphorylate and activate STATs, including STAT3. This made a logical connection with our previous results.

About the upstream elements that affect IL-6 and IL-8 expression, recently published data showed that Np63, RelA, and cRel members of the NF-κB family can interact together to affect transcription of NF-κB/Rel target genes, including IL-6 and IL-8 promoters. To test whether Np63 binds IL-6 and IL-8 promoter regions (which contain known NF-κB/Rel regulatory elements) in neuroblastoma and osteosarcoma cells, we conducted ChIP assays. As shown in Fig. 6B, we detected significant p63-binding activity on IL-6 and IL-8 promoters in neuroblastoma and osteosarcoma cell lines. We also examined the necessity of IL-6 and IL-8 for STAT3 activation in neuroblastoma and osteosarcoma cells. To test this, we first treated cell lines with either single or combined siRNAs-specific for IL-6 and IL-8.

Transcriptional activity of IL-6 and IL-8 promoters is regulated by Np63α

We next investigated the underlying molecular mechanism of STAT3 activation by Np63 isoform. We compared the profiles of secreted cytokines in the presence or absence of Np63α using a human cytokine antibody array. As shown in Fig. 6A, in contrast with control cells, Np63α-overexpressing NHDF cells showed significantly higher expression of IL-6 and IL-8 proteins. Canonical IL-6 and IL-8 signaling occurs through Janus-activated kinases, which in turn phosphorylate and activate STAT3, including STAT3. This made a logical connection with our previous results.

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After confirming siRNA knockdown efficiency by ELISA (Supplementary Fig. S6), we performed immunoblot analysis for STAT3 phosphorylation (Tyr-705). As shown in Fig. 6C, only combined inhibition of IL-6 and IL-8 by specific siRNAs significantly reduced STAT3 phosphorylation level, indicating that either IL-6 or IL-8 activity induced by ΔNp63α is sufficient to keep STAT3 activated.

ΔNp63 expression is enhanced in osteosarcoma lung metastases

We suspected that ΔNp63 expression might endow these childhood tumors with greater metastatic potential. To address this question, we analyzed tissue samples taken from primary osteosarcoma tumors or lung metastases from patients with osteosarcoma were assessed for expression of IL-6, IL-8, and ΔNp63 by qRT-PCR. Results shown were normalized first to internal control housekeeping genes and then to the average expression in primary tumors. The y-axis shows fold expression of ΔNp63 relative to primary tumors. Statistical comparison was made using a nonparametric Mann–Whitney U test, which shows a significant difference between groups at the P = 0.024 level.

Discussion

Although the role of p63 in epithelial development has been well described, the mechanism by which p63 contributes to
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knockdown of

and provide data in supplementary Fig. S7, which shows that

form new vessels. We did make efforts to address this caveat

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independent of alterations in VHL protein levels. The precise

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shown data from human samples, suggesting a role for this

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demonstrated that

Np63 drives expression of IL-6 and IL-8, and have

demonstrated the importance of these pathways in growth,

p53, an oncogenic isoform of that gene in other tumor types (35). We anticipate that future studies will

delineate this process.

As tumor cells multiply, hypoxia develops. Tumor cells must

drive angiogenesis to progress further. The ability to activate

this angiogenic switch is considered a hallmark of malignancy. We have shown that Np63 drives tumors to express VEGF.

The ability to promote angiogenesis is likely to be one mechan-

ism that drives the selection for these cells among metastases. Although we wish to emphasize the potential role of Np63 expression in angiogenesis, we note that it is difficult to establish the degree to which in vivo tumor take was impaired by decreased cell proliferation compared with the inability to form new vessels. We did make efforts to address this caveat and provide data in supplementary Fig. S7, which shows that knockdown of Np63 results in G1 arrest. This suggests that

Np63 expression drives sustained chronic proliferation in these cell lines, making it difficult to separate in vivo effects on cell cycle from effects on angiogenesis. Indeed, the direct induction of angiogenesis by oncogenes that also drive proliferative signaling has been previously shown (e.g., Ras and Myc) illustrates the important principle that distinct hallmark capab-

ilities (proliferative as well as inducing tumor angiogenesis) can be coregulated by the same transforming agent (e.g., Np63, Ras, and Myc).

Previous studies have shown that Np63 promotes STAT3 through phosphorylation, which leads directly to transcriptional activation of the Np63 gene through binding to that gene’s promoter and generating a positive feedback loop (33). The mechanisms that led from Np63 to phosphorylation and activation of STAT3, however, had not been established. Here, we show that this occurs when Np63 binds both IL-6 and IL-8 promoters, and that these cytokines drive STAT3 phosphory-

ation in an autocrine loop. We also demonstrated that stabilization of HIF-1α protein by Np63 activates the necessary step driving VEGF production, which is dependent on STAT3 activation. We were interested to find that this process occurs independent of alterations in VHL protein levels. The precise mechanism, which links STAT3 to the stabilization of HIF-1α, remains to be elucidated. One area of future investigation may be to determine whether STAT3 directly binds to HIF-1α and recruits it to the human VEGF promoter.

We are intrigued by the finding that tumors excised from the lungs of patients with osteosarcoma show markedly high-level Np63 expression relative to the primary lesions from the same patients. We hypothesize that Np63 endows cells with greater metastatic efficiency, as would be suggested given the way that Np63 drives the production of proinflammatory (IL-6 and IL-

8) and angiogenic (VEGF) factors. Such a phenomenon would explain the profound selection for Np63-expressing cells that we observed in the lung metastases (Supplementary Fig. S8). Although the observations we report here do not provide conclusive evidence of this, they certainly support such a hypothesis and at the very least suggest a central role in the process.

Taken together, we demonstrate that Np63 promotes tumor growth in neuroblastoma and osteosarcoma by promoting chronic proliferation and later on tumor angiogenesis by increasing secretion of IL-6 and IL-8, leading to elevated phosphorylation of STAT3. Moreover, we found that elevated phosphorylation of STAT3 induced stabilization of HIF-1α protein, leading to increased VEGF secretion, which might be the driving force for tumor invasion and metastasis in these childhood tumors. Importantly, our clinical data provide the first evidence that analysis of Np63 expression in patients with osteosarcoma and with lung metastasis might be used as a prognostic factor. Furthermore, understanding the underlying molecular mechanism of lung metastasis by high-level Np63 expression will provide new therapeutic approaches targeting lung metastasis in patients with osteosarcoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.K. Bid, R.D. Roberts, M. Cam, A. Audino, P.J. Houghton, H. Cam

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