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Inhibitory effects of cytoplasmic-domain substituted epidermal growth factor receptor on growth, invasion and angiogenesis in human gastric cancer cells

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Abstract. Epidermal growth factor receptor (EGFR) blockade is a promising therapeutic approach for gastric cancer overexpressing EGFR. EGFR, with a cytoplasmic domain substituted by enhanced green fluorescent protein (DNEGFR-EGFP), can act as a dominant negative mutant receptor to block the EGFR signaling pathway by competing with endogenous EGFR for ligands. The aim of this study was to investigate the effects of DNEGFR-EGFP on the growth, invasion and angiogenesis of human gastric cancer cells, and to elucidate the possible mechanisms behind them. Using multiple cellular and molecular approaches such as gene transfection, MTT, flow cytometry, Western blotting, ELISA, invasion and angiogenesis assays, we found that DNEGFR-EGFP led to G_0/G_1 arrest by down-regulating cyclin D1 and CDK2 and up-regulating p27, and repressed the invasion and angiogenesis of SGC-7901 cells by inhibiting them from secreting MMP-2, MMP-9 and VEGF. These results indicate that the EGFR blockade strategy (termed dominant negative strategy targeting EGFR) may serve as a promising therapy for the treatment of EGFR-overexpressed gastric cancer.

Introduction

Gastric cancer is the fourth most common cancer worldwide and the second most common cause of death from cancer (700,000 deaths annually) (1). High-risk areas (age-standardized rate in men, more than 20/100,000) include East Asia (China, Japan), Eastern Europe and parts of Central and South America. Despite tremendous advances in surgery, chemotherapy and radiotherapy, the prognosis of gastric cancer is still poor, with the overall 5-year survival rate ranging from 5 to 15% (2). Therefore, there is a strong impetus to investigate new therapies to improve the outcome of patients afflicted with this deadly disease. Gene therapy is a promising candidate in the treatment of gastric cancer (3), and dominant negative strategy plays an important role in gene therapy, as dominant negative mutants of receptors compete with endogenous receptors for ligands, leading to the inhibition of receptor activation.

Increasing importance is being attached to the epidermal growth factor receptor (EGFR) in gastric cancer research. EGFR is a member of the ErbB family of receptors and is a 170-kDa protein that consists of an extracellular ligand-binding domain, a hydrophobic membrane-spanning region and an intracellular tyrosine kinase domain. Stimulation of EGFR by endogenous ligands, epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) results in a conformational change in the receptor, permitting it to enter into dimers with other oligomers (4). Dimerization results in the activation of intracellular tyrosine kinase and protein phosphorylation, and initiates signal transduction cascades which promote the cell division, invasion and angiogenesis of EGFR-overexpressed cancer cells (5,6). Therefore, EGFR blockade may be useful in inhibiting cell division, invasion and angiogenesis. The dominant negative EGFR (DNEGFR), a truncated receptor that lacks the tyrosine kinase domain, competes with endogenous EGFR for ligands, leading to the inhibition of receptor activation (7). Previous studies demonstrated that DNEGFR blocks EGFR signal transduction cascades in rat ovarian cancer NuTu-19 and human glioblastoma multiforme cells (7,8). EGFR is expressed in up to 47.2% of gastric cancers (9), which suggests that DNEGFR potentially has therapeutic potential for the treatment of the disease.

In this study, we constructed a pEGFPN1-DNEGFR vector, which harbored a fusion gene encoding EGFR with a cytoplasmic domain substituted by enhanced green fluorescent protein (EGFP). The fusion protein was designated DNEGFR-EGFP. EGFP allowed for the direct monitoring of DNEGFR. We evaluated the effects of DNEGFR-EGFP on the growth, invasion and angiogenesis of human gastric cancer cells, and investigated the possible molecular mechanisms involved in these effects.

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Materials and methods

Cell lines. Human gastric cancer SGC-7901 and human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen) and incubated at 37˚C in a humidified atmosphere of 5% CO_2/95% air.

Construction and identification of pEGFPN1-DNEGFR. Total RNA was isolated from SGC-7901 cells using TRIzol Reagent (Invitrogen) following the manufacturer's protocol. Reverse transcription was performed using the PrimeScript™ 1st Strand cDNA Synthesis kit (Takara, Dalian, P.R. China), with total RNA as a template according to the manufacturer's instructions. PCR amplification was subsequently carried out with Takara ExTaq® Hot Start Version (Takara) in a 50-μl total volume. The primer sequences were as follows: forward, 5'-AAAAGCTAGCACCATGCGACCCTCCGGGAC-3'; reverse, 5'-TAATCCGCGGTACGTACCGCATGAAGAGGC CGATCCC-3'. PCR products contained the 1-2004 bp of EGFR precursor cDNA (GeneBank accession, X00588). The *Nhe*I restriction site was incorporated into the forward primer, and the *Cfr*42I restriction site was incorporated into the reverse primer for cloning purposes. PCR conditions were as follows: 2 min at 94˚C for reverse transcriptase denaturation, followed by amplification of 30 cycles (94˚C for 30 sec, 70˚C for 30 sec and 72˚C for 2 min and 30 sec) and finally extension at 72˚C for 7 min. PCR products were purified with the Agarose Gel DNA Purification kit ver. 2.0 (Omega Bio-Tek, Norcross, GA, USA) restricted with *Nhe*I and Cfr42I (both from Fermentas International Inc., Burlington, Canada), then cloned into *Nhe*I and *Cfr*42I restriction sites of the pEGFP-N1 vector (Clontech, Mountain View, CA, USA). Clones of interest were identified by DNA sequencing in both directions (Takara). The pEGFP-N1 vector is expressed from the immediate early promoter of CMV (PCMV IE). The vector also contains the EGFP coding sequences and the neomycin resistance gene. The resultant expression vector was named pEGFPN1-DNEGFR.

Transfection of plasmids. Mediated by Lipofectamine 2000 (Invitrogen), SGC-7901 cells were transfected with the plasmids pEGFPN1-DNEGFR or pEGFP-N1 according to the manufacturer's protocol. In brief, one day before transfection, cells ($6x10^5$/well) were cultured in 2 ml of antibiotic-free growth medium in a 6-well plate. Cells were 90-95% confluent at the time of transfection. For transient transfection, cells in each well were treated with 10 μl Lipofectamine and 4 μg pEGFPN1-DNEGFR vector, and incubated in 2 ml Opti-MEM I reduced serum medium (Invitrogen) for 5 h. Cells were then transferred to 2 ml of full-growth medium at 37˚C in a CO_2 incubator. Cells stably transfected with the vectors were selected with G418 (350 μg/ml; Invitrogen) 48 h after transfection, and individual clones were isolated, expanded and maintained in G418 (100 μg/ml) for analysis. The cells were grouped as UC (untreated cells), EC (EGFP-expressing cells, which were stably transfected with pEGFP-N1) and DC (DNEGFR-EGFP-expressing cells, which were stably transfected with pEGFPN1-DNEGFR).

MTT assay. The effect of pEGFPN1-DNEGFR on the proliferation of SGC-7901 cells was evaluated using the MTT assay. Log-phase cells were seeded in 96-well plates ($5x10^3$ cells/well) for 24-72 h. Each group was analyzed every day in triplicate by the following method: 20 μl MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to each well, and cells were incubated for a further 4 h at 37˚C. Subsequently, the formazan crystals were solubilized with 150 μl of dimethylsulphoxide (DMSO; Sigma-Aldrich). The absorbance (A) value was measured at a wavelength of 570 nm on an ELISA plate reader (Bio-Rad Laboratories, Hercules, CA, USA) with DMSO as the blank. The growth curve of cells was plotted using time (day) as the x-axis and the A value as the y-axis.

Cell cycle assay. Cells were harvested, washed with PBS and fixed with cold 70% ethanol overnight at 4˚C, then washed and resuspended in PBS containing RNase A and stained with propidium iodide (PI) for 30 min at room temperature. PI-stained cells were detected using a flow cytometer (BD Biosciences, San Jose, CA, USA). Cell cycle distribution was calculated using the Modifit-3 program (BD Biosciences).

Western blot analysis. Western blot analysis was used to investigate cyclin D1, CDK2 and p27 at the protein level. Cells were washed once with ice-cold PBS, then lysed in lysis buffer [50 mmol/l Tris (pH 7.5), 100 mmol/l NaCl, 1 mmol/l EDTA, 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/l sodium orthovanadate, 10 μl/ml protease inhibitor cocktail and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF)] for 20 min on ice and centrifuged. The protein concentration of the clarified lysate was quantified by the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Protein samples (50 μg) were subjected to SDS/10% PAGE and transferred to PVDF membranes (Bio-Rad Laboratories). Blots were blocked in PBST with 1% BSA, incubated with the primary antibodies to cyclin D1, CDK2 and p27 (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 37˚C for 1 h, and washed and incubated with a goat anti-rabbit antibody-conjugated horseradish peroxidase (dilution 1:10000; Santa Cruz Biotechnology) at 37˚C for 1 h. Specific antibody-labeled proteins on the membranes were detected using Pierce ECL Substrate (Thermo Fisher Scientific) and visualized on the Gel Doc XR System (Bio-Rad Laboratories) according to the manufacturer's instructions. After being washed with stripping buffer (Thermo Fisher Scientific), the membrane was reprobed with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (1:1000 dilution; Santa Cruz Biotechnology) using the same procedures as described above. Each experiment was repeated three times. Densitometry was utilized to further assess each gel.

MMP-2, MMP-9 and VEGF assays. The log-phase cells were seeded in 6-well plates ($1x10^5$ cells/well) and incubated at 37˚C in a humidified atmosphere of 5% CO_2/95% air. After 48 h, the media were harvested and spun at 800 x g for 5 min at 4˚C to remove cell debris. The supernatant was assayed immediately

using ELISA kits (catalog nos. DMP200, DMP900, DVE00; R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol. In the meantime, the cell count was carried out after trypsinization. The experiments were repeated three times.

In vitro invasion assay. The invasion assay was performed using BD BioCoat Matrigel Invasion Chambers (BD, Franklin Lakes, NJ, USA) as previously described (10), with several modifications. The chamber contained an 8-μm pore-size PET membrane coated with a uniform layer of BD Matrigel Basement Membrane Matrix, the membrane separating the chamber into two compartments. Cells ($5x10^4$) to be assayed were placed in the upper compartment in 400 μl complete medium, and 600 μl 3T3 conditioned medium (as a chemoattractant) was placed in the lower compartment. The Matrigel invasion chamber was incubated for 24 h at 37˚C in a humidified atmosphere of 5% CO_2/95% air. Non-invading cells on the upper surface of the Matrigel were wiped off using a cotton swab. Invading cells on the lower surface of the membrane were fixed, stained with H&E (Sigma-Aldrich), and counted under a microscope by randomly selecting five fields per membrane (magnification x200). Experiments were performed in triplicate.

HUVEC tube formation assay. The HUVEC tube formation assay was performed using a slight modification of a previously described method (11). Cells were cultured in serum-free RPMI-1640 medium for 24 h. The conditioned media were collected, centrifuged, transferred to fresh tubes and stored at -20˚C. After being thawed on ice, growth factor-reduced Matrigel Matrix (BD) was diluted with RPMI-1640 medium (1:3). The 24-well plate was coated with diluted Matrigel Matrix (100 μl/well) and incubated at 37˚C for 30 min to allow the Matrigel Matrix to polymerize. HUVECs were trypsinized and seeded ($1x10^5$ cells/well) in triplicate with conditioned media (1 ml/well). The plate was incubated for 6 h. Each well was photographed at x200 magnification using an inverted microscope with digital camera. The assessment of the total length of the vessel perimeter in a field was calculated using the Image-Pro Plus 6.0 program (Media Cybernetics, Bethesda, MD, USA). The mean value of three random fields was analyzed.

In vivo growth assay. Each experimental group contained six nude mice. Nude mice (4-week-old males) were purchased from the Laboratory Animal Center of Chongqing Medical University, housed in a pathogen-free animal facility, and fed a commercial basal diet and water *ad libitum*. The Chongqing Administrative Committee of Laboratory Animals approved the experimental protocol for the use of the animals in the study. Trypsin (0.25%) with EDTA was used to harvest monolayers of SGC-7901 cells. These cells were washed twice with PBS and counted for cell number and viability with trypan blue exclusion, and then resuspended in PBS at a concentration of $5x10^6$ cells in 100 μl. Cell viability of at least 90% was required for experimental use. Cells ($1x10^6$) in PBS were injected subcutaneously into the flank region of nude mice. The animals were observed once a day, then sacrificed by CO_2 inhalation after 4 weeks. The subcutaneous tumors were then excised from the animals. The size of the tumors was determined by caliper measurement. Tumor volume was calculated according to the formula: 0.5 x length x $width^2$.

Assessment of immunohistochemistry and microvessel density. After being excised from the animals, subcutaneous tumors were fixed in 4% buffered paraformaldehyde and embedded in paraffin. The tumors were sectioned (5 μm), and the tumor tissue sections were heated at 58˚C for 2 h. Subsequently, the tissue sections were dewaxed with xylene twice for 10 min, followed each time by washing with 100, 95 and 75% ethanol and rinsing with PBS. After being incubated in 3% H_2O_2 to block endogenous peroxidase activity, the tissue sections were boiled in 10 mmol/l citrate buffer (pH 6.0) twice for 6 min for antigen retrieval. The anti-mouse CD34 antibody (1:200 dilution; Biosynthesis, Beijing, China) was applied to tissue sections, and the sections were further incubated for 2 h in a humidified chamber at 37˚C, then washed three times with PBS and incubated in Polymer Helper (Zhongshan, Beijing, China) for 20 min. After washing, the sections were incubated in a polyperoxidase-anti-rabbit IgG (Zhongshan) for 30 min at room temperature. The peroxidase was visualized with 3-3'-diaminobenzidinetetrahydrochloride (DAB; Sigma-Aldrich) solution, and then counterstained with hematoxylin (Sigma-Aldrich). The tissue sections were scanned at low magnification to select densely vascularized areas (hot spots). Microvessel counts were performed in three random fields of hot spots by two independent pathologists at x200 magnification, and the mean number of microvessels was analyzed (12).

Statistical analysis. Statistical differences were evaluated by one-way analysis of variance (ANOVA) followed by LSD multiple comparison tests using SAS Software (SAS Institute Inc., Cary, NC, USA). $P<0.05$ was used to indicate statistical significance.

Results

DNEGFR-EGFP inhibited cell growth in vitro and in a mouse model. The MTT assay indicated that DNEGFR-EGFP inhibited the growth of SGC-7901 cells *in vitro* ($P<0.05$) (Fig. 1A). As cell growth *in vitro* is often associated with cell growth *in vivo*, we next extended the *in vitro* assay to the mouse model to determine the inhibitory effect of DNEGFR-EGFP on the growth of subcutaneous mouse xenografts. Cells were injected subcutaneously into the flank region of nude mice. After 4 weeks, nude mice in the DC group developed smaller tumors compared with those in the UC and EC groups ($P<0.05$) (Fig. 1B). The mean volumes of the tumors were 1.07±0.17, 1.03±0.11 and 0.38±0.02 cm^3 in the UC, EC and DC groups, respectively (Fig. 1C). Compared with the UC group, the mean volume of tumors in the DC group decreased by 64.5%.

DNEGFR-EGFP induced G_0/G_1 arrest. To investigate the effect of DNEGFR-EGFP on cell cycle progression in SGC-7901 cells, cell cycle assay with PI staining and flow cytometry was performed. The G_0/G_1 phase fractions in the UC and EC groups were 50.03±2.01 and 49.61±0.49%,

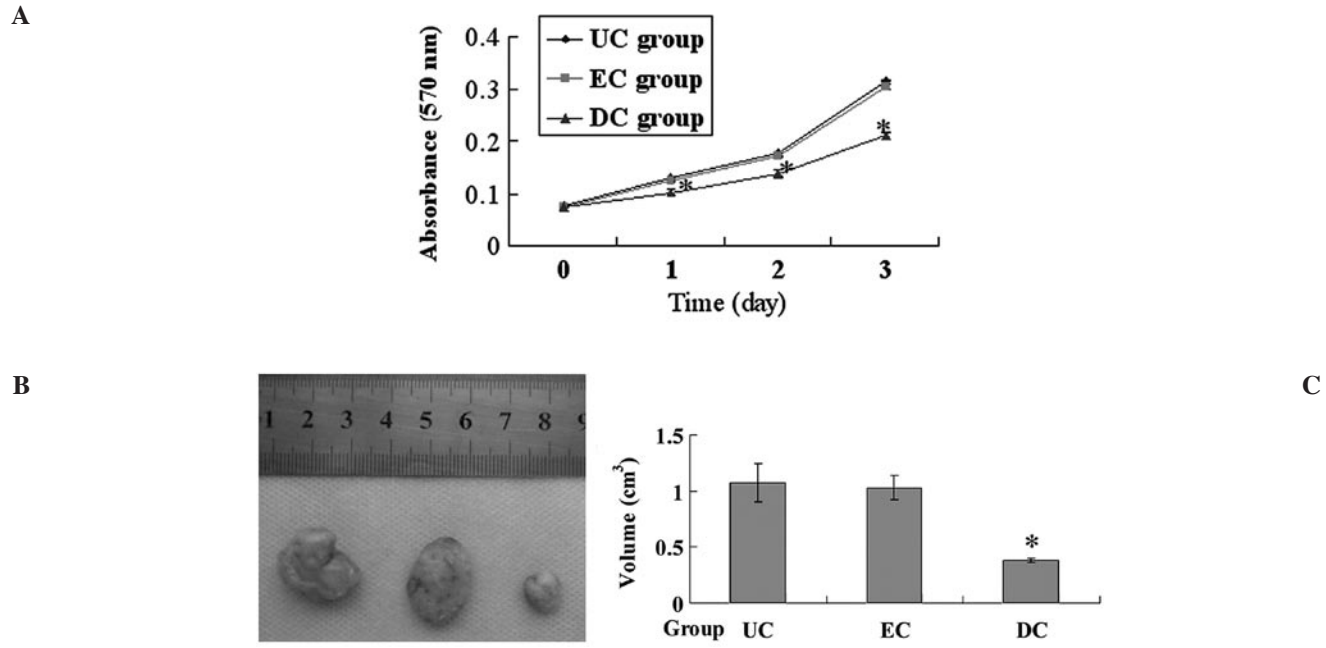

Figure 1. DNEGFR-EGFP inhibited cell growth *in vitro* and in a mouse model. (A) Growth curves of cells plotted using the MTT assay. Each group was analyzed every day in triplicate for 24-72 h. Each data point was the mean ± SD from three independent experiments; bars, SD. (B and C) The volume of each tumor was calculated according to the formula: 0.5 x length x width^2. Each experimental group contained 6 nude mice. Columns, mean values of 6 nude mice; bars, SD. *P<0.05 compared with the UC and EC groups.

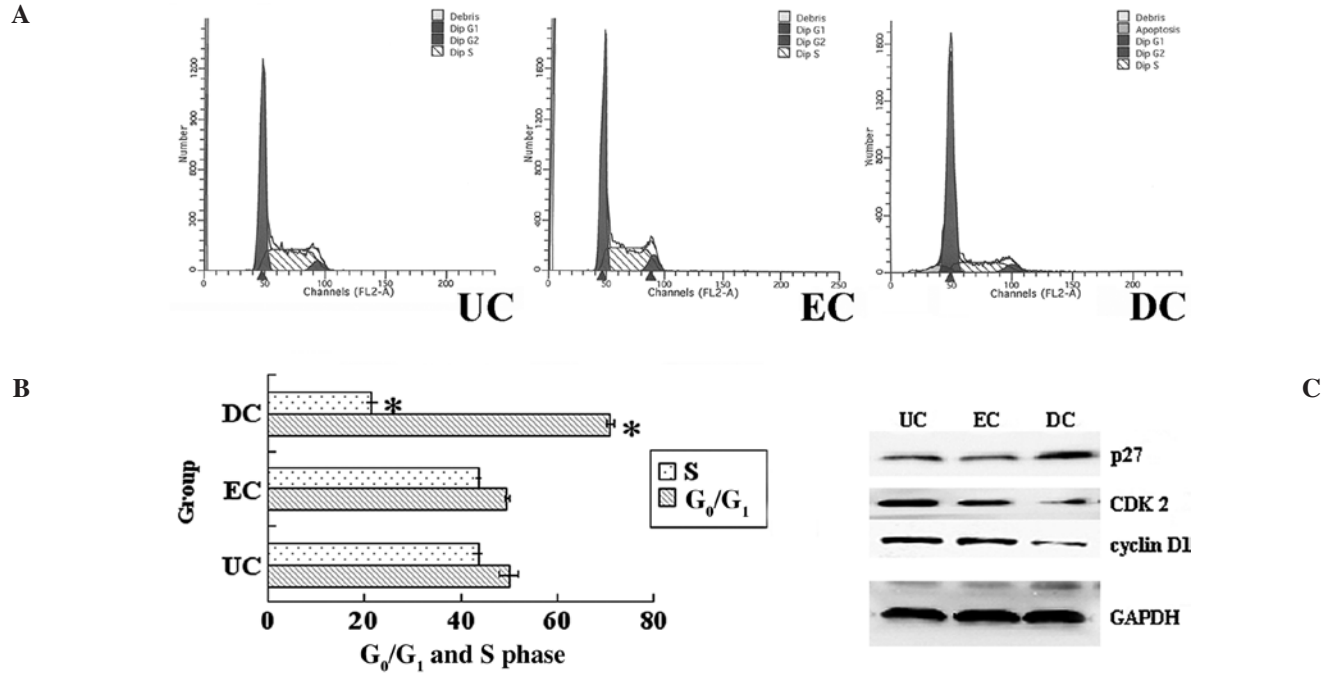

Figure 2. DNEGFR-EGFP induced G_0/G_1 arrest by down-regulating cyclin D1 and CDK2 protein and up-regulating p27 protein. (A and B) DNEGFR-EGFP-induced G_0/G_1 arrest. Columns, mean values of three samples; bars, SD. *P<0.05 compared with the UC and EC groups. (C) DNEGFR-EGFP decreased cyclin D1 and CDK2 and increased p27 at the protein level. The expression levels of cyclin D1, CDK2 and p27 were detected by Western blot analysis. Experiments were repeated three times.

respectively, and increased to 70.88±0.85% in the DC group. The S phase fractions in the UC and EC groups were 43.63±1.26 and 43.63±0.64%, respectively, and decreased to 21.58±1.40% in the DC group (P<0.05) (Fig. 2A and B). These results indicate that DNEGFR-EGFP led to the G_0/G_1 arrest of the SGC-7901 cells.

To further elucidate the mechanisms of G_0/G_1 arrest, we examined the effects of DNEGFR-EGFP on the expression of several key regulators of the G_1-S phase transition. As shown in Fig. 2C, the protein levels of cyclin D1 and CDK2 decreased, while those of p27 increased (P<0.05). These results indicate that the down-regulation of cyclin D1 and

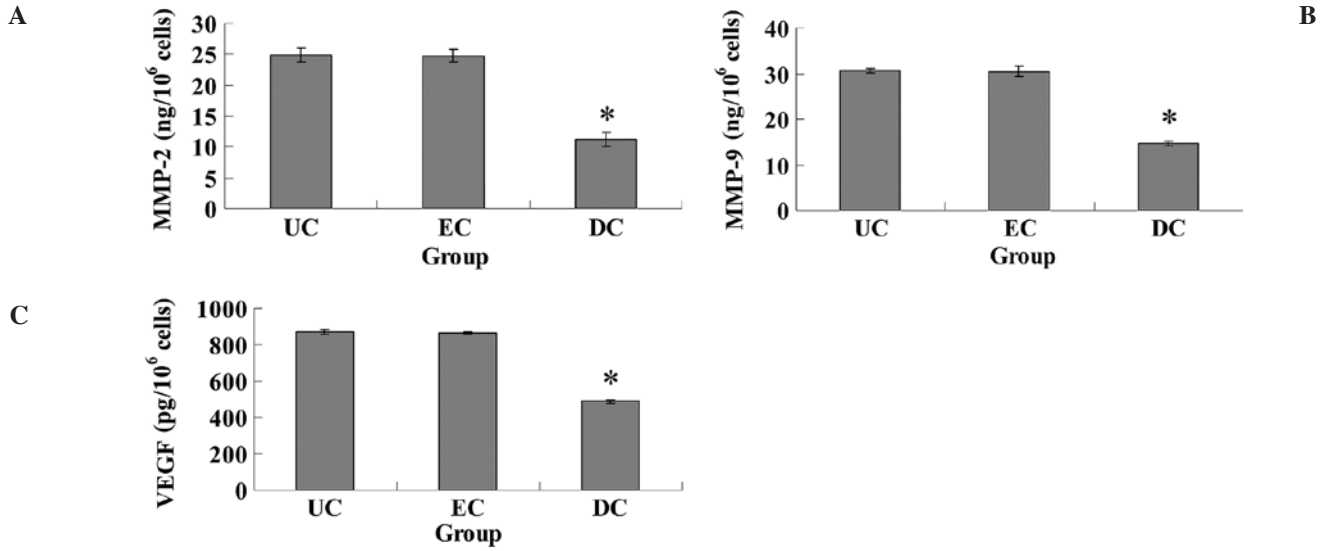

Figure 3. DNEGFR-EGFP inhibited cells from secreting (A) MMP-2, (B) MMP-9 and (C) VEGF. The cell supernatant was assayed using ELISA kits following the manufacturer's protocol, and a cell count was performed. The experiments were repeated three times. Columns, mean values of three samples; bars, SD. *P<0.05 compared with UC and EC groups.

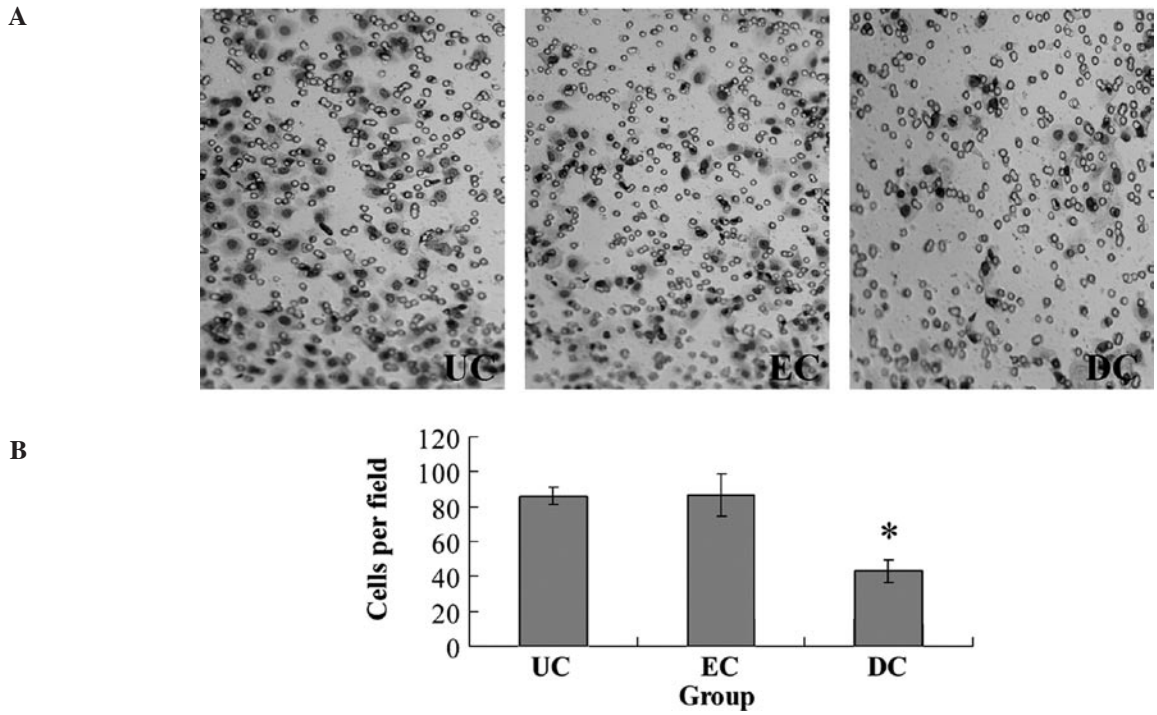

Figure 4. DNEGFR-EGFP decreased cell invasion. (A) Representative photomicrographs (magnification x200) of the *in vitro* invasion assay. Invading cells on the lower surface of the membrane were fixed, stained and counted under a microscope by randomly selecting five fields per membrane (x200). Experiments were performed in triplicate. (B) The mean number of cells was summarized. Columns, mean values of three independent experiments; bars, SD. *P<0.05 compared with UC and EC groups.

CDK2 protein expression and the up-regulation of p27 protein expression may be responsible for G_0/G_1 arrest induced by DNEGFR-EGFP.

DNEGFR-EGFP decreased the levels of MMP-2, MMP-9 and VEGF. Since protein levels of MMP-2, MMP-9 and VEGF are closely linked to invasion and angiogenesis in gastric cancer (13), we investigated the effects of DNEGFR-EGFP on the protein levels of MMP-2, MMP-9 and VEGF. As shown in Fig. 3, the protein levels of MMP-2, MMP-9 and VEGF secreted in culture media in the DC group decreased compared with those of the UC and NC groups (P<0.05). These results indicate that DNEGFR-EGFP inhibited cells from secreting MMP-2, MMP-9 and VEGF.

DNEGFR-EGFP decreased cell invasion. MMP-2 and MMP-9 are thought to be critically involved in the process of tumor cell invasion. Since DNEGFR-EGFP inhibited the secretion

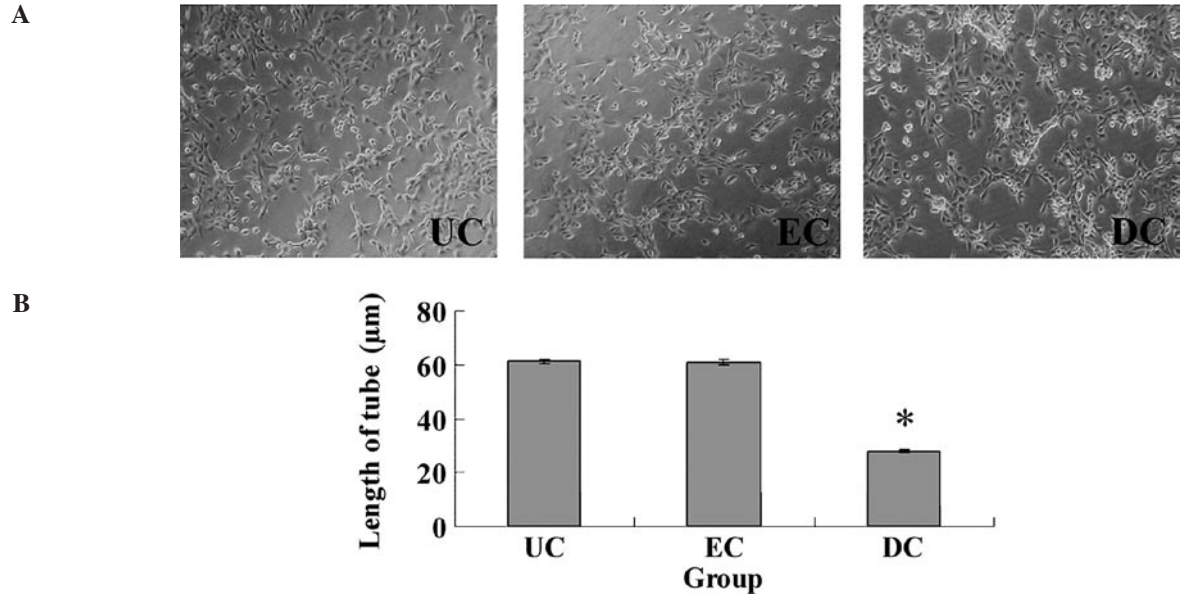

Figure 5. DNEGFR-EGFP inhibited HUVEC tube formation. (A) Representative photomicrographs (magnification x200) of HUVEC tube formation in conditioned media. HUVECs were seeded ($1x10^5$ cells/well) on Matrigel-coated 24-well plates with 1 ml of conditioned media per well in triplicate and incubated for 6 h. (B) The mean tube length was summarized. Values were presented as the mean ± SD of three independent experiments. Bars, SD. *P<0.05 compared with the UC and EC groups.

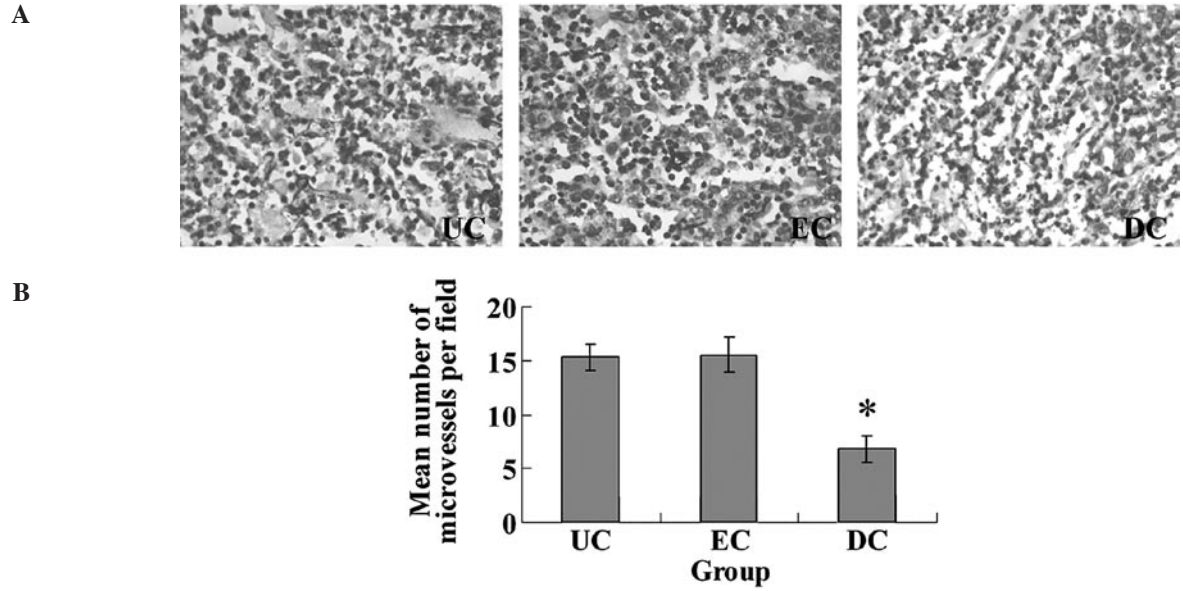

Figure 6. DNEGFR-EGFP inhibited angiogenesis *in vivo*. (A) Representative photomicrographs (magnification x200) of microvessels of tumor tissue sections. (B) Summarization of microvessel density, calculated by averaging the number of microvessels in the primary tumors of each group (n=6). Bars, SD. *P<0.05 compared with the UC and EC groups.

of MMP-2 and -9, we further tested the effect of DNEGFR-EGFP on cell invasion. Invasive potential was determined on the basis of the ability of cells to invade the Matrigel-coated membrane containing mainly laminin and collagen type IV, which are major components of the extracellular matrix (ECM). As illustrated in Fig. 4, cells in the DC group (43.0±6.0 cells/field) showed a lower level of penetration through the Matrigel-coated membrane compared with cells in the UC (86.0±5.0 cells/field) and NC (86.3±12.1 cells/field) groups (P<0.05). These results indicate that DNEGFR-EGFP decreased cell invasion.

DNEGFR-EGFP inhibited HUVEC tube formation in vitro and angiogenesis in vivo. VEGF is a key mediator of tumor angiogenesis (14-16). Since DNEGFR-EGFP inhibited the secretion of VEGF, we investigated whether conditioned media in the DC group reduced the tube formation of HUVECs, an indirect measure of angiogenesis. Conditioned media in the DC group significantly reduced tube formation compared with media in the UC and NC groups (P<0.05) (Fig. 5). As a direct method, microvessel density (MVD) assessment of tumor sections is considered the gold standard for measuring angiogenesis (17). To further test the effect of DNEGFR-

EGFP on angiogenesis *in vivo*, microvessel counts of tumor sections were performed. A significant reduction in MVD was noted in mice in the DC group compared with those in the UC and NC groups ($P<0.05$) (Fig. 6).

Discussion

EGFR overexpression has been confirmed in gastric cancer (9). The EGFR signaling pathway is involved in cell division, invasion and angiogenesis, and correlates with the poor prognosis of patients with EGFR-overexpressed gastric cancers. Therefore, EGFR blockade is considered to be a promising therapeutic approach for EGFR-overexpressed gastric cancers. EGFR-targeted monoclonal antibodies, tyrosine kinase inhibitors, have been accepted as cancer therapy strategies (18-21). DNEGFR may thus become a novel therapy strategy for the treatment of EGFR-overexpressed gastric cancer. It was found that DNEGFR blocked EGFR signal transduction cascades and inhibited the tumorigenicity of rat ovarian cancer NuTu-19 cells and human glioblastoma multiforme cells (7,8). However, the effects of DNEGFR on the growth, invasion and angiogenesis of human gastric cancer cells remain unknown.

In this study, we constructed a pEGFPN1-DNEGFR vector. After being transfected with the vector, SGC-7901 cells expressed DNEGFR-EGFP. Since DNEGFR-EGFP inhibited cell growth *in vitro* and in a mouse model, we examined whether this cell growth inhibition was due to cell cycle arrest in any specific phase of the cell cycle. Cell growth inhibition was found to result from G_0/G_1 arrest. To investigate the molecular mechanisms behind this, we determined the protein levels of cyclin D1, CDK2 and p27. Protein levels of cyclin D1 and CDK2 were decreased, while those of p27 were increased. The results indicate that DNEGFR-EGFP leads to G_0/G_1 arrest by down-regulating cyclin D1 and CDK2 and up-regulating p27, consistent with previous reports using EGFR monoclonal antibody cetuximab in squamous cell carcinoma cells (22).

DNEGFR-EGFP inhibited cells from secreting MMP-2, MMP-9 and VEGF. MMPs are critically involved in the process of tumor cell invasion (23,24); MMP-2 and -9 are thought to be key enzymes involved in the degradation of type IV collagen, a component of the ECM. High levels of MMP-2 and -9 in tissues are associated with tumor cell invasion (25). We investigated the effect of DNEGFR-EGFP on cell invasion using invasion chambers. The results indicate that DNEGFR-EGFP repressed cell invasion by inhibiting cells from secreting MMP-2 and -9. VEGF is a central mediator of angiogenesis (15,16). We postulated that DNEGFR-EGFP inhibits tumor angiogenesis by inhibiting cells from secreting VEGF, and therefore tested the effect of DNEGFR-EGFP on angiogenesis *in vitro* by HUVEC tube formation assay. DNEGFR-EGFP was found to decrease the tube formation of HUVECs. To further investigate the effect of DNEGFR-EGFP on angiogenesis in a mouse model, microvessel counts of tumor sections were performed. The MVD of the tumor sections was decreased, which is consistent with the results of the HUVEC tube formation assay. Angiogenesis is fundamental to tumor growth and invasion (17). For a cancer to grow more than 2-3 mm^3, it requires its own blood supply to meet the demands of tumor cell metabolism (26). DNEGFR-EGFP inhibited angiogenesis, and the inhibition of cancer cell angiogenesis results in the inhibition of tumor growth and invasion. The inhibition of tumor growth decreases the secretion of VEGF, thus inhibiting angiogenesis in turn. In addition to stimulating angiogenesis by VEGF paracrine loops, VEGF promotes the growth of cancer cells expressing VEGF receptors by autocrine loops (27,28). Therefore, DNEGFR-EGFP may simultaneously inhibit the growth of SGC-7901 cells by VEGF autocrine loops. Other EGFR blockade strategies, such as cetuximab, were more effective in combination with radiotherapy or chemotherapy for patients with EGFR-overexpressed cancers compared with radiotherapy or chemotherapy alone (18,29,30). Whether DNEGFR-EGFP can enhance the radiosensitivity and chemosensitivity of SGC-7901 cells remains to be confirmed through further study.

Taken together, DNEGFR-EGFP led to G_0/G_1 arrest by down-regulating cyclin D1 and CDK2 and up-regulating p27, and also repressed the invasion and angiogenesis of SGC-7901 cells by inhibiting them from secreting MMP-2, MMP-9 and VEGF. These observations indicate that the EGFR blockade strategy (termed dominant negative strategy targeting EGFR) may serve as a promising therapy for the treatment of EGFR-overexpressed gastric cancers.

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