VP22 mediates intercellular trafficking and enhances the *in vitro* antitumor activity of PTEN

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Abstract. PTEN acts as a phosphatidylinositol phosphatase with a possible role in the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. Mutations in *PTEN* are frequent and their presence is associated with poor prognosis in breast cancer, which is the most common type of non-cutaneous malignancy in females. Delivery of the tumor suppressor *PTEN* gene represents a powerful strategy for breast cancer therapy, but a present limitation of gene therapy is the ability to deliver sufficient quantities of active proteins to target cells. The capacity of HSV-1VP22 fusion proteins to spread from the primary transduced cell to surrounding cells could improve gene therapeutics, particularly in cancer. To assess the potential efficacy of *VP22* as a gene therapy for breast cancer, expression vectors for N- and C-terminal PTEN-VP22 fusion proteins were constructed. VP22-mediated intercellular transport and antitumor efficacy in BT549 (PTEN-null) breast tumor cells were investigated. The results showed that PTEN-VP22 has the same spreading abilities as VP22. In cell proliferation and apoptosis assays, *PTEN-VP22* gene transfer induces a stronger anti-proliferative effect and apoptotic activity compared with *PTEN* gene transfer alone. In addition, VP22 enhanced the PTEN-mediated decrease in the level of phosphorylated AKT. The results show that PTEN-VP22 can spread *in vitro* and *PTEN-VP22* gene induces significantly greater antitumor activity than the *PTEN* gene alone. This study confirms the utility of VP22-mediated delivery *in vitro* and suggests that *PTEN-VP22* may have applications in breast cancer gene therapy.

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Introduction

Breast cancer is the most common type of non-cutaneous malignancy in females and is the second (only to lung carcinoma) most common cause of cancer-related mortality (1). In the United States, females have an estimated 12% lifetime risk of being diagnosed with breast cancer; the risk of breast cancer-related mortality is estimated at 2.82%, even after optimal treatment (2). Conventional anticancer therapeutics have reached the limit of their utility, necessitating a novel therapeutic strategy to improve outcomes. *PTEN* (phosphatase and tens in homolog on chromosome ten) is a tumor suppressor and encodes a dual-specificity phosphatase (3). Its primary substrate is the second messenger phosphatidylinositol 3,4,5 trisphosphate (PIP3) (4). PTEN antagonizes the phosphatidylinositol 3-kinase (PI3K)/AKT pathway and affects cellular processes including growth, proliferation and survival (5). *PTEN* is mutated in numerous types of cancer; the high frequency of monoallelic mutations of *PTEN* has been demonstrated in endometrial carcinoma, glioblastoma, and prostate, breast, colon and lung tumors (6). Complete loss of *PTEN* is generally associated with advanced cancer and metastases in endometrial cancer and glioblastoma (6).

In breast cancer, a recent study revealed that PTEN loss is a common event in breast cancers caused by *BRCA1* mutations (7). PTEN has also been investigated for its prognostic power in several types of human malignancy. Loss of PTEN expression is associated with the poor survival of patients with basal-like breast cancer (8), and data from preclinical and clinical studies implicate PTEN loss in constitutive PI3K/AKT/mTOR signaling and *de novo* resistance to Herceptin 2-targeted therapy (9). Delivery of the tumor suppressor *PTEN* gene represents a powerful strategy for breast cancer therapy, although virus-mediated gene therapy is associated with safety problems and non-virus-mediated gene therapies are inefficient (10).

Previous studies have shown that VP22 proteins from HSV-1 have the capacity to cross cell membranes (11). In addition, VP22 proteins are capable of transducing heterologous proteins, such as p53, p27, CD, GFP and Hsp70 (12), across the cell membrane, although the delivery mechanisms have not been fully characterized. It was hypothesized that introducing VP22 proteins as well as PTEN may improve cell penetration and increase antitumor efficacy. In this study, VP22 was conjugated

to the C terminus of PTEN and the growth-inhibitory activity of the fused proteins was observed in a breast carcinoma cell line.

Materials and methods

Cell lines and cell culture. The BT549 cell line was provided by Dr Bao Qian Jin (North Sichuan Medical College, Sichuan, China). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% bovine serum (Beyotime Biotechnology, Shanghai, China).

Eukaryotic expression vector construction. The vector pcDNA3-PTEN for the expression of wild-type human PTEN was generated by PCR subcloning using a full-length wild-type human *PTEN* cDNA as template. The *PTEN* amplicon was digested with *Hin*dIII/*Xho*I (Takara Biotechnology Co., Ltd., Dalian, China) and subcloned into a eukaryotic expression vector pcDNA3 (Invitrogen LIfe Technologies, Carlsbad, CA, USA). pcDNA3-VP22, which expressed HSV-1 VP22, was constructed as described previously (13). pcDNA3-PTEN-VP22 was constructed for the expression of N-terminal VP22-fused PTEN (PTEN-VP22) by overlapping extension PCR. Briefly, human *PTEN* cDNA (Fisher Scientific, Hanover Park, IL, USA) was amplified with forward (5'-GTCGAATTCATGACAGCCATCATC-3') and reverse primers (5'-GAGAGGTCATGACTTTTGTAATTTGTGT-3'). *VP22* was amplified with forward (5'-TACAAAAGTCA TGACCTCTCGCC-3') and reverse primers (5'-AATGAAT TCTCACTCGACGGGC-3'). The reaction mixture (final volume, 50 μl) consisted of 25 μl Pfu PCR Master Mix (Tiangen Biotech Co., Ltd., Beijing, China), 2 μl templates and 1 μl each primer (forward and reverse). The thermal cycling conditions were: 94˚C for 3 min, followed by 30 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 1 min, and a final step at 72˚C for 5 min. The extension was then performed with the *PTEN* and *VP22* fragments as primers, with the following conditions: 94˚C for 3 min, followed by 10 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 1 min, and a final step at 72˚C for 5 min. After extension, upstream (5'-GTCGAATTCATGACAGCCATCATC-3') and downstream (5'-AATGAATTCTCACTCGACGGGC-3') primers were added, and 30 PCR cycles were performed. The resulting chimeric *PTEN-VP22* gene was digested with *Eco*RI (Takara Biotechnology Co., Ltd. and subcloned into pcDNA3.

Identification of the eukaryotic expression vectors. Successful clones were identified by restriction digestion with *Hin*dIII/*Xho*I (pcDNA3-PTEN) and *Hin*dIII/*Xba*I (pcDNA3-PTEN-VP22) at 37˚C for 4 h. pcDNA3-PTEN and pcDNA3-PTEN-VP22 were sequenced by GenScript Co., Ltd. (Nanjing, China).

Cell transfection. BT549 cells were grown to 70% confluence and then washed twice with phosphate-buffered saline prior to transfection in serum-free DMEM containing *Trans*It-LT1 Transfection Reagent (Mirus Bio LLC, Madison, WI, USA), as described by the manufacturer. Cells were transfected with the same quantity of pSV-β-Galactosidase (Promega Corporation, Madison, WI, USA) per well to account for deviations generated by different transfection efficiencies.

Western blot analysis. After transfection (72 h), the cells were harvested in RIPA lysis buffer (Tiangen Biotech Co., Ltd.), homogenized, and centrifuged at 15,000 x g for 10 min at 4˚C; protein concentration in the supernatants was measured by the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and standardized. Proteins were separated by 10% SDS-PAGE and immunoblotted with rabbit anti-PTEN (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and rabbit anti-phospho-AKT (Ser^{473}) (Cell Signaling Technology Inc., Beverly, MA, USA) polyclonal antibodies. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology Inc.) was used as the secondary antibody for the DAB Detection system (Wuhan Boster Biological Technology, Ltd., Wuhan, China). Antibodies for β-actin (Boster) or total AKT (Cell Signaling Technology Inc.) were used as loading controls.

Immunofluorescence and quantitation. At 48 h post-transfection, cells were washed with phosphate-buffered saline, fixed in cold methanol for 10 min at room temperature (RT), and then permeabilized with 0.2% Triton X-100 for 90 min at RT. After washing in PBS and blocking for 30 min in 5% non-fat milk at RT, cells were incubated with rabbit anti-PTEN (1:200) antibody (Santa Cruz Biotechnology Inc.) at 4˚C overnight. After three washes, the secondary antibody fluorescein isothiocyanate-conjugated sheep-anti-rabbit IgG (Santa Cruz Biotechnology Inc.) was added for 1 h at RT. Cells were then analyzed on a microplate reader (Fluoroskan Ascent FL; Thermo Fisher Scientific, Waltham, MA, USA) and images were captured by inverted fluorescence microscopy (TCS SP2; Leica Microsystems, Wetzlar, Germany).

Cell proliferation assay. Cell proliferation was measured with the Cell Counting kit-8 (CCK8) assay (Beyotime Biotechnology) according to the manufacturer's instructions. Briefly, transfected cells were harvested at 10 h and plated in 96-well plates at a density of 3,000 cells/well for each treatment condition. At 24, 48, 72 and 84 h after transfection, 10 μl WST-8 dye (Beyotime Biotechnology) was added to each well, then incubated at 37˚C for 1 h, and absorbance (A) was measured at 450 nm using an iMark bio microplate reader (Bio-Rad Laboratories, Inc.). Cell survival was determined as $A_{treated}/A_{control}$.

Apoptosis analysis. At 72 h post-transfection, cells were harvested, washed with PBS, stained with Annexin V and propidium iodide, and apoptosis was measured by flow cytometry (acquired 10,000 cells/cell; FACSVantage SE; BD Biosciences, Franklin Lakes, NJ, USA) .

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Statistical analysis was performed across multiple groups using analysis of variance (ANOVA) and confirmed between individual groups using Student-Newman-Keul's method. $P<0.05$ was considered to indicate a statistically significant difference.

Results

VP22 mediates PTEN intercellular trafficking in BT549 cells. The trafficking ability of a fused PTEN-VP22

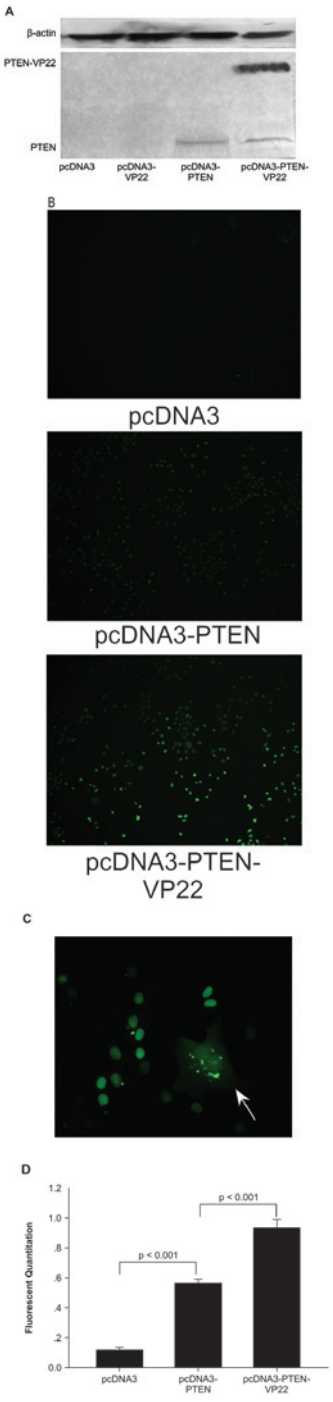

Figure 1. VP22 mediates PTEN intercellular trafficking in BT549 cells. (A) PTEN-VP22 fusion protein expression in BT549 cells. BT549 cells were transfected with 5 μg pcDNA3-PTEN-VP22, 5 μg pcDNA3-PTEN, 5 μg pcDNA3-VP22 or 5 μg pcDNA3, and protein expression was detected by western blot analysis 72 h later. (B) PTEN-VP22 fusion protein and PTEN distribution in BT549 cells. BT549 cells were transfected with 2.5 μg pcDNA3-PTEN-VP22, pcDNA3-PTEN, or pcDNA3 and immunostained 48 h later with an anti-PTEN antibody. Magnification, x20. (C) PTEN-VP22 fusion protein distribution with a typical VP22 pattern. BT549 cells were transfected with 2.5 μg pcDNA3-PTEN-VP22 and immunostained 48 h later with an anti-PTEN antibody. Arrow indicates primary transfected cells that are surrounded by recipient cells. Magnification, x60. (D) Fluorescence quantitation of PTEN-VP22 and PTEN in BT549 cells.

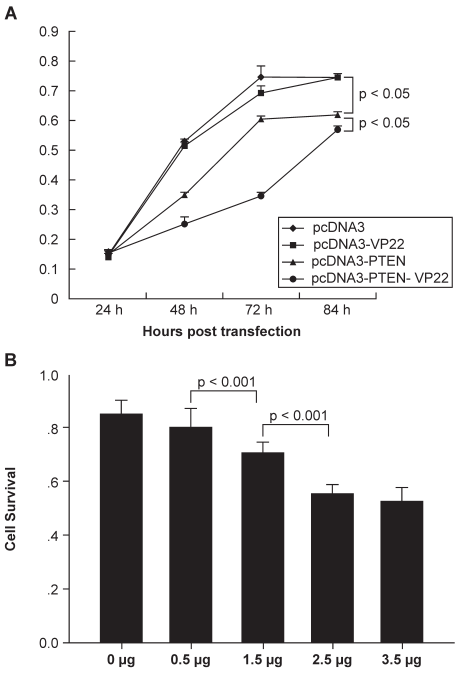

Figure 2. VP22 enhances PTEN-mediated antiproliferative activity in BT549 cells. (A) PTEN-VP22 inhibits BT549 over time. BT549 cells were transfected with 2.5 μg plasmid and cell survival was determined as $A_{treated}/A_{control}$. (B) PTEN-VP22 inhibits BT549 in a dose-dependent manner. BT549 cells were transfected with pcDNA3-PTEN-VP22. The quantity of transfected DNA was brought to 3.5 μg by supplementing with pcDNA3 in every well. Cell survival was determined as $A_{treated}/A_{control}$.

recombinant protein was measured. *PTEN* cDNA was fused to the N-terminal of the *VP22* cDNA to produce the pcDNA3-PTEN-VP22 fusion protein expression vector. Expression of the fusion protein was monitored by western blotting. In transiently transfected BT549 cells, a PTEN-null breast carcinoma cell line (3), an anti-PTEN antibody clearly detected the full-length PTEN-VP22 fusion protein at its expected size (~90 kDa), as well as PTEN (~60 kDa) (Fig. 1A). In pcDNA3-PTEN-VP22 transfected cells, western blotting showed high expression of full-length PTEN-VP22 as well as a truncated product with the molecular weight of PTEN, indicating cleavage of the two proteins.

To investigate the trafficking property of the fusion protein, BT549 cells were transfected with pcDNA3-PTEN-VP22 or pcDNA3-PTEN, and spreading was observed by fluorescence microscopy (Fig. 1B). The results showed that only a few cells per field were positive 48 h post-transfection with PTEN (Fig. 1B). By contrast, when cells were transfected with pcDNA3-PTEN-VP22, a larger number of positive cells (Fig. 1B) with a typical VP22 pattern (i.e., primary transfected cells with cytoplasmic and nuclear staining surrounded by recipient cells with nuclear staining) were observed (Fig. 1C). This phenomenon was confirmed by fluorescence quantitation after immunofluorescence with the anti-PTEN antibody. The results showed that the fluorescence of cells expressing PTEN-VP22 48 h after transfection was 0.927±0.0196 versus 0.558±0.0105 in cells expressing PTEN alone ($P<0.001$; Fig. 1D). Therefore, the fusion protein PTEN-VP22 appears to have the same spreading abilities as VP22.

In addition, fluorescence microscopy of the BT549 human breast carcinoma cell line expressing exogenous PTEN or PTEN-VP22 fusion protein revealed fluorescent PTEN or PTEN-VP22 in the cytosolic and nuclear compartments,

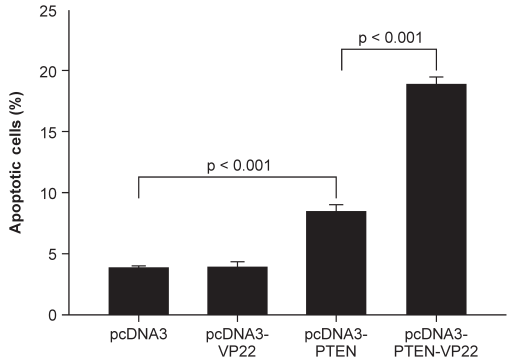

Figure 3. VP22 enhanced PTEN-mediated apoptotic induction in BT549 cells. BT549 cells were transfected with 5 μg plasmid and apoptosis levels were analyzed 72 h later.

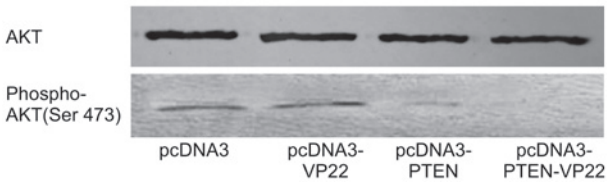

Figure 4. VP22 enhanced PTEN-mediated decreases in the level of phosphorylated AKT. BT549 cells were transfected with 5 μg plasmid and protein levels were detected by western blot analysis 72 h later.

although PTEN and PTEN-VP22 were predominantly nuclear in localization (Fig. 1B and C).

VP22 enhances PTEN-mediated antiproliferative activity in BT549 cells. To determine whether fusion of PTEN to VP22 affected its biological activity, the CCK-8 assay was used to measure the effect on tumor cell proliferation. The results (Fig. 2) showed that addition of VP22 to the C-terminus of PTEN enhanced antiproliferative activity in general. Growth was similar between pcDNA3-VP22 and pcDNA3 transfected cells, indicating that VP22 expression is non-toxic (Fig. 2A). Growth was inhibited over time when BT549 cells were treated with 2.5 μg pcDNA3-PTEN or pcDNA3-PTEN-VP22. The CCK-8 assay revealed that pcDNA3-PTEN and pcDNA3-PTEN-VP22 did not inhibit BT549 proliferation 24 h post-transfection. By contrast, pcDNA3-PTEN and pcDNA3-PTEN-VP22 exhibited significant antiproliferative activity at 48, 72, and 84 h compared with pcDNA3-transfected cells (pcDNA3-PTEN, P<0.001 at 48 and 84 h and P<0.01 at 72 h versus pcDNA3 at the same time points; pcDNA3-PTEN-VP22, P<0.001 at 48, 72, and 84 h, versus pcDNA3 at the same time points). Furthermore, the efficacy of pcDNA3-PTEN-VP22 inhibition of proliferation was greater than that of pcDNA3-PTEN (P<0.001 at 48 and 72 h; P<0.05 at 84 h). These results indicated that the conjugation of VP22 to the C-terminus of PTEN may enhance the basal antiproliferative activity in the BT549 breast carcinoma cell line.

To confirm these results, the antiproliferative activity of various doses of PTEN-VP22 at 48 h post-transfection were compared. As shown in Fig. 2B, cell growth was dose-dependent when BT549 cells were treated with 0.5, 1.5, and 2.5 μg pcDNA3-PTEN-VP22 (P<0.001). Thus, addition of VP22 increased the antiproliferative activity of PTEN in PTEN-deficient breast carcinoma cells.

VP22 enhances PTEN-mediated apoptotic induction in BT549 cells. Previous studies have shown that transduction of the wild-type PTEN gene into cancer cells induces apoptosis (14-16). To investigate whether intercellular spread of PTEN-VP22 enhances its apoptotic capacity, apoptotic induction by PTEN-VP22 and PTEN was compared in BT549 cells (Fig. 3). It was observed that 5 μg pcDNA3-VP22 did not induce apoptosis compared with 5 μg pcDNA3 (the negative control); however, apoptotic rates in cells transfected with 5 μg pcDNA3-PTEN differed significantly from those for the negative control (P<0.001), revealing that transfection of pcDNA3-PTEN induced apoptosis. In addition, a significant increase in apoptosis was detected in cells transfected with 5 μg pcDNA3-PTEN-VP22 versus 5 μg pcDNA3-PTEN (P<0.001), indicating that VP22-mediated spreading of PTEN-VP22 is associated with an enhanced rate of PTEN-mediated apoptosis in BT549 cells.

VP22 enhances PTEN-mediated decreases in the level of phosphorylated AKT. AKT is activated following phosphorylation by PIPs and AKT phosphorylation is inversely associated with *PTEN* expression. The correlation between AKT phosphorylation state and PTEN-VP22 expression was investigated in BT549 cells. As expected, levels of phospho-AKT were the same in pcDNA3 and pcDNA3-VP22 transfected cells and the high level of phospho-AKT was abrogated in *pcDNA3-PTEN* transfected cells, whereas the higher level of phospho-AKT was abrogated in pcDNA3-PTEN-VP22 transfected cells (Fig. 4). These results suggest VP22-mediated spreading of PTEN-VP22 is associated with decreased expression of phosphorylated AKT.

Discussion

A present limitation of gene therapy is the ability to deliver sufficient quantities of active proteins to target cells. While secreted proteins can overcome this limitation to a certain extent, it is particularly a problem for nonsecreted proteins, such as PTEN as these proteins are only active in the cells that they are initially delivered to. It has been suggested that VP22 fusion proteins may increase distribution through intercellular transport. Thus increased numbers of cells may reach therapeutic steady state, leading to an overall increase in drug efficacy in the target cell population.

Only N-terminal fusion was investigated as the C-terminal extremity of VP22 is essential for cell-to-cell transport (11,17). Expression vectors for wild-type PTEN and PTEN-VP22 were constructed and their activities were compared in the PTEN-null BT549 breast carcinoma cell line. Wild-type PTEN protein and high levels of PTEN-VP22 fusion protein expression *in vitro* were observed (Fig. 1A); both proteins were transcriptionally active (Fig. 1B). The present study shows that VP22 transduces PTEN across the cell membrane, resulting in a wider distribution in the BT549 cells (Fig. 1B and C). In addition, VP22 does not change the characteristics of PTEN localization in cells, and PTEN-VP22 was predominantly nuclear in localization, which is similar to PTEN (Fig. 1B).

Furthermore, it was demonstrated that this fusion protein is functional and that the *PTEN-VP22* gene transfer induces a stronger antiproliferative effect than *PTEN* alone in a time- and dose-dependent manner *in vitro* (Fig. 2A and B). VP22 did not display toxicity in these experiments (Fig. 2A), suggesting that the increased activity is solely due to the transport properties of VP22.

PTEN acts as a phosphatidylinositol phosphatase with a possible role in the phosphatidylinositol 3-kinase (PI3K)/ AKT pathway (5). Introduction of PTEN into PTEN-deficient cells inhibits the activation of AKT, which is a serine-threonine kinase downstream in the PI3K pathway, is involved in proliferative and anti-apoptotic pathways and exhibits tumor suppressive properties (18). In order to demonstrate how *PTEN-VP22* gene transfer could induce this antiproliferative effect, it was evaluated whether higher protein transport levels were correlated with increased apoptotic activity and decreased levels of phosphorylated AKT in BT549 cells. It was observed that *PTEN-VP22* transfection enhanced apoptosis relative to *PTEN*, while *VP22* did not alter apoptotic activity (Fig. 3). These results suggest that PTEN-VP22 can induce apoptosis in BT549 cells and VP22-mediated spreading of PTEN correlates with enhanced apoptosis. PTEN decreased the expression of phospho-AKT; however, the phosphorylation status of AKT was lower in the presence of *PTEN-VP22* versus *PTEN*. The phospho-AKT states of BT549 cells were not altered by VP22 alone (Fig. 4). Thus, this demonstrated that PTEN-VP22 can effectively block the (PI3K)/AKT pathway and VP22-mediated spreading of PTEN-VP22 is correlated with the enhanced rate of PTEN-mediated decreases in phospho-AKT status and induction of apoptosis in BT549 cells.

In conclusion, VP22-mediated transport of the PTEN tumor suppressor protein could enhance the biological functions of PTEN, providing a strategy for enhancing the efficacy of gene therapy of breast cancer.

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