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Construction and Expression of Adenoviral Shuttle Plasmids Carrying Different Kringles of Human Angiostatin in HEK293T Cells

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Abstract

Angiogenesis, the process of new blood vessel formation, is critically modulated by various angiogenic factors. Consequently, the vascular system remains quiescent in the adult. However, dysregulated angiogenesis is generally associated with tumorigenesis. Angiostatin, a proteolytic fragment of plasminogen, was demonstrated to inhibit tumor angiogenesis, thereby affecting tumor growth. Angiostatin is now in clinical trials for various cancer therapy. Angiostatin produced using traditional method (prokaryotic expression system) might not have the right modification and lose the activity easily. Gene therapy using a recombinant adenovirus has recently been proved to be an effective method to treat various diseases. We constructed adenoviral shuttle plasmids containing various fragments of angiostatin including K1-3, K1-4.5, K1-5, and K5 and confirmed their expression in HEK293T cells. Our work provides the raw material for the further construction of recombinant adenovirus (Ad) encoding secretory angiostatin, which could be useful for cancer therapy.

Keywords: Angiostatin ; cancer therapy; recombinant adenovirus

INTRODUCTION

Angiogenesis is a critical biological process in human physiological or pathological circumstances. Under normal physiological condition, angiogenesis is highly controlled and remains quiescent. However, cases such as chronic inflammation, arthritis, and other pathological changes would lead to uncontrolled angiogenesis. Therefore, various angiogenic factors or angiogenesis inhibitors participate in the regulation of tumor angiogenesis.

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Angiostatin, naturally formed from the internal cleavage of plasminogen, was initially isolated from the serum of mice bearing a murine Lewis lung carcinoma. Angiostatin specifically inhibits the endothelial vascular cell proliferation and induces apoptosis, but exerts no direct influence on *in-vitro* tumor proliferation. In the past decades, the approaches of applying angiostatin on lung cancer, breast cancer, glioma sarcoma, fibrosarcoma, and gastric cancer have already been reported.

Plasminogen is activated by proteolysis and converted into angiostatin and plasmin. Angiostatin contains its first 4 kringles and 80% of the kringle 5 (K1-4.5). Among the five kringles, K1-4.5 and K1-3 have been proved to have angiogenesis inhibitory activity, while only K4 displayed no significant effect on inhibiting vascular growth. Interestingly, the fifth kringle (K5) has been identified to have even higher inhibitory activity.

Recombinant angiostatin protein produced by prokaryotic expression system might not be stable enough or have the right eukaryotic modification. Gene therapy using a recombinant adenovirus has recently been proved to be an effective method to treat various diseases. Adenoviral system is useful for gene transfer, because adenovirus can transduce both non-dividing and dividing cells. Adenovirus have a large capacity of 8 kb. Compared with lentivirus vector, adenovirus will not integrate its gene into the genome, reducing the carcinogenic risk. Moreover, replication-deficient adenoviruses lose the ability to replicate themselves in normal cells, which increases safety in clinical treatment.

In this study, we constructed adenoviral shuttle plasmids encoding different kringles of human angiostatin. Different angiostatin cDNA fragments (kringles 1-3, kringles 1-4.5, kringles 1-5 and kringles 5) was amplified through PCR respectively and inserted in shuttle plasmids (PDC316) carrying a secretory signal peptide. The right recombinant plasmids were then confirmed using electrophoresis and DNA sequencing. We then transfected human embryonic kidney cells 293T (HEK293T) with these vectors and confirmed the expression level using real-time PCR (RT-PCR). Overall, our work laid good foundation for further construction of recombinant adeno-virus (Ad) encoding secretory angiostatin and provided insights in tumor therapy.

METHODS

Cell lines and culture conditions

HEK293T cells were cultured in DMEM medium (Gibco, 10566016) with 10% (vol:vol) fetal bovine serum (Gibco, 10270160) and 1% glutamine (Gibco, 25030081). Cells were incubated in a 37°C incubator with 5% CO_2.

Polymerase chain reaction

The four indicated fragments of angiostatin were cloned with distinct primer combination. K1-3 was amplified with K1F and K3R; K1-4.5 was amplified with K1F

and K4.5R; K1-5 was amplified with K1F and K5R; K5 was amplified with K5F and K5R.

Primers are as follows:

K1-F: cgggatccATGTACCTGAGCGAGTGCAAGACC

K3-R: cccaagcttCTAGCTGTCACAAGAGGGGATCT

K5-F: cgggatccATGGTGGCACCACCACCTGTG

K4.5-R: cccaagcttTCATCTGGGGTTTGTGGTATAGC

K5-R: cccaagcttTCAATCGAAGCTAGGGGCGGC

The PCR reaction system are listed below:

10×KOD buffer	5 ⌈L
dNTP	5⌈L
template (angiostatin)	0.5 ⌈L
Foward primer	1.5uL
Reverse primer	1.5uL
KOD enzyme	1 ⌈L
ddH_2O	up to 50 ⌈L

Reaction procedures are as follows:

predenature	95 °C, 5 min
replication (30 cycles)	95 °C, 30 s 58 °C, 30 s 68 °C, 1 kb/min
prolongation	68 °C, 5 min

Colony PCR

Single, clear-edged bacteria colony from the overnight cultural plate was picked out and put into a new tube with 500 ⌈L Amp^+ LB medium. The bacterial solution was then

put in a 37°C incubator for 3~6 h. 3 ⌈L bacterial solution was then used as templated in a PCR. The reaction system and procedures were similar to a normal PCR shown previously.

DNA electrophoresis

DNA PCR products are identified using agarose gel electrophoresis (AGE), and then recollected using Gel Recollection Kit.

Restriction enzyme digestion

The PCR products or vectors were incubated with the same two restriction enzyme BamHI and HindIII. The reaction system are as follows:

Cutsmart buffer	5 ⌈L
BamHI	1 ⌈L
HindIII	1 ⌈L
Vector/PCR product	1.5 ⌈g
ddH_2O	up to 50 ⌈L

The mixture was then incubated at 37°C for 3 h. The digested products were then extracted using gel extraction kit.

DNA ligation

The digested vectors and inserted genes were then ligated using T4 DNA ligase. The reaction system are as follows:

T4 DNA ligase	2 ⌈L
T4 DNA ligase	1 ⌈L
vector	need to be calculated
inserted gene	need to be calculated
ddH_2O	up to 20 ⌈L

The volume of vector or inserted gene is determined through the following formula, presuming mole ratio as 1:n（1:3 ~ 1:10).

Inserted genes: 0.0125×n×345.4×2×? kb / concentration

vector: 0.0125×1×345.2×2×? kb / concentration

The mixture was then incubated under room temperature for 3 h.

Transformation

10 ⌈L ligation product was gently added into competent cells (E.coli DH5〈) . The DH5a was then placed in ice for 30 min followed by 42°C water bath for heat shock. Then the DH5a would be transferred to LB plates and kept in a 37°C incubator overnight.

Plasmids extraction

Plasmids extraction kit (Omega) was employed to extract indicated plasmids from bacteria. Firstly, 6 mL bacteria solution was centrifugated at 4500 rpm for 10 min. Supernatant was then removed and 250 ⌈L Buffer N1 (containing RNase A) was added into each tube. The resolved bacteria were then transferred to a new tube followed by 250 ⌈L Buffer N2. The solution would be gently turned up and down 8 times before being added with 125 ⌈L cold Buffer N3. After white flocculent precipitation completely appeared, the mixture should be centrifugated at 12000 rpm for 10 min. The supernatant was then collected and added into column. The column would be washed with different wash buffer for several times. Then the plasmids would be eluted with 50~100 ⌈L elution buffer. The concentration of plasmids would be measured using Nanodrop.

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Cell transfection

To prepare cells for transfection, HEK293T cells were digested with trypsin and resuspended with DMEM containing 10% fetal bovine serum. Cell suspension (4×10^5/mL) was then seeded in 12-well plate. Cells were then incubated in a 37°C incubator with 5% CO_2 overnight before transfection. To transfect cells with plasmids, 500 ng plasmids were incubated with 4 ⌈L PEI in 100 ⌈L opti-MEM for 15 min. The mixture was then added in the 12-well plate. Cells were then incubated for 24 h before collection.

RNA extraction

500 ⌈L TRIzol was added in cells followed by adding 200 ⌈L chloroform. The mixture was then centrifugated at 12000 rpm for 5 min. The supernatant was then transferred to

a new tube before being mixed with equal volume of isopropanol. The mixture was then transferred to the column and eluted with elution buffer. The concentration of extracted RNA was measured with Nanodrop.

Reverse transcription-polymerase chain reaction (RT-PCR)

The reverse transcription reaction was followed by protocols of HiScript II Q Selecct RT SuperMix (Vazyme). The reaction system was as follows:

HiScript II Select qRT SuperMix	4 ⌈L
Oligo $(dT)_{23}VN$	1 ⌈L
RNA template	1 ⌈g
ddH_2O	up to 20 ⌈L

Reaction procedures are as follows:

50 °C	15 min
85 °C	30 s

The product can be used for subsequent PCR, which is similar with normal PCR system and procedures.

DNA sequencing

1000 ng of indicated plasmids or 10 ⌈L of cDNA collected from transfected cells were sent to Guangzhou Sangon for DNA sequencing. Results received would then be compared with the theoretical sequence.

RESULTS

Gene cloning of four different kringle domains of angiostatin

To increase the expression level, we firstly synthesized the sequence of plasminogen after codon optimization according to the codon frequency of human (Figure 1A). We

then designed and synthesized four pairs of primers and employed polymerase chain reaction (PCR) to get the four different fragments of angiostatin (Figure 1B). We cloned the fragment of K1-3 (~800 bp), K1-4.5 (~1500 bp), K1-5 (~1500 bp), and K5 (~500 bp) (Figure 1C). The product was then confirmed using DNA electrophoresis. As shown in Figure 1C, the PCR products were all in accordance with theoretical size. Taken together, we successfully amplified the targeted fragments.

Ligation of kringles and adenoviral shuttle vectors.

PCR products and adenoviral shuttle vectors (Figure 2A) were cut with two specific restriction enzyme – BamHI and HindIII to create the sticky ends. The digested kringles and vectors were then ligated using T4 DNA ligase before being transformed into E.coli DH5〈 (Figure 2B).

Identification of the recombinant adenoviral shuttle plasmids.

Single bacterial colony was picked out and identified (Figure 3A). We have successfully inserted the different kringle genes into the shuttle vector since size of these bans were in accordance with the theoretical value (Figure 3B). Therefore, the confirmed bacterial colony was applied to the following plasmids extraction. To confirm whether the right sequence was inserted into the vectors, the extracted plasmids were sent to undergo DNA sequencing. And results proved that we have successfully constructed the recombinant adenoviral shuttle vectors carrying different kringles with right sequence (Figure 3C).

Eukaryotic expression of the recombinant shuttle plasmids carrying kringles.

To confirm the eukaryotic expression, HEK293T cells were then transfected with these recombinant adenoviral shuttle plasmids encoding different kringles of angiostatin. 24 h later, cells were collected to extract RNA. The RNA extracted from 293T cells was reverse-transcripted using reverse transcriptase to get cDNA (Figure 4A). To confirm whether right sequence was expressed in HEK293T cells, cDNA was identified using PCR (Figure 4B) and DNA sequencing (Figure 4C). PCR results showed that right size of kringles expressed in cells (Figure 4B). Meanwhile, DNA sequencing results proved that the expressed mRNA is in accordance with the theoretical sequence (Figure 4C). Taken together, we have constructed the right recombinant adenoviral shuttle plasmids carrying different kringles which can successfully express in eukaryotic cells.

DISCUSSION

The growth and metastasis of tumors require the formation of new blood vessels to provide enough nutrients, transport wastes or initiate distal transfer. When tumor grows to a certain size, transcription factors are activated in the host environment of local hypoxia, promoting vascular growth factors and suppressing inhibitory factors,

initiating angiogenesis. Angiogenesis is adjusted by multiple factors, including oxygen, pH value, tumor surface shedding, oncogenes and vascular growth regulation factors, among which the balance of vascular growth stimulating factors and inhibitory factors determines the developmental directions of blood vessels.

Traditional tumor therapeutical methods include tumorectomy, radiation therapy and chemotherapy. Chemotherapy covers all cytotoxic medication that kills tumor cells in vitro, while having two major drawbacks that urge researchers to invest in new therpaies: low selectivity that bring harms to the body and genetical instability that leads to resistance.

Therefore, vascular growth inhibitors are considered as an effective indirect therapy towards tumor, curing not by targeting the tumor cells themselves but by suppressing vessel growth and blocking nutrient flow. The advantages of such method are listed as follow: not adhere to a certain type of tumor cells but instead theoretically applicable to all solid tumor; since endothelial cells has better gene stability, drug resistance is better prevented; angiogenesis inhibitors has much less effect on normal vascular cells since they target specific rapid-growing endothelial cells. Previous studies on angiostatin gene therapy and radiation therapy combined also showed good clinical effect; recombinant protein and gene transfection also significantly inhibited the growth and metastasis of various tumors in vivo, indicating angiostatin's potential value in clinical application.

Due to the high cost and difficulties to obtain the angiostatin with natural structure, gene therapy might become a better choice to transfer effective angiostatin into patients. In this study, we chose to work with adenoviral shuttle plasmids encoding different kringles of human angiostatin. Recombinant adenoviruses are prominent in multiple aspects of application, including gene transfer in vitro, vaccination in vivo, and gene therapy, as a result of its following advantages: adenoviruses are capable of transferring genes to various cell types without having to depend on active cell division; human cells are easily infected with adenovirus vectors and consequently yield high levels of the transgene expression; thirdly, the development of gutless adenoviral vectors allows the anti-adenoviral vector immunity to be avoided; last but not the least, being widely applied and well documented in experiments, researchers have accumulated enough experience to experiment with adenovirus effectively and safely. Therefore, building adenoviral shuttle plasmids is a crucial step and effective method in the process of gene therapy — applying genetic engineering strategies to secrete key molecules such as angiostatin in human body, thus contributing to the curing of cancer and tumors.

In conclusion, we successfully constructed recombinant adenoviral shuttle plasmids carrying different kringles of angiostatin and confirmed their eukaryotic expression.

Our products would have potential value in angiogenesis gene therapy and lay foundation for further studies on its mechanism and promoting clinical application.

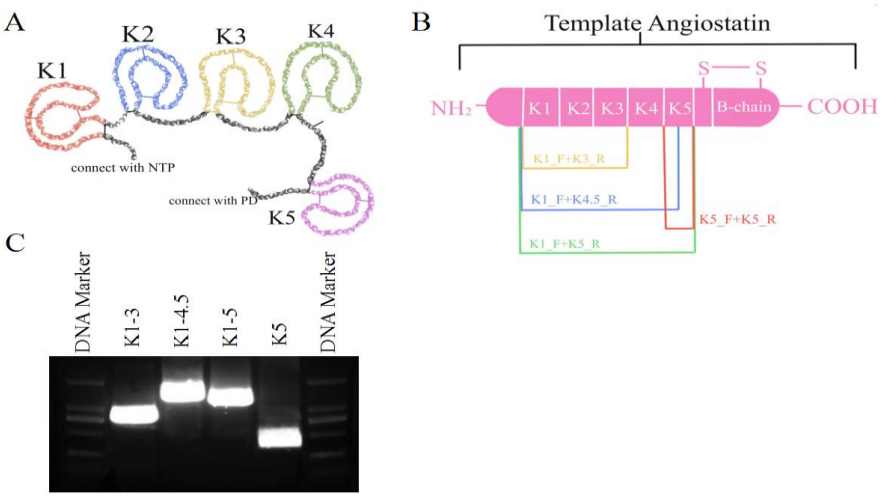

Figure 1. Gene cloning of four different kringle domains of angiostatin

(A)Visualization of the kringles-domain structure of angiostatin. Angiostatin contains the structure of kringles 1-4 and 80% of the amino sequence of k5.

(B) Schematic diagram of angiostatin template and primers.

(C) Electrophoresis result of PCR according to different primer combinations.

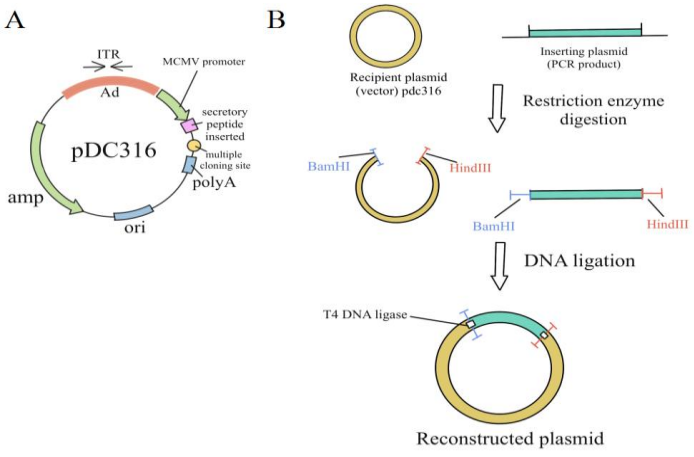

Figure 2. Ligation of kringles and adenoviral shuttle vectors.

(A) Illustration of the structure of pDC316 vector. Compared with common pDC316 vectors, the vector applied in this experiment is optimized through inserting a secretory peptide as well as a multiple cloning site after MCMV promoter.

(B) Visualization of the enzyme digestion and DNA ligation process to construct a recombinant shuttle plasmid. After both digested by restriction enzymes BamHI and HindIII, the recipient plasmid (pDC316) and the donor plasmid (four categories of PCR product originated from angiostatin) was combined under the catalyst of T4 DNA ligase.

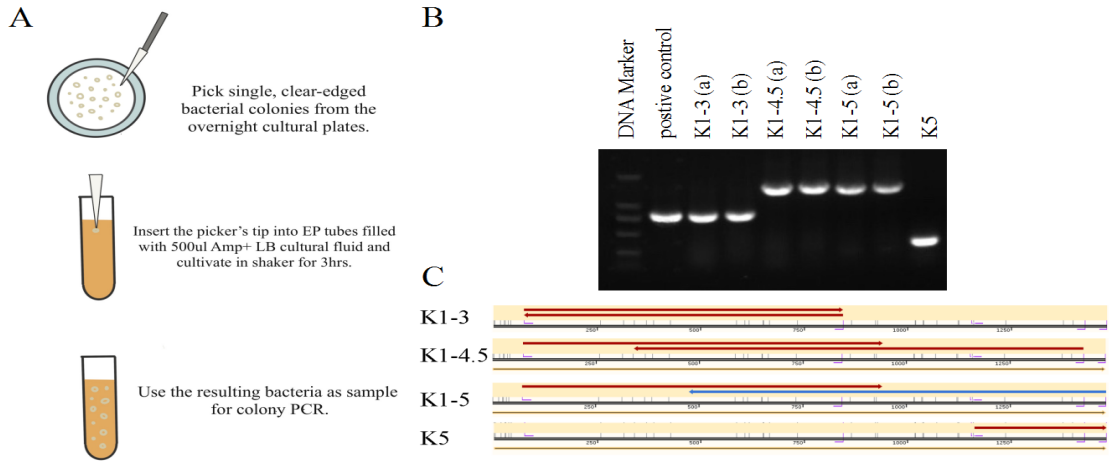

Figure 3. Identification of the recombinant adenoviral shuttle plasmids.

(A) Process of picking bacteria colonies, amplification, and colony PCR. Picking clear-edged, single colonies ensures that each PCR sample only contains one DNA sequence, as genetic information could vary between different colonies on one cultural plate. Picked bacteria was cultured for another 3 hours in cultural liquid to further amplify their DNA before colony PCR was applied.

(B) Electrophoresis result of colony PCR.

(C) DNA sequencing of the extracted plasmids.

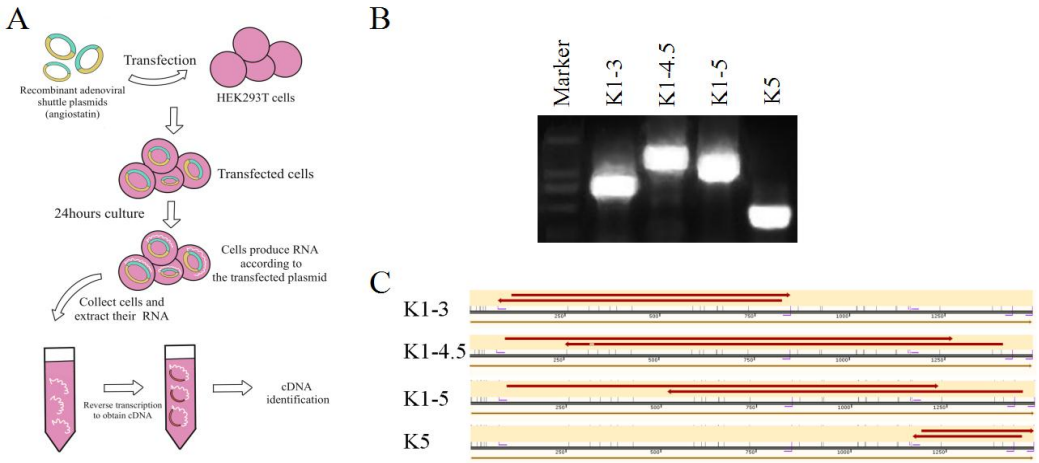

Figure 4. Eukaryotic expression of the recombinant shuttle plasmids carrying kringles.

(A)Diagram of cellular transfection and reverse transcriptase-PCR.

(B)electrophoresis result of RT-PCR.

DNA sequencing of the products of RT-PCR.

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