Regulation of cancer-related growth factor expression by artificial zinc-finger proteins

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ABSTRACT
One attractive approach to anticancer therapy is repression of expression of vascular endothelial growth factor (VEGF) gene, which is a potent target for prevention of tumor growth. To achieve this, artificial transcription factors (ATFs) designed for VEGF gene regulation were fused to cell-penetrating peptides (CPPs). We demonstrated ATFs fused to CPPs, designated CPP-ATFs or designed regulatory proteins (DRPs), could penetrate into mammalian cells and transiently repress expression of a reporter gene, which was under control of the VEGF promoter/5'-UTR. We discuss gene-regulatory properties of CPP-ATFs in detail.

INTRODUCTION
The development of a blood supply is crucial to the rapid growth of cancer cells. The factors involved in this process are complex, however tumor hypoxia and macrophage infiltration are responsible for the synthesis of pro-angiogenic cytokines such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). These factors stimulate proliferation of vascular endothelial cells, the synthesis such as urokinase type plasminogen activator and the matrix metalloproteases, which result in digestion of the extracellular matrix and allow endothelial cell invasion.

The concept that inhibition of angiogenesis results in starvation of tumors was proposed by Folkman in 1971. Inhibition of angiogenesis in tumours is now an attractive therapeutic approach. Physiological angiogenesis only occurs in specific situations such wound healing and during the menstrual cycle. It is therefore expected that therapies targeted at pathological angiogenesis could be highly specific and be little toxic.

Among many factors responsible for tumor angiogenesis, VEGF is one of potent therapeutic targets. The overexpression of VEGF has been described in a variety of solid tumors such as lung, kidney and breast carcinomas. Direct evidences for a role of VEGF in tumorigenesis were obtained from results that anti-VEGF monoclonal antibodies inhibited VEGF-induced angiogenesis in vivo and in vitro.

Previously we have reported a method to rationally design artificial zinc-finger proteins (AZPs) targeting diverse DNA sequences using a nondegenerate recognition code table. AZPs have been used to create artificial transcription factors (ATFs), which activated or repressed the VEGF gene, depending on the type of a transcriptional effector domain that was fused to the AZP, when introduced as transgenes in mammalian cells.

We also demonstrated VEGF gene regulation by introduction of ATF fusion proteins with cell-penetrating peptides (CPPs), designated CPP-ATFs or designed regulatory proteins (DRPs), into mammalian cells. Since trans-activating transcriptional activator (TAT) from HIV type I was first reported to cross mammalian cell membrane when exogenously added to culture medium, various CPPs have been identified so far and it has been shown that various proteins in size fused to CPPs can penetrate into mammalian cells and their biological properties can be retained there.

We first evaluate the properties of a DRP containing PTD4, a cell-penetrating peptide that is reported to be more efficient in cell penetration than TAT, for gene regulation in detail. We will also examine efficacies of additional CPP-ATF variants.

RESULTS AND DISCUSSION
PTD4-ATF was consisted of PTD4, a nuclear localization signal (NLS) from simian virus 40 large T antigen, an AZP with six fingers that binds the 19bp DNA sequence that coincides with +516 to +534 in the human VEGF gene, where +1 is the transcription start site, and a Krüppel-associated box (KRAB) domain as a transcription repression domain. A DNA fragment encoding PTD4-ATF (Fig. 1) was introduced into an Escherichia coli expression plasmid.

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<th>PTD4</th>
<th>NLS</th>
<th>AZP</th>
<th>KRAB domain</th>
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Fig. 1 Schematic representation of ATF fused to PTD4.

An E. coli strain was transformed with the E. coli expression plasmid of PTD4-ATF. After expression of the PTD4-ATF protein and ultrasonication of the E. coli strain,
the protein was purified as previously described. We analyzed the purity of the PTD4-ATF protein by SDS-PAGE (Fig. 2).

![Fig. 2 SDS-PAGE analysis of purified PTD4-ATF. Lane 1: Extracts of an E. coli strain alone. Lane 2: Extracts of an E. coli strain transformed with the E. coli expression plasmid of PTD4-ATF after incubation for 3h at 37°C with 1 mM IPTG. Lane 3: Purified PTD4-ATF.](image)

Next, we examined whether the purified PTD4-ATF can repress gene expression in transient reporter assays where a reporter plasmid containing luciferase reporter gene under the control of the VEGF promoter/5' UTR (-2279 to +1038 relative to the transcription start site) was used. A total of 1 x 10^5 HEK293 cells per well was plated onto a poly(D-lysine)-coated 96-well plate with 100 μl of DMEM supplemented with 0.1 mM nonessential amino acids and 10% FBS. After incubation for one day, the purified PTD4-ATF was added into culture medium. Following incubation for 1h, the reporter plasmid was cotransfected with pCMV-β-galactosidase plasmid and Lipofectamine 2000. The transfected cells were harvested every twelve hours and luciferase activities were measured to evaluate the efficacy of PTD4-ATF to repress gene expression. Luciferase activities were normalized to β-galactosidase activities. We confirmed PTD4-ATF could repress the luciferase reporter gene efficiently.

We also examined PTD4-ATF concentration dependency for repression of the luciferase reporter gene. Concentrations of PTD4-ATF used were ranged from 0.1 μM to 1 μM and transfected cells were harvested one day after transfection. In this experiment, clear concentration dependency was observed.

**CONCLUSION**

We examined the efficacy of CPP-ATF designed for regulation of human VEGF gene in transient luciferase-reporter assays. This system allowed us to access the efficacy of CPP-ATFs for gene regulation quantitatively and promptly. We will screen CPP-ATF variants by using this system.

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**REFERENCE**