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Original Article

The overexpression of uPA promotes the proliferation and fibrinolytic activity of human umbilical vein endothelial cells

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Abstract: The purpose of this article is to study whether the overexpression of urokinase-type plasminogen activator (uPA) can promote the proliferation and fibrinolytic activity of human umbilical vein endothelial cells (HUVECs). The recombinant adenovirus vectors containing the human uPA gene were constructed and transfected into HUVECs. In this study, the mRNA of uPA was detected by qPCR, and the uPA protein was measured by Western blot. The cell proliferation was measured using MTT. The fibrinolytic activity of uPA was quantified using a colorimetric assay. We also measured MMP2 (metalloproteinase-2), MMP9 (metalloproteinase-9), and VEGF (vascular endothelial growth factor) proteins using ELISA. The results showed that the levels of the uPA mRNA and the protein in the overexpression group were significantly higher compared to the other groups, ($P < 0.05$). The cell proliferation and uPA activity were increased significantly in the overexpression group, compared to the other groups, ($P < 0.05$). The secretions of MMP2, MMP9, and VEGF in the overexpression group were significantly higher than they were in the other two groups ($P < 0.05$). In conclusion, we successfully transfected a recombined adenovirus vector carrying uPA into a HUVEC. The exogenous uPA gene could transcribe and secrete the uPA protein in the HUVECs. The overexpression of uPA can increase cell proliferation and uPA activity. It can improve the invasion and angiogenesis ability in HUVECs by promoting their secretions of MMP2, MMP9, and VEGF.

Keywords: Overexpression, uPA, proliferation, HUVEC, MMP2, MMP9, VEGF

Introduction

Deep venous thrombosis (DVT) is a serious cardiovascular disease. Without proper treatment, DVT can easily develop into post-thrombotic syndrome, femoral bruising, femoral white swelling, and even death due to pulmonary embolism [1]. Every year, millions of people suffer from DVT, and the incidence of DVT is getting higher and higher [2]. At present, the treatment for DVT in the lower extremities is mainly anticoagulant therapy, thrombolytic therapy, interventional therapy, surgical thrombectomy and so on, but these treatments are risky, expensive, and have many disadvantages.

Therefore, it is of great significance to find a new treatment method, which enables the body itself to produce cytokines that respond to venous thrombosis, that is, to ablate the thrombus, to promote the remodeling of venous wall fibrosis, and not to cause significant negative effects on other tissues of the body.

UPA is a second generation thrombolytic agent and belongs to the serine proteases. It has two forms: one is an inactive single-chain structure, the other binds to urokinase-type plasminogen activator receptors (uPAR), enzymatic hydrolysis of membrane-bound plasminogen and other proteases and produces an active double-chain form, which does not require fibrin as a cofactor and can directly activate plasminogen to become plasmin [3-10]. Plasmin can degrade fibrin clot proteolytic enzymes, which is an important component of the fibrinolytic system. Once

the body produces a coagulation reaction, it activates the fibrinolytic system and removes excess thrombus [11]. UPA synthesis has been used in thrombolytic therapy in vitro, such as with acute myocardial infarction, cerebral embolism, peripheral arteriovenous thrombosis, etc.

However, there are few studies on promoting uPA secretion by human vein endothelial cells for thrombolytic therapy in vivo. Therefore, we transfected the adenovirus carrying the uPA gene into human umbilical vein endothelial cells and measure the secretion of uPA, so as to lay a foundation for the later thrombosis treatment of uPA in vivo.

Materials and methods

Cell lines, cell cultures and reagents

HUVECs were obtained from the American Type Culture Collection (Manassas, VA). Lipofectamine 2000, Rabbit monoclonal anti-human antibodies against uPA, and β-actin were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). VEGF, MMP2, and MMP9 were purchased from Boster Biological Technology., LTD (Wuhan, China). LightCycler®96 Real-time Fluorescence Quantitative PCR (qPCR) is manufactured by the Roche Company in Switzerland.

Construction of recombinant adenovirus vectors

The recombinant adenovirus vectors containing exogenous uPA gene were designed and synthesized by the Wuhan Cell Marker Biotechnology Co., Ltd. in Wuhan, China. The gene-specific uPA primer pair was as follows: Forward, 5'-CAAGCTTGCCACCATGAGAGCCCTGCTGGCG-3' and reverse, 5'-CAAGCTTCCCGAGGGCCAGC42CATTCTCTTC-3'.

Cell culture and transfection

The HUVECs grew in a 10% fetal bovine serum (FBS) DMEM solution containing 100 mg/L streptomycin and 100 units/mL penicillin. It was cultured continuously at 37°C and in a 5% carbon dioxide cell incubator. About 2000 cells were inoculated in a six-well plate the day before the transfection. According to the lipofectamine-2000 instructions, the cells were transfected with an adenovirus vector.

The experiment was divided into three groups: a blank control group (PBS), an empty vector group (blank adenovirus vector), and an overexpression group (recombinant adenovirus vector containing uPA gene sequence), and each experiment was repeated three times.

qPCR

48 hours after the transfection of the HUVECs, the culture medium was removed, and then the total RNA was extracted from the HUVECs using the Trizol Reagent (Invitrogen, California, USA). A PrimeScript RT kit (Kangwei Century Biotechnology Co., Ltd.) was used to synthesize the cDNA. According to the instructions, SYBR Green dye was used for qPCR. The reaction conditions were as follows: 10 min 95 pre-denaturation, 40 cycles (95 15 S; 60 30 S); 72 30 S); and the primers used in the qPCR were as follow: uPA, 5'-CTACTACGGCTCTGAAGTCACCAC-3'; (sense) and 5'-GTAGACGCCTGGCTTGTCCT-3' (antisense); In each reaction, 10 μl SYBR® Premix Ex Taq™, 0.4 μM reverse and forward, 1 μl cDNA in a total volume of 20 μl were used. The standard $2^{-\Delta\Delta}$ CT method was used to calculate the results.

Western blot analysis

A Western blot analysis was performed with reference to the reported literature [15]. In short, RIPA containing protease inhibitors and phosphatase inhibitors cleaved human umbilical vein endothelial cells, 10% SDS-PAGE electrophoresis, then the cells were transferred to a PVDF membrane, 3% BSA blocked 1 hour later, then the membrane was incubated with Rabbit monoclonal anti-human antibodies (uPA or β-actin 1:1000) overnight, After washing with TBST for 1 hour, we added goat anti-rabbit secondary antibody (1:8000) for 1 h, followed by a TBST wash, luminescent solution, exposure imaging, and β-actin was used as a protein load measurement.

MTT assay

MTT was performed with reference to the reported literature [16]. In short, HUVEC was put into 96-well plates the night before transfection. The density of the plates was 2000 cells/well, and we added a 100 μL medium containing 10% FBS into each well. The next day, four 96-well plates were transfected with the cells.

Then one plate was taken out after being transfected for 24 h, 48 h, 72 h, 96 h, and then 20 μL MTT (5 mg/ml) was added into each well. After 4 h, 150 μL/well of dimethyl sulfoxide (DMSO) was added to the culture solution, the wells were shaken for 10 min, and the optical density (OD) of the sample was measured on a microplate spectrophotometer (Thermo Multiscan MK3, USA) at 490 nm.

Colorimetric assay

The uPA activity was determined using a colorimetric assay Kit for uPA (Shanghai Zhenao Biotechnology Co., Ltd.). Briefly, the cells were cultured in a 60 mm cell culture dish (1 to 5 × 10^7 cells), then they were carefully mixed with 3ml cleaning solution, then we covered the cell surfaces and carefully removed the cleaning solution. We then used a cell-scraping rod to gently scrape off the cells, we added 3 ml Reagent A, and we mixed the cells into the 15 ml conical centrifuge tube. Next, we put it into a 4°C centrifuge for 5 min. carefully removed the supernatant and added Reagent B 500 μL. We then mixed it and transferred it to a pre-cooled 1.5 ml centrifugal tube with strong whirlpool oscillation for 15 s. Then we incubated it in an ice tank for 30 min and centrifuged it in a 4°C miniature centrifuge for 5 min at a speed of 16 000 G. Next we carefully removed the 500 ml supernatant to the new pre-cooled 1.5 ml centrifugal tube. 96-well plates were taken out and marked with reference wells and sample wells. 85 μL Reagent C, 5 μL Reagent D and 10 μL Reagent E were added to the wells and incubated at 37 C for 60 minutes. Then they were transferred to a 100 μL colorimetric dish and tested with a spectrophotometer.

Enzyme linked immunosorbent assay (ELISA)

After 48 hours of transfection, the cell supernatants were collected and centrifuged for 10 minutes at 3000 r/min and stored at -20°C. We covered them with a flat plate and incubated them overnight at 4°C. The next day, the washing and coating solution was gradually diluted, and a negative control was set up. The supernatant was added to each pore and incubated for 2 hours. We then abandoned the liquid, patted the supernatant dry and washed it three times, then we added the detection antibodies uPA, MMP2, and MMP9, then we fully washed the solution, we added the substrates A and B liquid 50 μL to each well, and then we stopped the reaction after 20 minutes, then we tested 450 nm A value on the microplate spectrophotometer.

Statistical analysis

The experimental results were expressed as the mean ± the standard error of the mean (S.E.M). SPSS 18.0 was used for the statistical analysis. The statistical analyses were performed with one-way ANOVA and Student's *t*-test. Differences of data were considered statistically significant when a *P*-value < 0.05.

Results

The recombined adenovirus vector carrying uPA was successfully transfected into HUVECs

The recombined adenovirus vector carried the enhanced green fluorescent protein (EGFP) gene, which could be seen under a fluorescence microscope after it was transfected. Most of the cells in the overexpression group and the empty vector group showed green fluorescence, but no green fluorescence was observed in the blank control group after 48 h of transfection (MOI 50). It is suggested that the transfection was successful, and the transfection efficiency was about 85±2.6% (**Figure 1**).

The overexpression of uPA promoted uPA mRNA expression in the HUVECs

The expressions of the uPA mRNA were measured using a qPCR analysis after the cells were transfected for 48 hours. The expression of uPA mRNA in the overexpression group was significantly higher than the expression of the blank control and empty vector groups ($P < 0.05$), but there was no significant difference between the blank control group and the empty vector group ($P > 0.05$, **Figure 2A**).

The overexpression of uPA promoted uPA protein expression in the HUVECs

The expressions of the uPA protein were detected by Western blot analysis after the cells were transfected for 48 hours. The uPA protein in the HUVECs in the overexpression group was significantly higher than it was in the blank control group and the empty vector group ($P < 0.05$). However, there was no significant difference

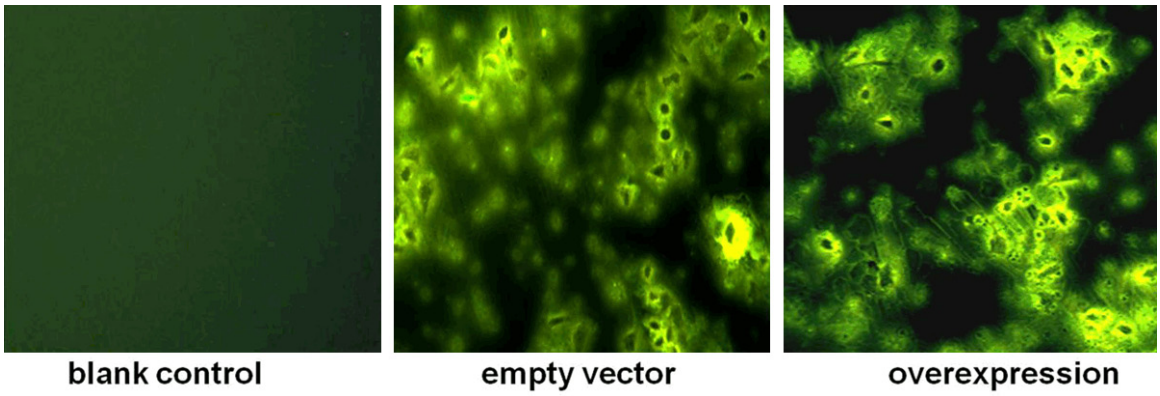

Figure 1. A recombined adenovirus vector carried the enhanced green fluorescent protein (EGFP) gene, which could be seen under a fluorescence microscope after they were transfected. Most of the cells in the overexpression group and the empty vector group showed green fluorescence, but no green fluorescence was observed in the blank control group 48 h after transfection (× 200).

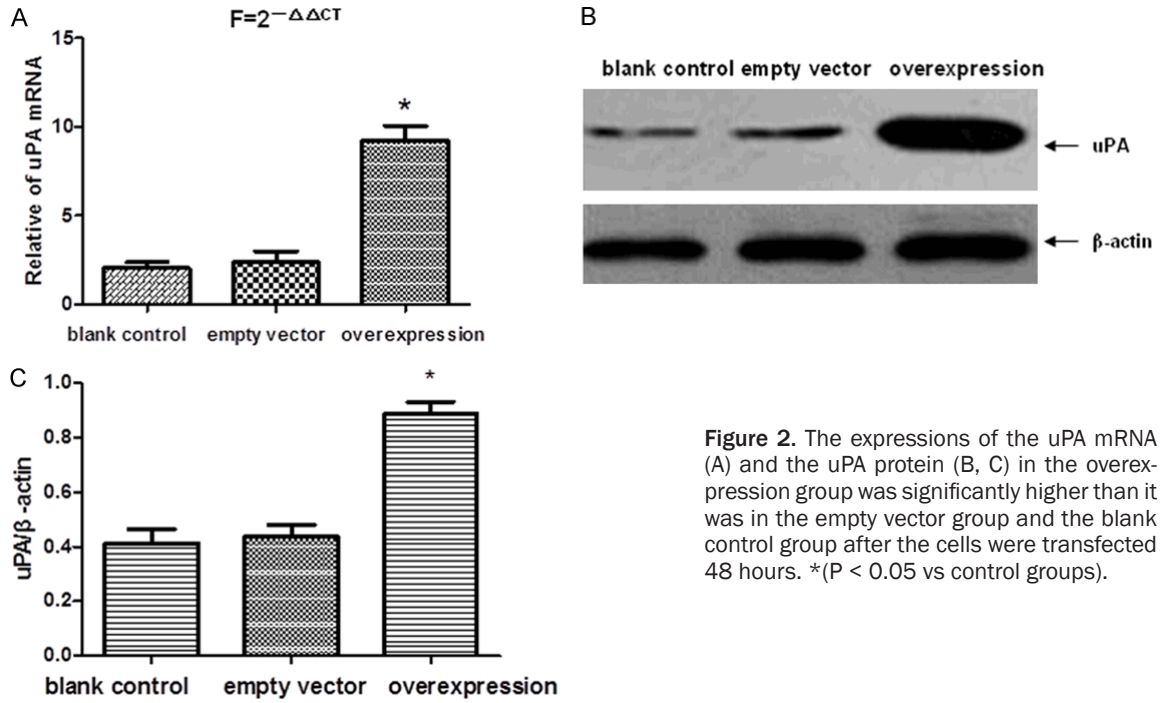

Figure 2. The expressions of the uPA mRNA (A) and the uPA protein (B, C) in the overexpression group was significantly higher than it was in the empty vector group and the blank control group after the cells were transfected 48 hours. *(P < 0.05 vs control groups).

between the blank control group and empty vector group (P > 0.05, **Figure 2B** and **2C**).

The overexpression of uPA promoted proliferation in the HUVECs

The MTT results showed that the proliferation of HUVECs in the overexpression group was significantly higher than it was in the empty vector and blank control groups after the cells were transfected 48 hours. (P < 0.05), but there was no significant difference between the blank control group and the empty vector group (P > 0.05, **Figure 3**).

The overexpression of uPA promoted the secretions of MMP2, MMP9 and VEGF in the HUVECs

The secretions of MMP2, MMP9, and VEGF were detected by ELISA after the cells were transfected for 48 hours. The secretions of MMP2, MMP9, and VEGF in the overexpression group were significantly higher than they were

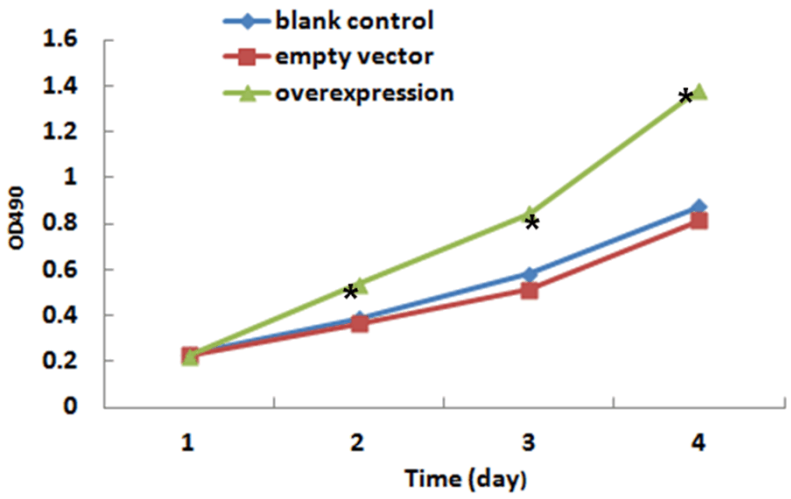

Figure 3. The cell proliferation curve in the overexpression group was significantly higher than it was in the empty vector group and the blank control group after the cells were transfected 48 hours. *(P < 0.05 vs control groups).

in the HUVECs in the blank control and empty vector groups (P < 0.05). However, there was no significant difference between the blank control group and the empty vector group (P > 0.05, **Figure 4**).

The overexpression of uPA promoted uPA activity

The uPA activity in the overexpression group (15343.830±716.499 IU/mg) was found to be significantly increased compared with the activity in the blank control group (2107.040± 133.512 IU/mg) group and the empty vector group (2265.441±119.687 IU/mg) (P < 0.05). However, there was no significant difference between the blank control group and the empty vector group (P > 0.05, **Figure 5**).

Discussion

In this study, the results showed that the levels of uPA mRNA and the proteins in the overexpression group were significantly higher, compared to the other groups, (P < 0.05). The cell proliferation and uPA activity were increased significantly in the overexpression group, compared to the other groups, (P < 0.05). It is suggested that we had recombined the adenovirus vector carrying uPA and successfully transfected it into HUVECs. Exogenous uPA gene can transcribe and secrete uPA proteins in HUVECs. The overexpression of the uPA gene promoted the proliferation and fibrinolytic activity of the HUVECs.

The mechanism of DVT is complex, and the important reason behind DVT's complexity is the injury of the vascular wall. Vein endothelial cells, as the main component of the vascular wall, can regulate the relationship between blood and blood vessels by providing physical barriers on the one hand, and by playing an anti-thrombotic role by secreting fibrinolytic cytokines (such as uPA) [17]. Moreover, uPA, when combined with uPAR in vivo, can activate the signal transduction pathways that mediate various cellular physiological processes and participate in cell migration, adhesion, proliferation, and differentiation [18]. UPA is one of the most suitable thrombolytic agents in vivo. Gene-level therapy for venous thrombosis has advantages that traditional therapy does not have. It can promote the secretion of endogenous uPA by its own tissue, leading to thrombolytic ablation. In the treatment of venous thrombosis, it is necessary not only to restore the patency of blood vessels, but also to restore the endothelial cell intima of the venous wall [13]. Vascular endothelial growth factor (VEGF) is a growth factor that can specifically act on vascular endothelial cells and is a functional protein that promotes vascular endothelial cell proliferation [19, 20]. It is involved in thromboembolization and in the intimal repair of venous thrombosis [21].

Stepanova V studies have shown that uPA promotes the secretion of VEGF [22]. Waltham et al. transfected the expression plasmid containing the vascular endothelial growth factor gene into the animal model of inferior vena cava thrombosis and established a model of vascular recanalization after embolization [23]. Matrix metalloproteinases (MMPs) promote cell invasion by degrading the extracellular matrix. MMP 2 and MMP9 are important members of its family and promote cell invasion and metastasis [24, 25]. Studies have shown that uPA can promote cell invasion and angiogenesis by degrading the extracellular matrix and basement membrane components, such as fibronectin, laminin and collagen, when tissue secretes a

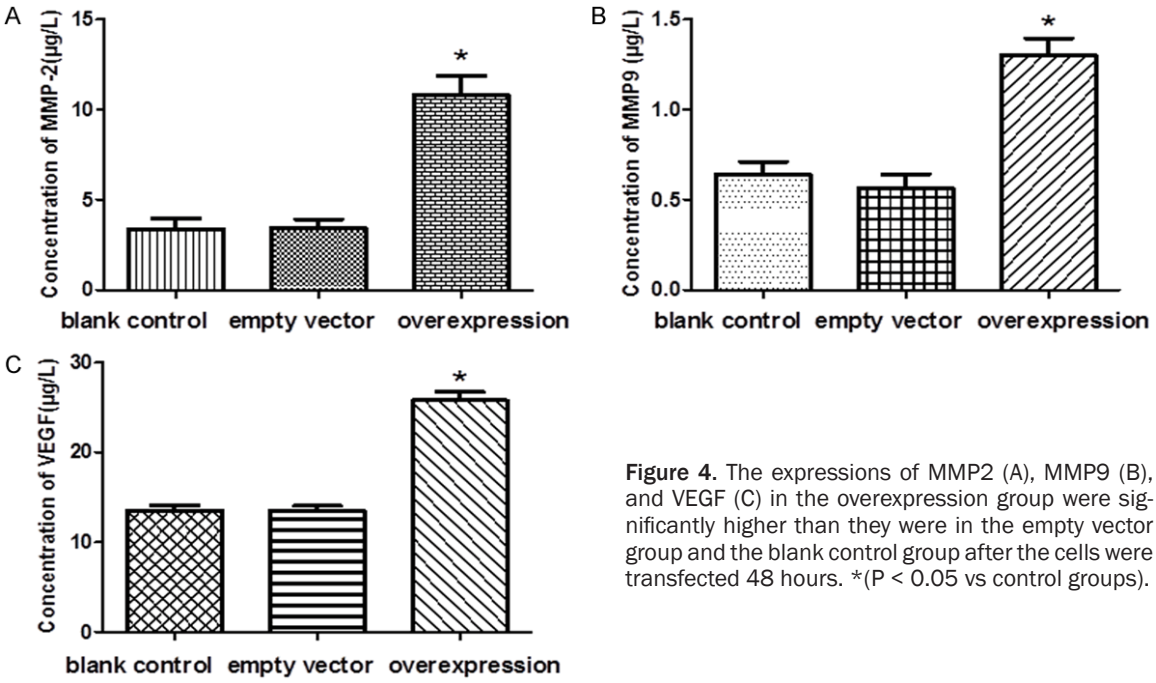

Figure 4. The expressions of MMP2 (A), MMP9 (B), and VEGF (C) in the overexpression group were significantly higher than they were in the empty vector group and the blank control group after the cells were transfected 48 hours. *($P < 0.05$ vs control groups).

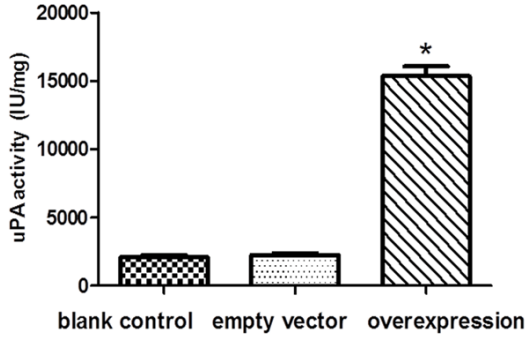

Figure 5. The UPA activity in the overexpression group was significantly higher than it was in the empty vector group and the blank control group after the cells were transfected 48 hours. *($P < 0.05$ vs control groups).

large amount of uPA [26-29]. Our study showed that the secretions of MMP2, MMP9 and VEGF in the overexpression group were significantly higher than those in the other two groups ($P < 0.05$). These results suggest that the overexpression of uPA can improve the invasion and angiogenesis abilities in HUVECs by promoting their secretions of MMP2, MMP9, and VEGF.

Conclusion

We recombined an adenovirus vector carrying uPA and successfully transfected it into HUVECs. The exogenous uPA gene can transcribe and secrete the uPA protein in HUVECs. The overexpression of uPA can increase cell proliferation and uPA activity. It can improve the invasion and angiogenesis abilities in HUVECs by promoting their secretions of MMP2, MMP9, and VEGF. However, the thrombolytic process is very complex in vivo. We have not carried out animal experiments, and the specific effect is not clear. We will further study the thrombolytic effect on animal models.

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Disclosure of conflict of interest

None.

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