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Original Article

Angiogenin negatively regulates the expression of basic fibroblast growth factor (bFGF) and inhibits bFGF promoter activity

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Abstract: Research focused on angiogenin and basic fibroblast growth factor (bFGF) was expected to identify means to inhibit tumor growth and metastasis. However, interactions exist between the two angiogenic factors, and the overall mechanism is still not clear. To explore the mechanism by which angiogenin regulates the expression of bFGF, RT-PCR and western blot were performed to analyze bFGF expression. Nuclear translocation of angiogenin was investigated by immunofluorescence staining and immune electron microscopy. Site-directed mutagenesis, reporter gene assays, and electrophoretic gel mobility shift assay (EMSA) were applied to investigate the promoter activity. The results showed that angiogenin negatively regulates bFGF expression in HeLa cells. Angiogenin undergoes nuclear translocation, and an angiogenin binding site (CTCTCTCT) on the bFGF promoter was identified. bFGF promoter activity was inhibited by angiogenin. Angiogenin and bFGF are two potent and representative angiogenesis factors, that play an important role in tumor angiogenesis and development. These mechanisms guide us to further investigate the effect of angiogenin and bFGF on tumor growth and development.

Keywords: Angiogenin, bFGF, expression, promoter activity

Introduction

The growth and metastasis of solid tumors depend on angiogenesis [1] and many angiogenic factors have been isolated and identified. Two such factors that are prominent in tumor growth and metastasis are basic fibroblast growth factor (bFGF) and angiogenin.

Angiogenin was first isolated from the medium of human colon cancer HT-29 cells because of its angiogenic activity [2]. Expression of angiogenin is up-regulated in a variety of cancers [3, 4]. Angiogenin participates in many cellular events that are necessary for angiogenesis. For example, it induces blood vessel formation in the rabbit cornea and chicken embryo chorioallantoic membrane [2]. Angiogenin interacts with a 42 kDa binding protein or a 170 kDa putative receptor on the surface of endothelial cells to activate cell-associated proteases [3, 4]. In addition to its angiogenic activity, angiogenin also has a direct effect on cancer cell proliferation, migration, and invasion [5, 6].

Angiogenin is a member of the ribonuclease superfamily. Although the ribonucleolytic activity of angiogenin is much lower than that of pancreatic ribonuclease, this action is necessary for angiogenin to participate in the process of angiogenesis [7]. Angiogenin undergoes nuclear translocation in endothelial cells, and the process is rapid and strictly dependent on cell density, but is independent of microtubules and lysosomes [8-10]. Inhibition of nuclear translocation of angiogenin in endothelial cells abolishes its angiogenic activity [8-10]. Nuclear translocation of angiogenin has not been observed in normal non-blood vessel cells or confluent endothelial cells [9-11]. Except for ribonucleolytic activity in endothelial cells, the function of angiogenin in the nucleus is unclear in cancer cells.

bFGF is one of the most potent angiogenic factors and its expression is up-regulated in numerous types of cancer cells and tissues, such as cervical cancer [12-14]. bFGF is also a potent mitogen for different cell types. bFGF

induces proteinase production and expression of specific integrins and, therefore, plays an important role in cell proliferation and migration in cultured endothelial cells [15-17]. bFGF is also involved in development and differentiation of various organs [18-20].

In recent years, research focused on angiogenic factors and angiogenic inhibitors was expected to identify a means to inhibit tumor growth and metastasis. However, numerous interactions exist between different angiogenic factors, and the overall mechanism of angiogenesis is still not clear. We have previously reported that bFGF affects angiogenin expression and cell proliferation in human melanoma A375 cells. In this study, we further investigated the interaction between the two factors and the mechanism by which angiogenin regulates the expression of bFGF.

Materials and methods

Cell culture and plasmids

Human cervical cancer cell lines HeLa and human embryonic kidney cell lines 293T were acquired from the Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS.

The plasmids pCI-Ang(+) and pCI-Ang(-), which contain human angiogenin coding region in sense and antisense orientations, were kindly provided by Dr Guo-fu Hu. The plasmid containing fragments of the human bFGF gene (-1,800/+314) was a gift from Michal K Stachowiak. We constructed luciferase expression vectors using promoterless pGL3-enhancer (Promega Biotec, Madison, WI). Cell transfection were done by means of Lipofectamine (GIBCO, Invitrogen Corporation, Grand Island, New York, USA).

Semi-quantitative reverse transcriptase polymerase chain reaction assay (RT-PCR)

TRI reagent (Invitrogen Corporation, Grand Island, NY, USA) was used for total RNA extraction. RT kit (Promega Corporation, Madison, WI, USA) was used to synthesize first-strand cDNA. Primer sequences for bFGF: 5'-ATGGCAGCCGGGAGCATCACC-3' (forward primer) and 5'-CACACACTCCTTTGATAGACACAA-3' (reverse primer). Sequences for angiogenein: 5'-CATCATGAGGAGACGGGG-3' (forward primer) and 5'-TCCAAGTGGACAGGTAAGCC-3' (reverse primer).

Western blot analysis

Cells were harvested and lysed with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 100 mM β-mercaptoethanol, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate). Equal amounts of total proteins were separated by 12% SDS PAGE and transferred to nitrocellulose membrane (Bio-Rad, Hercules, California, USA). After blocking with 5% non-fat dried milk in TBS, membranes were incubated with an anti-bFGF polyclonal antibody (R&D Systems) and an anti-actin monoclonal antibody (Sigma-Aldrich, St. Louis, Missouri, USA) overnight at 4°C. Membranes were then incubated with HRP-linked secondary antibodies (Beijing Zhongshan Golden Bridge Biotechnology, China) for 1 h at 37°C. The signals were detected by Super Signal West pico chemiluminescent Substrate (Pierce, Rockford, Illinois, USA).

Immunofluorescence staining

Cells were washed and fixed with cold methanol for 20 min, and permeabilized with 0.1% Triton X-100 for 15 min. After blocking in 5% bovine serum albumin for 30 min, cells were incubated with anti-bFGF monoclonal antibody overnight at 4°C. The cells were then washed with PBS and stained with FITC-conjugated secondary antibodies (1:200 dilution; Proteintech, Chicago, IL, USA) for 1 h in the dark. Images were observed by confocal laser microscopy (Olympus, FV1000, Tokyo, Japan).

Immune electron microscopy

Cells were washed and incubated with 1 μg/ml angiogenin for 1 h at 37°C and then fixed in 2.5% glutaraldehyde and 4% paraformaldehyde for 2 h. After washing, cells were dehydrated in an ethanol series and permeabilized with 100% ethanol, and embedded in Lowicryl K4M for 24 hours under UV irradiation at -35°C, and for another 2-3 days at room temperature. Ultra-thin Lowicryl sections were mounted on Formvar nickel grids, blocked in 1% BSA, and then incubated with an anti-angiogenin monoclonal antibody 26-2F (provided by Dr. Guofu Hu, Harvard Medical School) at 4°C overnight. Anti-mouse IgG conjugated with gold particles

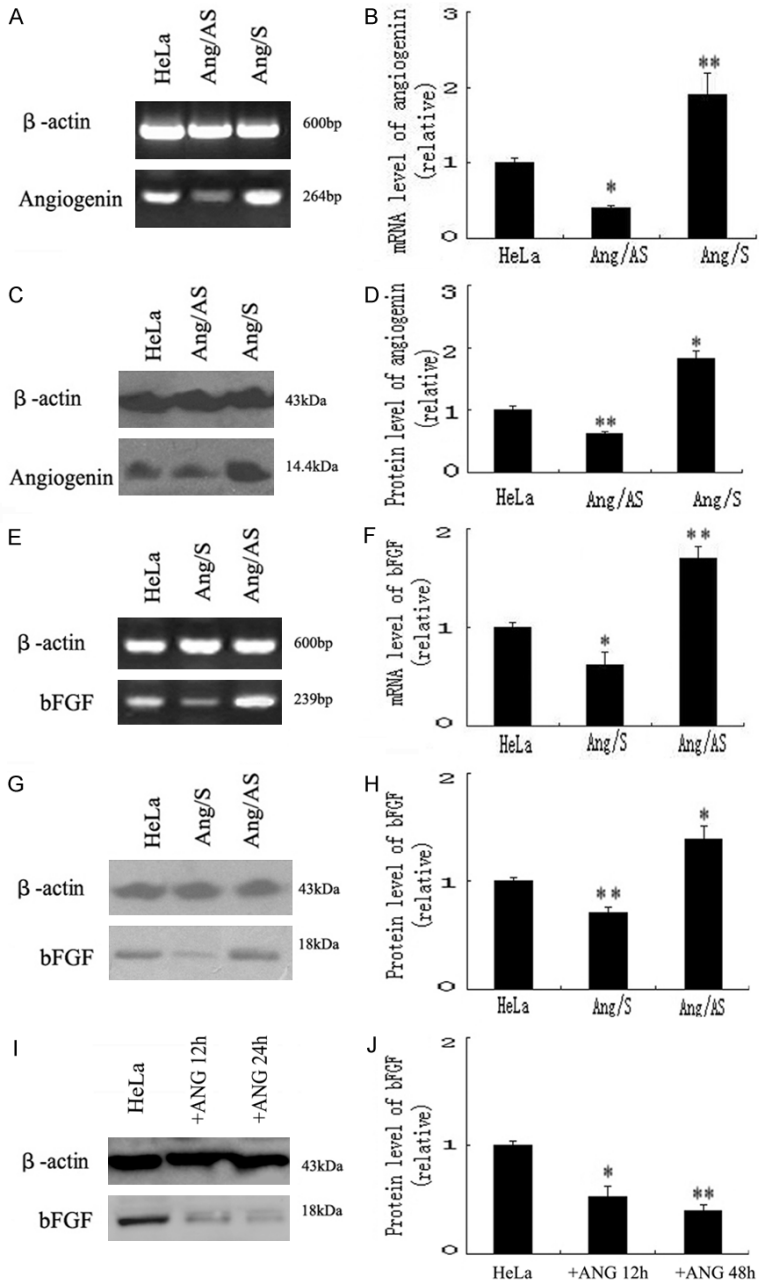

Figure 1. The effect of angiogenin on basic fibroblast growth factor (bFGF) expression. A. Angiogenin mRNA level in angiogenin over-expressing/under-expressing cells was detected by RT-PCR. B. Densitometric analysis of the ratio of angiogenin mRNA to Beta-actin mRNA. (*P=0.018 compared with control, **P=0.009 compared with control). C. Angiogenin protein level in angiogenin over-expressing/under-expressing cells was detected by western blot. D. Densitometric analysis of the expression levels of angiogenin. (*P=0.015 compared with control, **P=0.024 compared with control). E. bFGF mRNA level in angiogenin over-expressing/under-expressing cells was detected by RT-PCR. F. Densitometric analysis of bFGF mRNA expression. (*P=0.016 compared with control, **P=0.037 compared with control). G. bFGF protein level in angiogeninover-expressing/under-expressing cells was detected by western blot. H. Densitometric analysis of bFGF protein expression for picture G. (*P=0.014 compared with control, **P=0.009 compared with control). I. bFGF protein level in exogenous angiogenin stimulated cells was detected by western blot. J. Densitometric analysis of bFGF protein expression for picture I. (*P=0.032 compared with control, **P=0.015 compared with control).

(Biocell, Cardiff, UK) was used to reveal antigen-antibody complexes. The sections were washed and stained with 5% uranyl acetate, and observed under an electron microscope (Hitachi EM H-600-2).

Site-directed mutagenesis

The sequence CTCTCTCT of the bFGF promoter was mutated to CGCGCTCT. Site-directed mutagenesis was performed using QuikChange II XL kit according to the manufacturer's instructions (Stratagene, USA) and the oligo designed from human FGF-2 exon 1 genomic clone sequence (accession no. X04431) is 5'-GGATTTACAGAAAATAACGCGCTCTCCAAGAAATGC-3'.

Reporter gene assays

Luciferase activity of transfected cells were analyzed using a Dual-Luciferase Reporter Assay System (Promega, USA) and detected by Turner Designs TD20/20 Luminometer (Sunnyvale, CA, USA). The activity of Renilla luciferase was analyzed as control.

Electrophoretic gel mobility shift assay (EMSA)

Cells were harvested and nuclear protein was prepared. EMSA was performed using a LightShift® Chemiluminescent EMSA Kit (Pierce, MA, USA). Competitor double-stranded oligonucleotides were incubated with nuclear protein in binding buffer for 10 min and then biotin-labeled probe was added and incubated for 20 min. The reactions were separated on a 5% polyacrylamide gel and transferred to nylon membrane (Bio-Rad, Hercules, California, USA). Chemiluminescence was used to detect biotin-labeled DNA.

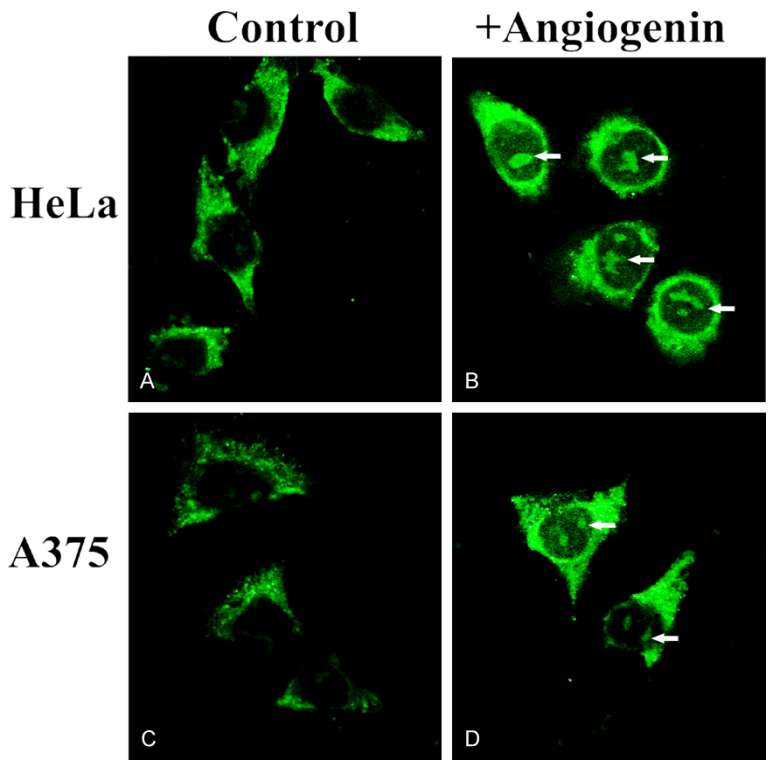

Figure 2. Angiogenin translocated to the nucleus. Nuclear translocation of angiogenin was detected by immunofluorescent staining. Cells were no-stimulated (A, C) or stimulated with 1 μg/ml of angiogenin (B, D) for 4 h, and fixed with methanol. Cells were incubated with anti-angiogenin monoclonal antibody and then stained with FITC-conjugated secondary antibodies. Images were observed by confocal laser microscope.

Statistics

Data analysis was performed using the Statistical Package for the Social Sciences, version 22 (IBM corp. Armonk, NY, USA). All the data are presented as means ± SD of at least three independent experiments and analyzed using the Student's *t* test. $P < 0.05$ was considered significant.

Results

The effect of angiogenin on bFGF expression

HeLa cells were stably transfected with the plasmids pCI-Ang(+) and pCI-Ang(-) to obtain angiogenin over-expressing and under-expressing cells (**Figure 1A-D**). bFGF is a key mediator of proliferation in many cancers; therefore, we examined bFGF expression in angiogenin transfectants. RT-PCR and western blot analysis were performed to determine mRNA and protein levels. As shown in **Figure 1E-H**, the angiogenin over-expressing cells expressed a lower amount of bFGF than controls. However, the angiogenin under-expressing cells expressed a higher amount of bFGF compared with controls. To further confirm the effect of angiogenin, exogenous angiogenin was used to stimulate HeLa cells and the result showed that the expression of bFGF decreased in exogenous angiogenin stimulated cells (**Figure 1I**, **1J**).

Translocation of angiogenin to the nucleus

Immunofluorescence staining showed exogenous angiogenin was taken up and translocated into the nucleus of HeLa and A375 cells (**Figure 2**). Nuclear translocation of angiogenin was also detected by immune electron microscopy (**Figure 3**). An anti-angiogenin monoclonal antibody and 5 nm colloid gold-conjugated IgG showed the main region to which angiogenin translocated was dense fibrillar components (DFCs) near fibrillar centers (FCs).

Angiogenin inhibits bFGF promoter activity

To assess whether angiogenin can influence bFGF promoter activity, HeLa cells were co-transfected with pGL3-bFGF Luc (-1800/+314) and the angiogenin expression vector pCI-Ang(+). As a positive control, pREP7-RLuc and a P53 expression vector were also cotransfected into cells. Significant inhibition of (-1800/+314) bFGF Luc expression was observed with angiogenin over-expression (**Figure 4A**). The inhibition was enhanced with increased angiogenin expression (**Figure 4B**).

Involvement of the sequence CTCTCTCT in the angiogenin inhibition of bFGF promoter activity

As shown in **Figure 5A**, the sequence CTCTCTCT in the bFGF promoter was mutated to

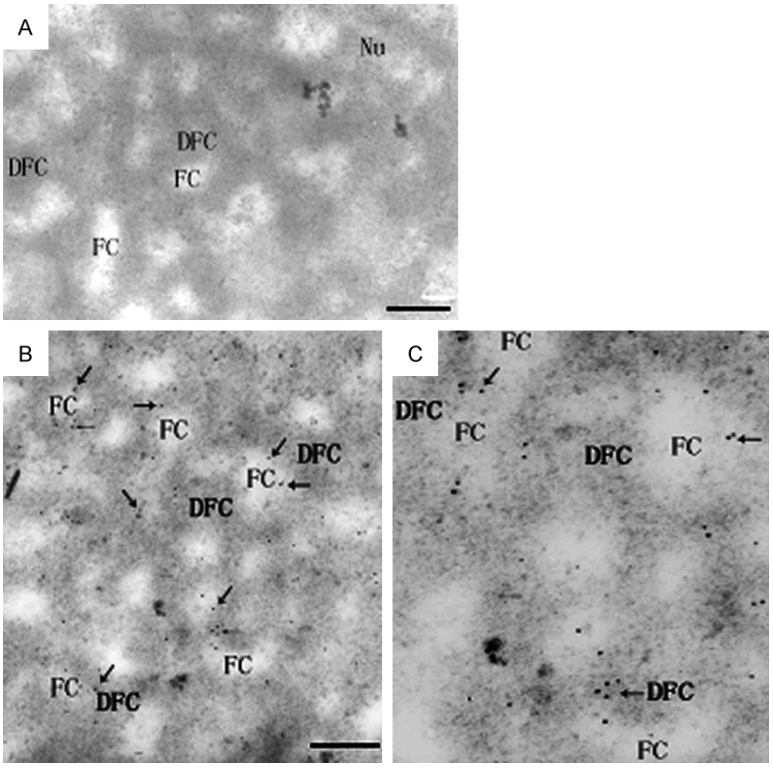

Figure 3. Immune electron microscopy showed the nuclear translocation of angiogenin in A375 cells. A375 cells were cultured in DMEM supplemented with 10% FBS for 24 h and then washed with serum-free medium and incubated with 1 μg/ml angiogenin at 37°C for 1 h. After treatment, cells were embedded and sliced, and then the sections were incubated with anti-angiogenin monoclonal antibody 26-2F at 4°C overnight. Antigen-antibody complexes were revealed with anti-mouse IgG conjugated with 5 nm gold particles (Biocell, Cardiff, UK). The sections were then stained with 5% uranyl acetate, and observed under a Hitachi EM H-600-2 at 75 KV. (A) Nucleolus of cells unstimulated with angiogenin. (B) Nucleolus of cells stimulated with angiogenin. (C) Magnification of (B). (Nu: nucleolus. FC: fibrillar center. DFC: dense fibrillar component. Bar: 0.2 μm).

CGCGCTCT using the oligo 5'-GGATTTACAGAAAATAACGCGCTCTCCAAGAAATGC-3'. The mutated promoter construct, together with 4 μg angiogenin expression vector, were co-transfected into HeLa cells and luciferase activity was detected. Angiogenin produced less inhibition of bFGF promoter activity with the mutated promoter constructs compared with the control vector. The wild-type promoter activity was inhibited by 68.1% while the activity of the mutated promoter was inhibited by 22.9% (**Figure 5B**).

Binding of angiogenin to CTCTCTCT

Electrophoretic gel mobility shift assays revealed a specific protein-DNA complex between nuclear extracts from HeLa cells over-expressing angiogenin and a DNA fragment (5'-AATAACTCTCTCTCCAAGAAATGCAT-3') designed according to the bFGF promoter sequence and encompassing the CTCTCTCT sequence. A comigrating band was also obtained when angiogenin protein instead of HeLa nuclear extract was used (**Figure 5C**).

bFGF stimulates its own promoter activity

We confirmed that bFGF can stimulate its own promoter activity and that this stimulation is dose-dependent. As shown in **Figure 6A**, bFGF has maximum stimulation on its promoter activity at 1 μg/mL; the promoter activity increased by 79.1%. In a time-course study we found that at 24 h after transfection, the stimulation was maximal (**Figure 6B**). We also found that exogenous bFGF overcame angiogenin inhibition of bFGF promoter activity (**Figure 6C**). The angiogenin expression vector and pGL3-bFGFLuc (-1800/+314) reporter vector were co-transfected and then bFGF protein was added 5 h after transfection. The inhibition ratio of angiogenin on bFGF promoter activity was 41.2% when exogenous bFGF was absent, and the inhibition ratio was 23.1% in the presence of 0.1 μg/mL exogenous bFGF protein and 15.3% in the presence of 1 μg/mL exogenous bFGF protein.

Discussion

The growth and metastasis of solid tumors depends on angiogenesis. Tumors express a series of angiogenic factors. Among them, angiogenin and bFGF are potent and representative factors involved in tumor development. Many studies have focused on how they induce angiogenesis and effect tumor growth. In this study, we investigated the interaction between the two factors and the mechanism by which angiogenin regulates the expression of bFGF.

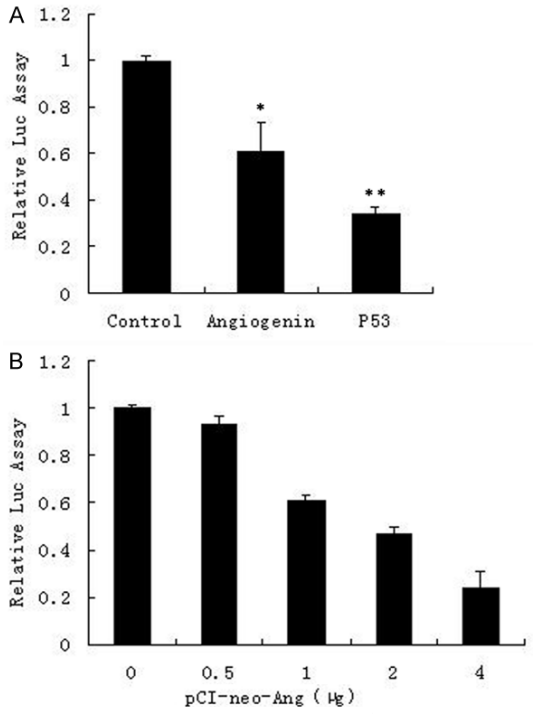

Figure 4. Inhibition of angiogenin on bFGF promoter activity. A. 1 μg of the angiogenin expression vector, together with pGL3 -bFGFLuc (-1800/+314) reporter vector were co-transfected into HeLa cells. Luciferase activities were determined by luciferase reporter assays and normalized to Renilla activity 24 h after transfection. P53 vector was as a positive control. *P=0.019, **P=0.008. B. The inhibition of angiogenin on bFGF promoter activity was dose-dependent. Different doses of angiogenin expression vector together with reporter vector were co-transfected into HeLa cells. Luciferase activities were determined 24 h after transfection.

The effect of angiogenin on bFGF expression was examined in HeLa cells. Angiogenin overexpressing cells actually expressed a lower amount of bFGF than controls, while the angiogenin under-expressing cells expressed a higher amount of bFGF. This result is consistent with our previous research in A375 cells [21]. Unlike melanoma, cervical cancer occurs only in females, and is the most common gynecological malignancy. It is conceivable that the regulatory mechanism between angiogenin and bFGF may be common to many types of cancer cell, and that investigation of this regulatory mechanism is important.

We have shown that angiogenin is constitutively translocated to the nucleus of HeLa cells and A375 cells and that it is mainly located in DFCs near FCs (**Figures 2**, **3**). The nuclear translocation of angiogenin is involved in rRNA transcription [4]. However, the mechanism of angiogenin nuclear translocation is still unknown, and it probably involves receptor-mediated endocytosis. Continuous nuclear translocation of angiogenin in cancer cells may certainly be a contributing factor to tumorigenicity and other processes.

It has been reported that inflammatory cytokines induce the expression of bFGF through the activation of AP-1 response elements in the bFGF promoter, and early growth response-1 protein plays a role in α1-Adrenergic stimulation of bFGF promoter activity in cardiac myocytes [22]. Angiogenin plays a role as a transcription factor in the synthesis of ribosomal RNA both in intact endothelial cells and isolated endothelial nuclei [4]. To verify if angiogenin regulates the expression of bFGF at the transcription level, we examined the effect of angiogenin on bFGF promoter activity. bFGF promoter activity was inhibited by 39.1% when 1 μg angiogenin expression vector was cotransfected and the inhibition was enhanced with increased expression of angiogenin (**Figure 4**).

We then found that a CTCTCTCT sequence was involved in angiogenin-induced inhibition of bFGF promoter activity and that angiogenin can directly bind to this site. An angiogenin-binding element (ABE: CTCTCTCTCTCTCTCTCCCTC) has been previously identified from the nontranscribed region of rDNA, and it is involved in rRNA synthesis [23]. In this study, we confirmed that four CT repeats can bind to angiogenin, which has an inhibition effect on promoter activity. Searching GenBank with a CTCTCTCTCTCT query sequence generated more than 5000 hits. It has been reported that CT repeats are involved in numerous biological events, including nucleosomal organization, recombination, and DNA replication [23]. According to these results, we presume that angiogenin may not only be related to bFGF expression but also may play a role in regulating the expression of other genes containing CT repeats.

However, when the CTCTCTCT sequence was mutated, inhibition of bFGF promoter activity by angiogenin decreased to 22.9%, but was not completely abolished. We also found that bFGF stimulates its own promoter activity in a dose-

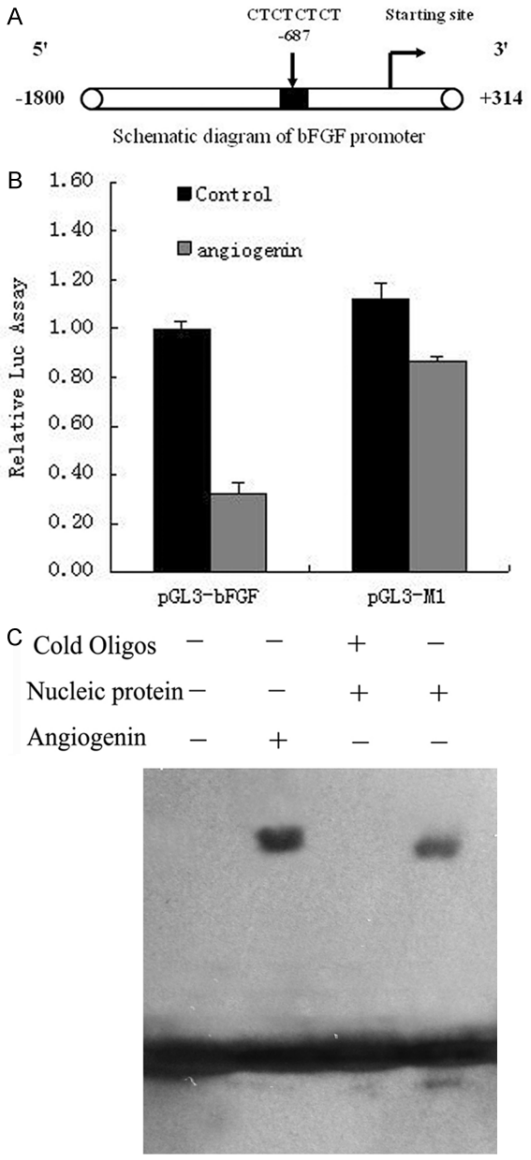

Figure 5. CTCTCTCT sequence was involved in the inhibition of angiogenin on bFGF promoter activity. A. Schematic diagram of bFGF promoter. B. Construction of bFGF promoter point mutation was detected by luciferase reporter assays. Angiogenin over-expressing vectors and bFGF promoter point mutation constructs were co-transfected into cells, and luciferase activities were depicted relative to the Renilla activity. C. Binding of angiogenin to CTCTCTCT. In vitro angiogenin was tested for binding to the site CTCTCTCT probe by EMSA. The nuclear protein was incubated with biotin-labeled DNA probe in binding buffer. For competition with DNA elements, competitor double-stranded oligonucleotides were added with nuclear extract for 10 min at room temperature and then biotin-labeled probe for an additional 20 min. The reactions were run through a 5% polyacrylamide gel and then transferred to nylon membrane. Cross-link transferred DNA to membrane using a UV-light. Biotin-labeled DNA was detected by chemiluminescence.

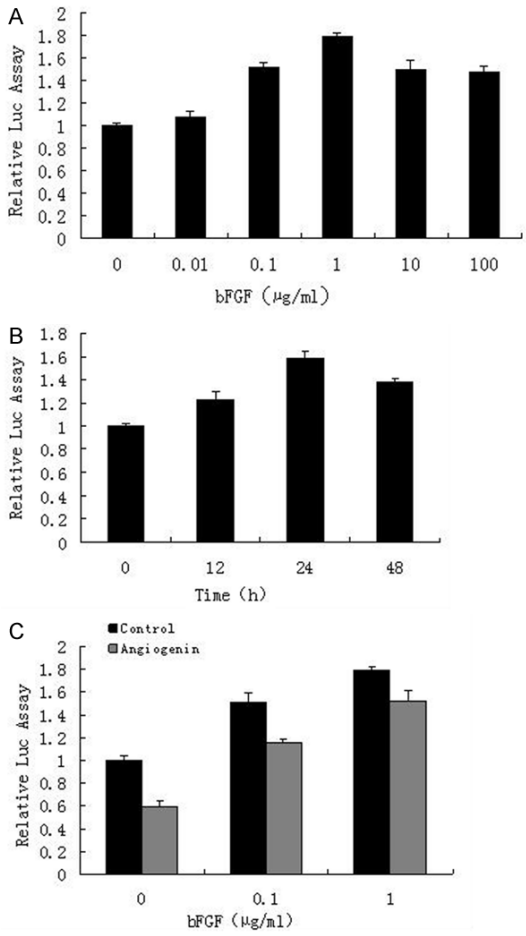

Figure 6. bFGF stimulates its own promoter activity. A. Stimulation of bFGF on its own promoter activity was dose-dependent. pGL3 -bFGFLuc (-1800/+314) reporter vector was transfected into HeLa cells and different dose of bFGF protein was added 5 h after transfection. Luciferase activities were determined and normalized to Renilla activity 29 h after transfection. B. pGL3 -bFGFLuc (-1800/+314) reporter vector was transfected into HeLa cells and 100 ng/mL bFGF protein was added 5 h after transfection. Luciferase activities were determined respectively 0 h, 12 h, 24 h, 48 h after stimulation. C. Exogenous bFGF compensated the inhibition of angiogenin on bFGF promoter activity. Angiogenin expression vector, together with pGL3 -bFGFLuc (-1800/+314) reporter vector were co-transfected into HeLa cells and bFGF protein was added 5 h after transfection. Luciferase activities were determined 24 h after stimulation.

dependent manner. Consequently, we suggest that the mechanism by which angiogenin regulates bFGF expression is two-fold: on one hand, angiogenin binds CTCTCTCT in the bFGF promoter to inhibit transcription, and on the other hand, when angiogenin is overexpressed, bFGF levels are decreased, resulting in decreased stimulation of its own promoter activity. To illu-

minate this complex feedback mechanism more clearly, it is necessary to further investigate the effect of endogenous and exogenous angiogenin separately and to provide further verification by *in vivo* experiments in mice. This is one limitation of this study and it will be carried out in our subsequent research.

In summary, angiogenin enters the nucleus and binds to CTCTCTCT in the bFGF promoter to inhibit bFGF expression. This mechanism prompted further investigation into the effect of angiogenin and bFGF on tumor growth and development. It is of particular interest that nuclear angiogenin may also regulate other genes containing CT repeats and, if so, future research needs to investigate possible other feedback mechanisms between angiogenin, bFGF and other angiogenic factors.

Acknowledgements

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Disclosure of conflict of interest

None.

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