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ΔNp73/ETS2 complex drives glioblastoma pathogenesis—targeting downstream mediators by rebastinib prolongs survival in preclinical models of glioblastoma

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Abstract

Background. Glioblastoma (GBM) remains one of the least successfully treated cancers. It is essential to understand the basic biology of this lethal disease and investigate novel pharmacological targets to treat GBM. The aims of this study were to determine the biological consequences of elevated expression of ΔNp73, an N-terminal truncated isoform of TP73, and to evaluate targeting of its downstream mediators, the angiopoietin 1 (ANGPT1)/tunica interna endothelial cell kinase 2 (Tie2) axis, by using a highly potent, orally available small-molecule inhibitor (rebastinib) in GBM.

Methods. ΔNp73 expression was assessed in glioma sphere cultures, xenograft glioblastoma tumors, and glioblastoma patients by western blot, quantitative reverse transcription PCR, and immunohistochemistry. Immunoprecipitation, chromatin immunoprecipitation (ChiP) and sequential ChIP were performed to determine the interaction between ΔNp73 and E26 transformation-specific (ETS) proto-oncogene 2 (ETS2) proteins. The oncogenic consequences of ΔNp73 expression in glioblastomas were examined by in vitro and in vivo experiments, including orthotopic zebrafish and mouse intracranial-injection models. Effects of rebastinib on growth of established tumors and survival were examined in an intracranial-injection mouse model.

Results. ΔNp73 upregulates both ANGPT1 and Tie2 transcriptionally through ETS conserved binding sites on the promoters by interacting with ETS2. Elevated expression of ΔNp73 promotes tumor progression by mediating angiogenesis and survival. Therapeutic targeting of downstream ΔNp73 signaling pathways by rebastinib inhibits growth of established tumors and extends survival in preclinical models of glioblastoma.

Conclusion. Aberrant expression of ΔNp73 in GBM promotes tumor progression through autocrine and paracrine signaling dependent on Tie2 activation by ANGPT1. Disruption of this signaling by rebastinib improves tumor response to treatment in glioblastoma.

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Key Points

1. Aberrant expression of deltaNp73 in GBMs promotes tumor progression through autocrine and paracrine signaling dependent on Tie2 activation by ANGPT1.
2. Rebastinib improves tumor response to treatment in highly vascularized tumors such as GBM.

Importance of the Study

GBM is a highly vascular and lethal brain tumor with a median survival of 15 months after diagnosis. Traditional treatment modalities have proven to be ineffective; thus, novel therapies are desperately needed. The ANGPT1/Tie2 signaling pathway has emerged as a potential target for therapeutic intervention. This study identified that ΔNp73 drives GBM pathogenesis through autocrine and paracrine pathways dependent on Tie2 signaling. Our preclinical studies identified that inhibition of Tie2 receptor by rebastinib reduced tumor growth and prolonged survival. Collectively, these results support that Tie2 inhibition improves tumor response and the initiation of clinical trials testing rebastinib or analogous therapy for patients with high-grade gliomas.

Protein 73 (p73) is a member of the p53 family of transcription factors, and contains 2 main isoforms of the protein: transactivating (TA) and deltaN (ΔN). In general, the TAp73 isoform functions similarly to p53 in regulating apoptosis, senescence, and genome stability,[1] while ΔNp73 expression has been associated with several cancers and to tumorigenesis.[2] In contrast to TAp73, ΔNp73 promotes neuronal survival by both p53-dependent and p53-independent mechanisms,[3] and subsequent studies with ΔNp73-deficient mouse models confirmed that ΔNp73 expression is required for neuronal survival and prevention of neurodegeneration.[4,5] These results indicate that the interplay between the main p73 isoforms and resulting biological impact may be much more complex than previously anticipated. Importantly, these observations suggest a critical role for ΔNp73 in neurogenesis and imply that precise regulation of this isoform is essential for its neuroprotective function.

The secreted glycoprotein angiopoietin 1 (ANGPT1) belongs to the angiopoietin family, playing important roles in vascular development and angiogenesis. ANGPT1 has the unique property of activating tunica interna endothelial cell kinase 2 (Tie2). Upon activation, Tie2 on endothelial cells activates several other effectors enhancing the stability of new blood vessels by recruiting surrounding mesenchymal cells and promoting their differentiation to vascular smooth muscle cells.[6] ANGPT1 is overexpressed in high-grade gliomas and has been shown to promote glioblastoma (GBM) angiogenesis by inducing vascular sprouting.[7] Although its receptor Tie2 is preferentially expressed on vascular endothelium and hematopoietic stem cells, studies have demonstrated that Tie2 signaling also functions in a paracrine and autocrine manner directly on cancer cells, including gliomas.[8,9] Importantly, increased Tie2 levels were found in the neovasculature of a number of human tumors, including GBM.[10,11] Nevertheless, the mechanism driving elevated ANGPT1 and Tie2 expression levels in these tumors remains largely unclear.

Here, we report that ΔNp73 expression is elevated in GBM compared with normal tissue. We further identified that ΔNp73 forms a complex with E26 transformation-specific (ETS) proto-oncogene 2 (ETS2) on ETS binding elements and plays a crucial role in GBM angiogenesis by directly increasing the expression of ANGPT1 and Tie2. Crucially, we demonstrated that inhibition of ΔNp73 by specific short hairpin RNA (shRNA) in GBM reduced tumor growth and increased survival. We explored the therapeutic effects of Tie2 inhibition by rebastinib, a selective small-molecule inhibitor of Tie2 kinase, in an orthotopic mouse glioma model with respect to tumor growth, angiogenesis, and survival. We determined that rebastinib treatment significantly inhibited tumor vascularization, decreased growth of established tumors, and prolonged survival.

Methods

Cell Lines and Reagents

The GBM cell lines Denver Brain Tumor Research Group (DBTRG)-05MG, SJGBM2, CHLA-200, and AM38 were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 15% fetal bovine serum (Thermo Fisher Scientific). Glioma sphere cultures from 7 clinical samples were cultured as described previously.[12,13] Pediatric xenograft GBM tumors were kindly provided by Peter J. Houghton (Greehey Children's Cancer Research Institute).[14] Additional details are described in the Supplementary Methods.

Western Blot and Immunoprecipitation

Cells were harvested in lysis solution and subjected to western blotting. Membranes were probed with the primary antibodies and developed as described in the Supplementary Methods. For immunoprecipitation (IP), protein extract from SJGBM2-ΔNp73 cells were incubated either with mouse monoclonal p73 (ER-15, Novus Biologicals) or with immunoglobulin G1 antibodies (BioLegend), including 50 µL Dynabeads Protein G (Thermo Fisher Scientific) overnight at 4°C. Subsequently, IP was performed as described in Thermo Fisher Scientific's protocol, and ETS2 binding was detected by western blotting using a rabbit polyclonal Ets-2 antibody (C-20) (Santa Cruz Biotechnology).

Lentiviral Constructs, Small Interfering RNA, and Plasmids

The lentiviral constructs and high-titer lentiviral stocks were generated as described in Addgene's pLKO.1 protocol. Small interfering RNA (siRNA) targeting ETS1 or ETS2 were purchased from Dharmacon. Detailed description including plasmids and cloning can be found in the Supplementary Methods.

Cell Viability and Clonogenicity Assays

Viable cells were quantified by Alamar blue (Thermo Fisher Scientific) and the capability of colony formation of cells was assessed in methylcellulose media as described in the Supplementary Methods.

Quantitative Reverse Transcription PCR, Chromatin Immunoprecipitation, and Sequential Chromatin Immunoprecipitation Assays

Quantitative reverse transcription PCR (qRT-PCR) was performed on an ABI Prism 7900HT Sequence Detection System using the TaqMan Universal Mastermix (Applied Biosystems Thermo Fisher Scientific). Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer's instructions (EZ ChIP Kit, Millipore). For sequential (re-)ChIP assays, cross-linked chromatin from SJGBM2-ΔNp73 cells was immunoprecipitated using antibody against p73 (ER-15, Novus Biologicals). Additional details are described in the Supplementary Methods.

Immunofluorescence and Immunohistochemical Staining

Immunofluorescence analysis was performed on monolayer cells. To study the expression level of ΔNp73 in patient tissues, 5 human brain GBM tissue samples and matched normal brain tissue samples were used for immunohistochemistry (IHC) analysis. Detailed method description can be found in the Supplementary Methods and Table 1.

Patient Samples

All patient samples were collected with informed consent in accordance with the institutional review board of the Children's National Health System in Washington, DC. Histological sections were reviewed by a neuropathologist, according to the World Health Organization (revised 2016) classification of tumors. Normal brain tissue samples were collected from the whole brain procured at post mortem. These samples were verified by a neuropathologist for the absence of infiltrating tumor cells by examining IHC of H3K27M (a mutation present in these tumors). In these patients, the primary tumor is in the pons. A detailed description of primary tumor sites and matched normal tissue locations is found in Supplementary Table 1.

Mice and Zebrafish Studies

All mouse experiments were approved by the Nationwide Children's Hospital Institutional Animal Care and Use Committee. Briefly, luciferase-labeled 5×10^5 DBTRG-shCtr, DBTRG-shΔNp73, SJGBM2-Ctr, or SJGBM2-ΔNp73 cells suspended in 2 µL of Opti-MEM were implanted into the caudate nucleus of 5-week-old Hsd: athymic nude-*Foxn1*^{nu} mice (Envigo). Ten mice for each group were used. For the survival studies with rebastinib, luciferase-labeled DBTRG or CHLA-200 cells were used. Detailed method description for the mice and zebrafish studies can be found in the Supplementary Methods.

Statistical Analyses

Data were graphed and analyzed using GraphPad Prism 7 software. Error bars represent mean ± standard deviation (SD) from triplicate measurements from 1 experiment. Most of the experiments were repeated at least 2 times with similar results. Differences between 2 groups were analyzed by Student's *t*-test. Survival curves were created using the Kaplan–Meier method with log-rank test of significance. *P*-values of ≤0.05 were considered statistically significant.

Results

Glioblastomas Aberrantly Express the N-terminal Truncated Isoform of p73

We examined the expression of the ΔNp73 isoform in GBM and found that ΔNp73 is transcriptionally overexpressed in the majority of glioma sphere cultures from adult clinical samples as well as pediatric xenograft GBM tumors and cell lines compared with control tissue (Fig. 1A–C). Interestingly, TA isoforms of p73 were not detectable by qRT-PCR in any of the analyzed samples confirming ΔNp73 to be the major isoform expressed in GBM (Fig. 1A–C and Supplementary Figure 1A–C). The expression of the ΔNp73 protein in these samples correlated with mRNA expression (Supplementary Figure 1D). Additionally, we performed IHC staining for ΔNp73 in 5 pairs of GBM patient sections and matched normal brain tissues. As shown in Fig. 1D, in

contrast to the paired normal tissues, the percentage of ΔNp73-positive cells in GBM tumors ranged from 20% to 55%. Together, our data demonstrate that GBMs aberrantly express the ΔNp73 isoform.

ΔNp73 Expression Prolongs Survival, Promotes Intracranial Growth, and Induces Tumor Vascularization of GBM

We next examined the biologic consequence of high levels of ΔNp73 expression in GBM. To determine how ΔNp73 expression in GBM affects survival, we first utilized a zebrafish orthotopic xenograft model. DBTRG-shCtr, DBTRG-shΔNp73, SJGBM2-Ctr, or SJGBM2-ΔNp73 GBM cell lines (Supplementary Figure 1E) were transplanted into the midbrain region of embryonic zebrafish and the larvae were analyzed over time. As shown in Fig. 2A, zebrafish implanted with DBTRG cells, expressing high levels of ΔNp73, had a significantly decreased survival compared with ΔNp73 knockdown (DBTRG-shΔNp73) implanted zebrafish. Conversely, overexpression of ΔNp73 in SJGBM2 cells was associated with decreased survival (Fig. 2B). Next, we evaluated whether ΔNp73 promotes intracranial growth of GBM. Luciferase-labeled DBTRG-shCtr, DBTRG-shΔNp73, SJGBM2-Ctr, or SJGBM2-ΔNp73 cells were implanted into the caudate nucleus of mice. Tumor growth has evaluated weekly by bioluminescence imaging. As shown in Fig. 2C, inhibition of ΔNp73 in DBTRG cells significantly reduced tumor growth. In contrast, overexpression of ΔNp73 in SJGBM2 cells significantly increased growth of intracranially injected tumors (Fig. 2D). Overall, our data indicate that aberrant expression of ΔNp73 in GBM plays a crucial role in the malignant growth of this deadly disease.

GBMs are among the most vascularized tumors in humans.[15] We consequently evaluated whether expression of ΔNp73 in GBM cell lines stimulates tumor vascularization (angiogenesis) in GBM. Using an endothelial tube formation assay, we demonstrated that stable overexpression of ΔNp73 in the SJGBM2 cell line significantly increased the number of endothelial tubes formed, conversely knockdown of ΔNp73 significantly decreased endothelial tube formation (Supplementary Figure 2A, B). To further investigate the role of ΔNp73 expression in tumor angiogenesis, IHC was performed using a CD34-specific antibody. As shown in Fig. 2E, inhibition of ΔNp73 resulted in a significant decrease of CD34-positive vessel formation in tumor tissues compared with the control group as determined by microvessel density (MVD). Furthermore, tumors derived from mice implanted with GBM cells overexpressing ΔNp73 displayed a significant increase of CD34-positive vessel formation (Fig. 2E). Together, our results provide strong evidence that aberrant expression of ΔNp73 plays a crucial role in GBM angiogenesis.

ΔNp73 Forms Complexes with ETS2 to Regulate ANGPT1 and Tie2 Expression in GBM

Given that p73 isoforms are proteins with sequence-specific DNA binding properties, we hypothesized that

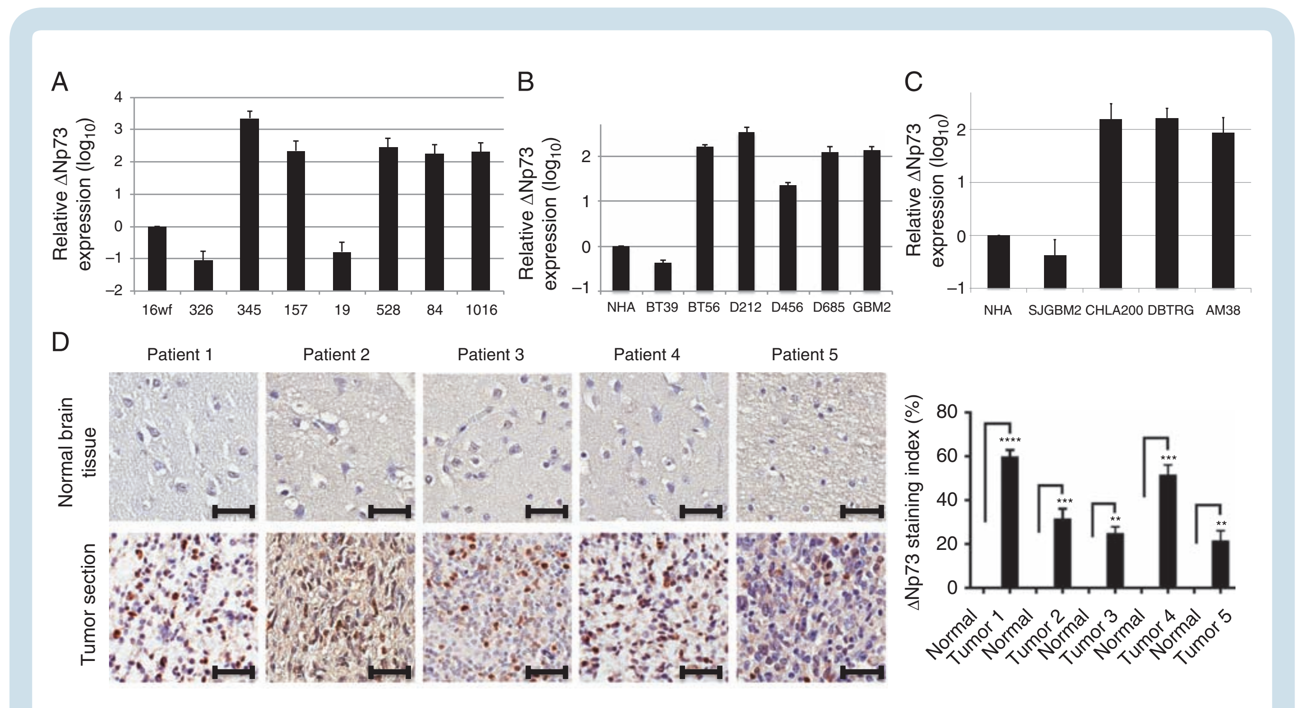

Fig. 1 GBM aberrantly expresses ΔNp73 isoform. (A–C) Quantitative RT-PCR was used to quantitate ΔNp73 mRNA levels in glioma spheres (A), compared with fetal brain-derived astrocytes (16wf), GBM tumors (B), and cell lines (C) compared with normal human astrocyte. (D) Representative ΔNp73 IHC staining of 5 pairs of GBM tumor and matched normal brain tissue are shown. Scale bar = 50 μm. Right, ΔNp73 staining index. *P*-values based on Student's *t*-test. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

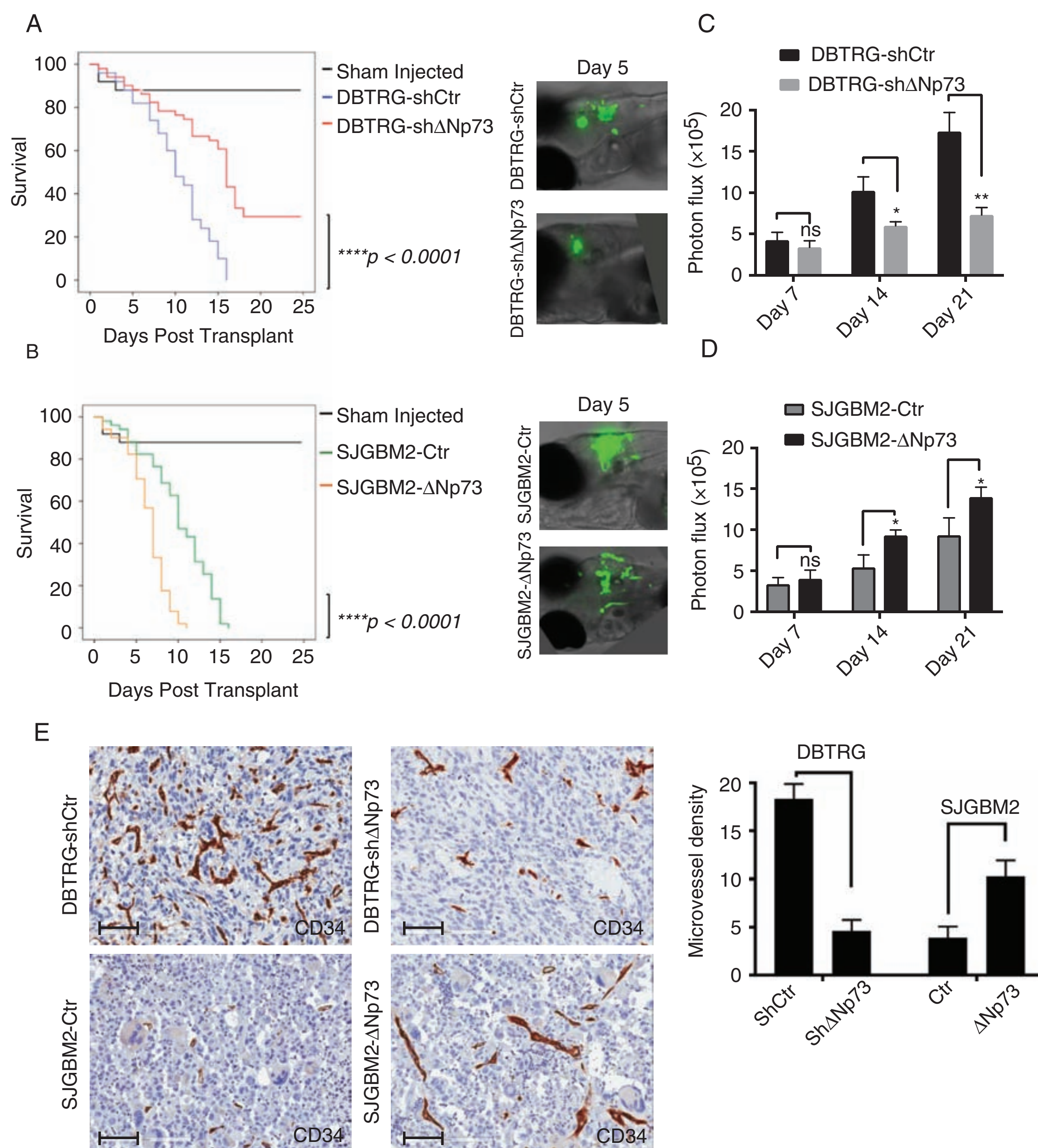

Fig. 2 ΔNp73 expression increases tumor progression. (A) Inhibition of ΔNp73 isoform in DBTRG cells significantly extended zebrafish survival. (B) Overexpression of ΔNp73 in SJGBM2 cells significantly reduced zebrafish survival. (A, B) Kaplan–Meier survival curves with log-rank test (****$P < 0.0001$). Right, representative confocal images of transplanted casper zebrafish 5 days posttransplant. (C, D) ΔNp73 promotes intracranial growth of gliomas in mice. Bioluminescent signal intensity was determined at the indicated days as described in the Methods section. Significant variations between 2 groups was determined by Student's *t*-test at the indicated days. ns, nonsignificant, *$P < 0.05$, **$P < 0.01$. (E) ΔNp73 expression in GBM induces tumor vascularization. Representative IHC images for CD34 staining in GBM sections are shown. Scale bar = 200 μm. Below, quantification of MVD in each type of intracranial glioma was determined as described in Materials and Methods. *P*-values based on Student's *t*-test. *$P < 0.05$, **$P < 0.01$.

ΔNp73 might alter the expression level of genes critical to angiogenesis. By using a human angiogenesis array, we identified 6 proteins, including ANGPT1 and Tie2, that were differently expressed in the presence or absence of ΔNp73 (Supplementary Figure 3A). Interestingly, several independent studies have shown that the activation of the Tie2 receptor by ANGPT1 is linked with GBM angiogenesis,[7,9,16,17] although the upstream mechanism of this deregulation remains unknown. As shown in Fig. 3A, both ANGPT1 and Tie2 gene expressions correlate with ΔNp73 in GBM cell lines. In addition, we found that secreted ANGPT1 levels (Supplementary Figure 3B) and Tie2 receptor expression in GBM cell lines correlate with ΔNp73 expression (Supplementary Figure 3C).

We next investigated the underlying molecular mechanism of ANGPT1/Tie2 regulation by ΔNp73 in GBM. As shown in Fig. 3B, both ANGPT1 and Tie2 promoters showed very high reporter activity when co-transfected with a ΔNp73 expression plasmid. Although the common view is that the dominant-negative function of ΔNp73 on p53-dependent activities is mainly the repression of p53 target promoters due to the lack of the TA domain in ΔNp73,[18] positive effects of these oncogenic isoforms on target gene expression have also been described.[19,20] Since only alpha isoforms of p73 possess a C-terminal sterile alpha motif (SAM) domain, a protein module involved in protein-protein interactions,[21,22] we sought to determine whether the increase of ANGPT1 or Tie2 by ΔNp73 is dependent on the SAM domain. We co-transfected ANGPT1 or Tie2 reporter constructs with either ΔNp73α (contains SAM, herein ΔNp73) or ΔNp73β (does not contain SAM) expression plasmids into 293T cells and assayed luciferase activity. ANGPT1 and Tie2 reporter activities were significantly enhanced only when coexpressed with the SAM-containing ΔNp73α isoform (Supplementary Figure 4A). Furthermore, when we employed constructs containing a mutation in the DNA binding domain of ΔNp73, ANGPT1 and Tie2 reporter induction was significantly decreased (Supplementary Figure 4B). These results suggest that both protein-protein interactions via the SAM domain of ΔNp73 and the DNA binding domain are essential for the positive regulation of both ANGPT1 and Tie2 promoters by ΔNp73.

To explore more precisely how ΔNp73 could enhance ANGPT1 and Tie2 transcription, the ANGPT1 and Tie2 promoters were analyzed using MatInspector 7.4 software (Genomatix Software). ETS binding elements were identified in both promoters, which supports our reporter experiments demonstrating that ETS elements within the promoter are essential for ΔNp73 transactivation (Supplementary Figure 4C, D). To establish whether the ΔNp73 complex binds to the ETS promoter region, we co-transfected ΔNp73 with ANGPT1 or Tie2 reporter constructs containing either wild-type or mutated ETS binding sites. As shown in Fig. 3C, ΔNp73 was able to increase transactivation of the wild-type ANGPT1 and Tie2 promoters; however, only a minimal increase in transactivation of the ETS mutated promoters was observed. These results provide further support that the ETS binding sites are required for the transactivation of ANGPT1 and Tie2 promoters by ΔNp73.

To further define the role of ΔNp73 in the regulation of ANGPT1 or Tie2 in GBM, we evaluated the ability of ΔNp73 to bind to putative ETS binding sites identified in the ANGPT1 and Tie2 promoters by performing ChIP. Following chromatin extraction from SJGBM2-ΔNp73 or

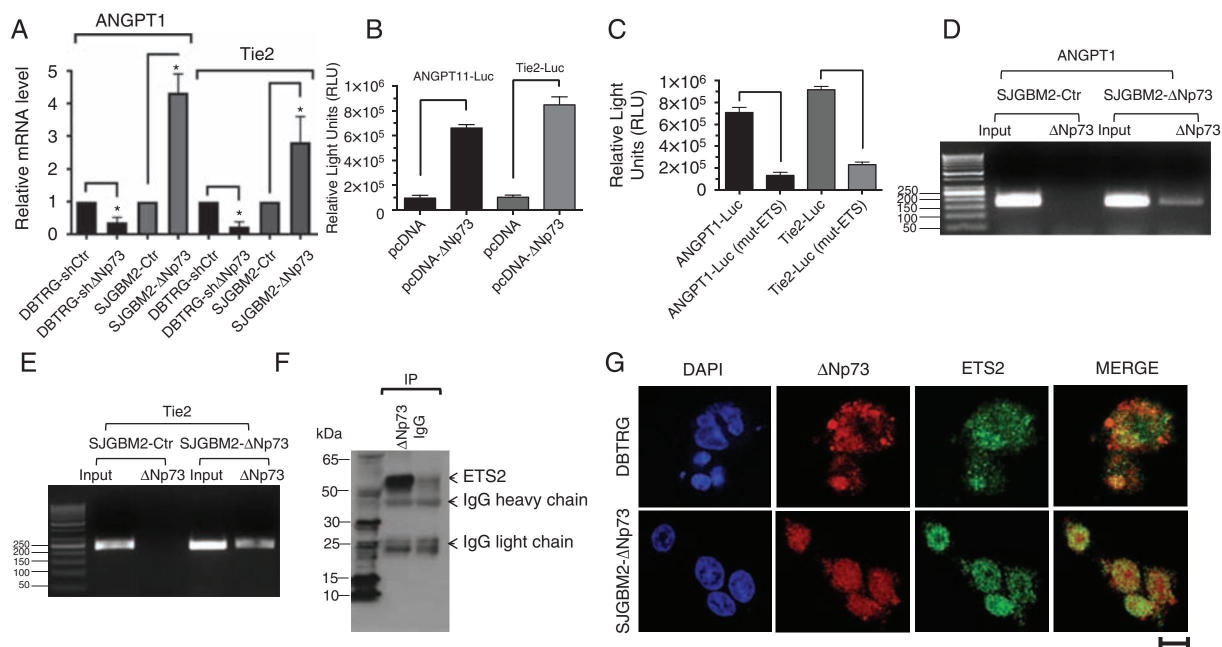

Fig. 3 ΔNp73 forms complexes with ETS2 to regulate ANGPT1/Tie2 expression in GBM. (A) ANGPT1 and Tie2 mRNA expressions were analyzed by TaqMan real-time qRT-PCR. (B) Luciferase assay of 293T cells co-transfected either with ANGPT1 or Tie2-responsive luciferase reporter constructs along with the plasmids encoding for ΔNp73 (pcDNA-ΔNp73) or control plasmid (pcDNA). (C) Indicated reporter plasmids were transfected along with pcDNA3-ΔNp73 into 293T cells. Luciferase assays were performed as described in Supplementary Methods. Data shown are mean ± SD of triplicate measurements from one representative experiment. (D, E) ChIP assays demonstrate binding of ΔNp73 on ANGPT1 and Tie2 promoters containing ETS sites. Immunoprecipitated samples from cell lines were analyzed by PCR using the primers as described in methods. (F) Co-IP assay of ΔNp73 and endogenous ETS2. ΔNp73 was immunoprecipitated from SJGM2-ΔNp73 cells. The presence of ETS2 in these immunoprecipitates was confirmed by western blotting with an antibody against ETS2. (G) Co-localization of ΔNp73 and ETS2 in glioblastoma cell lines. A representative image from each group is shown. Scale bar: 20 μm.

SJGBM2-Ctr cells and IP with a specific anti-p73 antibody, PCR was performed with primer pairs designed to amplify sequences that included only the ETS elements. As shown in Fig. 3D, E, binding of ΔNp73 to ETS sites on both ANGPT1 and Tie2 promoters suggests direct regulation of ANGPT1 and Tie2 promoters by ΔNp73. Therefore, we suspected that the ETS family of transcription factors are key regulators on both ANGPT1 and Tie2 promoters. ETS2, a member of the ETS family, has been shown to form a complex with gain-of-function mutant p53, enhancing p53 transcriptional activity, resulting in the misregulation of new target genes.[23,24] However, we could not detect any increase in ETS2 expression in GBM (Supplementary Figure 5). To evaluate the possibility that ΔNp73 directs ETS2 to the ANGPT1 and Tie2 promoters to increase their gene expression in GBM, thereby mimicking ETS2 overexpression, we performed a co-IP assay. As shown in Fig. 3F, western blot analysis of the immunoprecipitates with an ETS2 antibody revealed that ΔNp73 interacts with ETS2 in SJGM2-ΔNp73 cells. Moreover, IP with an ETS2 antibody followed by western blot with a p73 antibody also corroborated that ΔNp73 and ETS2 form complexes in these cells (data not shown). Next, we performed immunocytochemistry to determine the localization of ΔNp73 and ETS2. We found that ETS2 and ΔNp73 co-localize within the nucleus (Fig. 3G). In addition, we evaluated the localization of ΔNp73 and ETS2 on co-transfected 239T cells. As shown in Supplementary Fig. 6, both proteins are predominantly nuclear and were found to co-localize (Supplementary Figure 6D, yellow in merged panels). The overlapping expression pattern of ΔNp73 and ETS2 further strengthens the possibility that the 2 proteins associate, and their presence within the nucleus indicates that ETS2 and ΔNp73 play a key role as transcription machinery in GBM.

DNA regulatory elements frequently rely on the interaction of multiple transcription factors and cofactors to regulate gene expression. We therefore utilized re-ChIP to identify binding partners of ΔNp73 and ETS2 proteins on a single DNA sequence containing an ETS binding site. As shown, in Fig. 4A, B, re-ChIP confirmed that ΔNp73 and ETS2 form a complex at ANGPT1 and Tie2 promoters, suggesting ΔNp73 binds and/or recruits ETS2 to the ETS binding site, where both may participate in driving elevated gene expression. Lastly, one would predict that knocking down the ETS2 protein, which might be aberrantly recruited by ΔNp73 to the promoters should also reduce both ANGPT1 secretion and Tie2 receptor expression. As shown in Fig. 4C, D, ANGPT1 secretion and Tie2 receptor expression on GBM cell lines were significantly reduced following siRNA-mediated knockdown of ETS2 gene expression, supporting the idea that the ΔNp73-ETS2 complex on the ANGPT1 and Tie2 promoters is necessary to drive transcription. In summary, our data demonstrate that ΔNp73 regulates both ANGPT1 and Tie2 gene expression in GBM cells through an interaction with ETS2 at an ETS binding site motif within the promoter region. Furthermore, our results, together with the reported existence of an ANGPT1/Tie2 autocrine loop in GBM,[10] provide new insights into the previously unknown upstream molecular mechanism of this autocrine loop.

Rebastinib Inhibits Glioblastoma Growth and Extends Mouse Survival

Our data demonstrated that ΔNp73 stimulates tumor growth and angiogenesis by deregulating both ANGPT1 and Tie2 genes in GBM. These results, taken together with our in vivo experiments showing the existence of enhanced vascular and tumor growth in orthotopic xenograft brain tumors, led us to consider whether disruption of ANGPT1/Tie2 signaling by using a pharmaceutical inhibitor affects tumor cell proliferation and survival in vivo. Many compounds have been reported to inhibit Tie2 kinase, including Met inhibitors cabozantinib, foretinib, MGCD265 (glesatinib), and crizotinib; the BCR-Abl (breakpoint cluster region–Abelson murine leukemia) inhibitor ponatinib; the ALK/RON (anaplastic lymphoma kinase/receiver of origin Nantes) inhibitor crizotinib; and 2 other Tie2 inhibitors, pexmetinib and the preclinical tool compound SKBTie2. Interestingly, it has been shown that rebastinib is 17 to 5672-fold more potent and the most selective of these comparator inhibitors for inhibiting Tie2.[25] Therefore, we examined rebastinib inhibition of Tie2 signaling in GBM (Fig. 5A). To evaluate the inhibitory effect of rebastinib on cell proliferation, GBM cells expressing high levels of ΔNp73 (DBTRG, CHLA-200, and AM38) were treated with different concentrations of rebastinib for 72 hours, and then cell viability was measured with the Alamar blue assay. As shown in Fig. 5B, rebastinib potently decreased the cell viability of DBTRG, CHLA-200, and AM38 cells (half-maximal inhibitory concentrations = 34.7 nM, 40.3 nM, and 30.3 nM, respectively). In addition, we performed colony-formation assays to determine the effect of rebastinib on the anchorage-independent growth of DBTRG, CHLA-200, and AM38 cell lines. The colony-forming capabilities of all cell lines were inhibited by rebastinib in concentration-dependent manners (Fig. 5C). Next, we evaluated Tie2 downstream signaling in these 3 cell lines following rebastinib treatment. Previous studies have shown that Tie2 signaling exerts its proliferation and survival effect through activation of the phosphatidylinositol-3 kinase (PI3K)–Akt and mitogen-activated protein kinase (MAPK) signaling pathways.[26] As shown in Fig. 5D, the phosphorylated levels of downstream signaling molecules such as Akt and extracellular signal-regulated kinase 1 and 2 (ERK1/2) were decreased by rebastinib treatment in all 3 cell lines. In addition, we observed a decrease in Tie2 protein level in response to rebastinib. Importantly, we determined that GBM cells are not sensitive to rebastinib treatment and both phosphorylated (p-)Akt and p-MAPK levels decreased following inhibition of ΔNp73 (Supplementary Fig. 7A, B), suggesting that the effect of rebastinib is dependent mainly on ΔNp73 expression. Together, rebastinib actively suppressed the growth and proliferation of cells expressing high levels of ΔNp73 most likely by inhibition of the PI3K-Akt and MAPK signaling pathways. Lastly, treatment of GBM cell lines with rebastinib significantly reduced the endothelial tube formation supporting the role of Tie2 signaling in GBM angiogenesis (Fig. 5E).

We next examined rebastinib inhibition of Tie2 signaling in an orthotopic mouse glioma model. DBTRG or CHLA-200 GBM cells were injected into the caudate nucleus of nude mice. A week after injection, animals

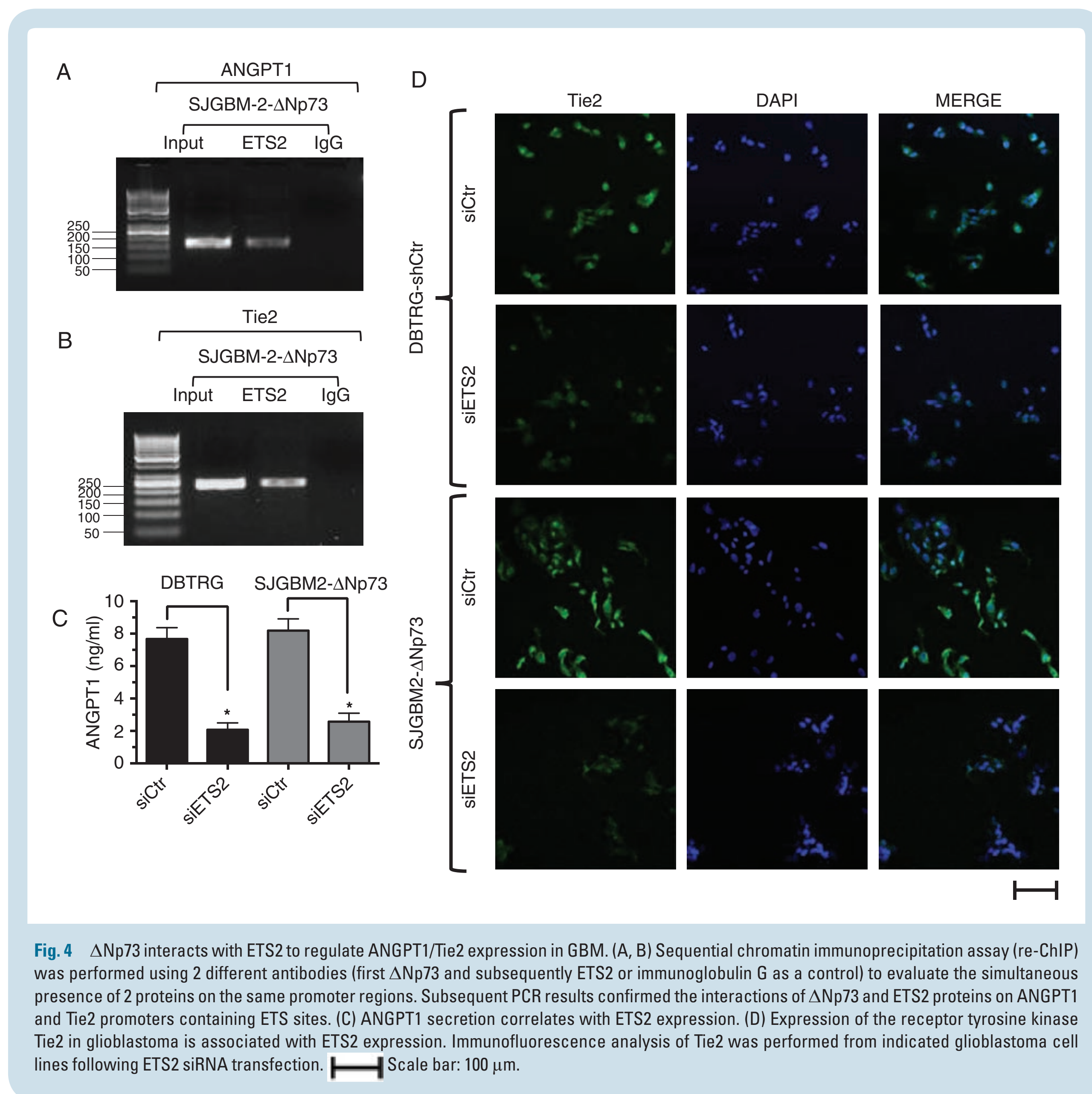

Fig. 4 ΔNp73 interacts with ETS2 to regulate ANGPT1/Tie2 expression in GBM. (A, B) Sequential chromatin immunoprecipitation assay (re-ChIP) was performed using 2 different antibodies (first ΔNp73 and subsequently ETS2 or immunoglobulin G as a control) to evaluate the simultaneous presence of 2 proteins on the same promoter regions. Subsequent PCR results confirmed the interactions of ΔNp73 and ETS2 proteins on ANGPT1 and Tie2 promoters containing ETS sites. (C) ANGPT1 secretion correlates with ETS2 expression. (D) Expression of the receptor tyrosine kinase Tie2 in glioblastoma is associated with ETS2 expression. Immunofluorescence analysis of Tie2 was performed from indicated glioblastoma cell lines following ETS2 siRNA transfection. Scale bar: 100 μm.

were treated daily with rebastinib or vehicle control for 2 weeks. To determine the primary tumor growth in vivo, bioluminescence imaging studies were conducted weekly as described in the Methods section. As shown in Fig. 6A, B, rebastinib significantly reduced primary tumor growth in both glioma models, suggesting rebastinib had a beneficial antineoplastic effect in vivo. To further compare the effects of rebastinib on intracranial tumors, all mice were terminated at day 21. As shown in Fig. 6C, treatment with rebastinib significantly reduced tumor vascularization measured by CD34 staining and confirmed by quantification of MVD in tumor tissues (Supplementary Figure 8A). Moreover, we found a significant decrease in Tie2 (Fig. 6D and Supplementary Figure 8B) and Ki67 staining (Fig. 6E and Supplementary Figure 8C) in tumors treated with rebastinib. Lastly, we evaluated the effect of rebastinib on survival of animals orthotopically implanted with DBTRG or CHLA-200 GBM cells. Animals were treated with rebastinib or vehicle control 1 week after implantation for 4 weeks. Rebastinib reduced tumor burden as determined by bioluminescent imaging (Supplementary Figure 9) and significantly increased survival of animals compared with vehicle treated animals (DBTRG, median survival 47 days treated vs 25 days vehicle; CHLA-200 median survival 50 days treated vs 29 days vehicle) (Fig. 6F). Together, our data demonstrated that Tie2 inhibition improved tumor response to treatment and supports further investigation of rebastinib therapy for patients with GBM.

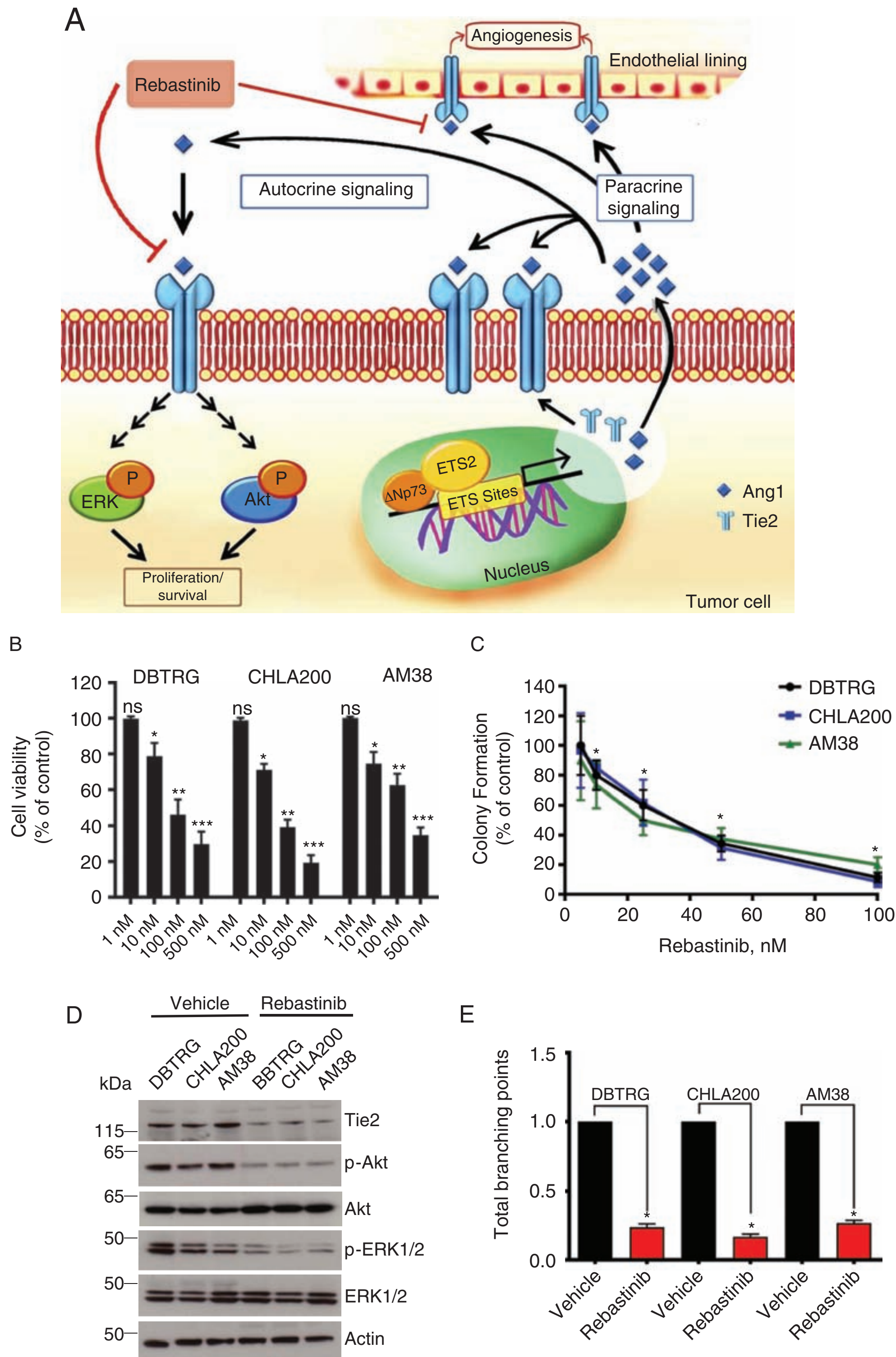

Fig. 5 (A) ΔNp73-ETS2 induced ANGPT1 secretion acts in a paracrine and autocrine manner. ANGPT1 contributes to the activation of Tie2 signaling on tumor cells (autocrine) promoting cell survival and proliferation, whereas activation of Tie2 signaling on endothelial cells (paracrine) stimulates angiogenesis. Rebastinib decreases cell viability (B) and clonogenicity (C) in a dose-dependent manner. (C) Figures represent mean values of at least 3 independent experiments. Asterisk (*) demonstrates levels of significance calculated between rebastinib treatment and related vehicle control. (D) Western blot demonstrating a decrease in Tie2, p-Akt, and p-ERK1/2 following rebastinib treatment (20 nM). (E) Rebastinib decreases GBM induced human umbilical vein endothelial cell tube formation.

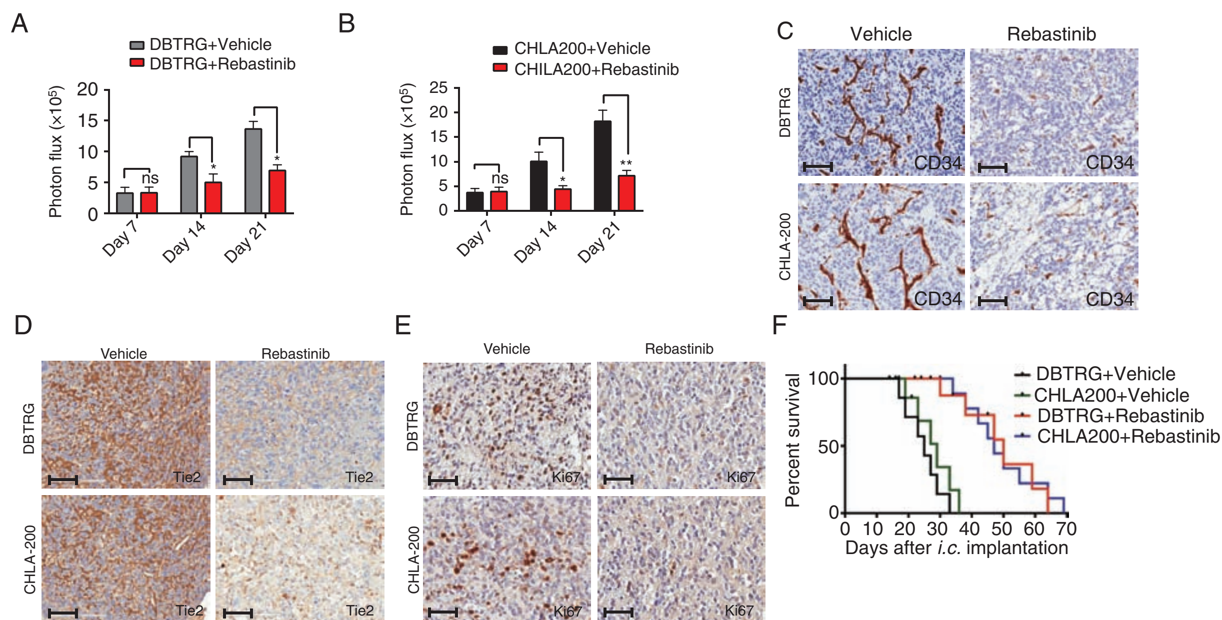

Fig. 6 Rebastinib inhibits GBM growth and extends mouse survival. (A, B) Bioluminescent imaging showing a decrease in tumor burden in DBTRG (A) and CHLA-200 (B) following rebastinib treatment. (C, D) Rebastinib treatment decreases CD34 (C), Tie2 (D), and Ki67 (E) expression. Scale bar = 200 µm. Quantification of MVD, staining area index of Tie2, and staining index of Ki67 are shown in Supplementary Figure 8.

Discussion

ΔNp73 deregulation has been demonstrated in a variety of cancers, implicating ΔNp73 in oncogenesis.[27] This is not surprising as ΔNp73 has been shown to play a critical role in neuronal survival and neurodegeneration.[3–5,28] In this study, we show that ΔNp73 is frequently overexpressed in GBM. Interestingly, we failed to determine a correlation between TP53 mutation and ΔNp73 overexpression (Supplementary Table 2), suggesting that ΔNp73 promotes tumorigenesis with a gain-of-function, rather than simply blocking transcriptional activity of p53, TAp63, and TAp73. Transcriptional analysis of ΔNp73 in brain tumor models available through the Pediatric Preclinical Testing Program showed that only GBM significantly expresses high levels of ΔNp73 (Supplementary Figure 10). Although the lineage of the tumor-initiating cell may contribute to the specific biological and genomic phenotype of GBM, the mechanisms that drive ΔNp73 deregulation in GBM remain to be elucidated.

Our studies show that ΔNp73 enhances gene expression of ANGPT1 and Tie2, and protein-protein interactions via the SAM domain of ΔNp73 are necessary for transactivation of ANGPT1 and Tie2 promoters. The data demonstrate that ΔNp73 transactivation activities require interaction with ETS2 on ETS binding elements. IP and knockdown analyses revealed that ETS2, but not ETS1 (data not shown), was responsible for the altered ANGPT1 and Tie2 gene expression induced by ΔNp73. ΔNp73 was found to enhance the interaction through binding of ETS2 to ETS sites. This suggests that induction of ANGPT1 and Tie2 genes by ΔNp73 is mediated by the formation of a tertiary ΔNp73-ETS2 complex at the ETS binding elements that seems to be more efficient in transactivation than a complex of ETS2 on the ETS sites on the promoters. Indeed, previous studies have revealed a novel trans-acting mechanism that leads to activation of ETS factors involving missense mutations in the gene encoding the tumor suppressor p53.[23,24,29,30] For instance, ChIP followed by ChIP sequencing performed in Li–Fraumeni syndrome cells demonstrated that ETS consensus motifs were frequently identified in promoters at which p53 mutants localized and formed complexes with ETS2.[23] Although our current data, as well as previous data with mutant p53 interactions, demonstrated ETS2 as the major binding partner for ΔNp73, the involvement of other ETS family members cannot be ruled out.

ANGPT1/Tie2 signaling pathways have emerged as candidate escape mechanisms responsible for relapse or progression following standard therapies in various types of cancer, including GBM.[31] Angiopoietin neutralizing peptibody and specific monoclonal antibodies have been developed[32,33] and tested in clinical studies showing increased progression-free survival in patients with metastatic breast cancer, ovarian cancer, and other solid cancers.[34,35] However, there are additional ligands, including Ang4, which activate Tie2,[36] and extracellular signals, including integrins[37,38] and lysyl oxidase,[39,40] which can activate Tie2-mediated signaling and internalize Tie2 signals in the DNA damage response.[40] Monotherapy involving the

Tie2 kinase inhibitor rebastinib resulted in a significant survival benefit in 2 GBM xenograft models. However, our approach was limited as we targeted only a single angiogenic factor. In the future it may be more appropriate to consider the synergistic effect of multiple factors involved in tumor angiogenesis (eg, targeting vascular endothelial growth factor signaling). In addition, recent studies in breast and pancreatic neuroendocrine tumors have revealed that rebastinib reduces tumor growth and prolongs survival by blocking recruitment and function of Tie2-expressing macrophages.[25] Whether a similar mechanism exists in GBM following rebastinib treatment warrants further investigation.

In conclusion, aberrant expression of the ΔNp73 isoform promotes GBM pathogenesis via its interaction with the ETS2 protein. We provide the first evidence that the ΔNp73-ETS2 complex directly activates both ANGPT1 and Tie2 gene expression in tumor cells. This contributes to the autocrine activation of Tie2 signaling on tumor cells with subsequent cell survival and proliferation as well as paracrine activation of Tie2 signaling on endothelial cells, stimulating angiogenesis and promoting tumor growth. Targeting angiogenesis has been shown to be effective in many preclinical models with multiple agents but there has not been a therapeutic benefit demonstrated in patients. The reason for this lack of translation is not clear. We also demonstrate the preclinical utility of rebastinib in glioma animal models, suggesting that rebastinib may be a promising therapy for the treatment of GBM. However, our results should be interpreted with caution.

Supplementary Material

Supplementary data are available at *Neuro-Oncology* online.

Keywords

ANGPT1 | deltaNp73 | ETS2 | GBM | Rebastinib | Tie2

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