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Original Article

Targeting NF-κB and PUMA prevents heart injury in heart transplantation

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Abstract: Objective: Activation of NF-κB and p53-upregulated modulator of apoptosis (PUMA) is associated with ischemia-reperfusion injury (I/R) of donor heart. The current study investigated whether targeting NF-κB and PUMA could preserve heart grafts in heart transplantation in mice. Methods: Heart grafts from BALB/c mice were preserved in Wisconsin solution (UW) solution (control) or UW solution containing siRNAs targeting NF-κB (p65) and PUMA for 48 hours and subsequently transplanted into syngeneic recipients. p65 and PUMA mRNA and protein was detected by qRT-PCR and Western blot assay; NF-κB activity was detected by EMSA assay; Heart histology was assessed. Apoptosis was detected by TUNEL staining. Results: (p65 and PUMA) siRNA solution/treated hearts exhibited improved histology, decreased cell apoptosis and diminished neutrophil and lymphocyte infiltration compared with control solution-treated organs. Furthermore, the mean heart graft survival times of the control siRNA solution or UW solution groups was 8 day and 9 days, and 56 days in siRNA solution groups. Conclusions: Incorporation of siRNA targeting p65 and PUMA into organ storage solution provide greater protection from apoptosis, ischemia/reperfusion (I/R) injury, and prolonging graft survival.

Keywords: Heart transplantation, ischemia/reperfusion, NF-Kb, p53-upregulated modulator of apoptosis, small interfering RNA

Introduction

Ischemia reperfusion (I/R) injury is an unavoidable event occurring during heart transplantation, leading to primary graft failure (PGF) and lower long-term survival rate of the recipient [1-3]. Therefore, minimizing I/R injury could improve graft function and survival, decrease the immune response, and may also allow safe extension of cold preservation times.

Many studies have found that during I/R of organs, pro-inflammatory cytokines TNF, IL-1β, IL-6 and IFNγ were increased, leading to tissue damage mediated by neutrophils and monocytes [4]. The pro-inflammatory cytokines TNF, IL-1β, IL-6 and the neutrophil chemoattractants MCP-1 (Monocyte Chemotactic Protein 1) and MIP-1α (Macrophage Inflammatory Protein 1-α) are gene targets of NF-κB. During I/R injury, NF-κB activation is important both in leukocytes and in the parenchymal cells of the transplanted organ [5], thus making inhibition of NF-κB activation during I/R injury could prevent or attenuate I/R injury.

A considerable amount of research has focused on the role that apoptotic mediators play in I/R injury. In particular, activation of prodeath Bcl2 proteins such as Bax, Bak, Bid, Puma, and BNIP3 and their upregulation, translocation, and integration into mitochondrial membranes have been reported in ischemically damaged tissues [6, 7]. The p53-upregulated modulator of apoptosis (Puma), a BH3-only member of the Bcl-2 protein family, is required for p53-dependent and -independent forms of apoptosis and has been implicated in the pathomechanism of several diseases, including cancer, acquired immunodeficiency syndrome, and ischemic brain disease. Toth et al. has found that Puma as an essential mediator of cardiomyocyte death upon I/R injury and offers a novel therapeutic target to limit cell loss in ischemic heart

disease [8]. Nickson et al. has found that Puma is a critical component of ER stress-induced apoptosis in cardiac myocytes, and inhibition of Puma activity may be used to treat cardiac infarcts or prevent heart failure by blocking ER stress-induced apoptosis [9]. In cerebral astrocytes,PUMA is invovled in the apoptosis of cerebral astrocytes upon I/R injury [10].

Other studies have also reported that PUMA was upregulated during I/R, and PUMA was the target for the treatment of I/R injury by inhibiting apoptosis [11-13]. We therefore suggested that targeting NF-κB and PUMA could inhibit pro-inflammatory cytokines and decrease infiltration of neutrophils into ischemic organs upon I/R on the one hand, and inhibit cell apoptosis to prevent I/R injury on the other hand.

RNA interference (RNAi) can be used to inhibit the expression of specific genes *in vitro* and *in vivo*, thereby providing an extremely useful tool for investigating gene function. In addition, RNAi is currently progressing from basic research to potential therapeutic applications. In the present study, we investigated the feasibility of silencing both NF-κB and PUMA expression by siRNA treatment on ischemia/reperfusion injury in rat hearts.

Methods and material

Mice

Male 8-10 week old BALB/c mice were purchased from Shanghai laboratory animal research center. Animals were housed under conventional conditions at the Animal Care Facility, the affiliated hospital of Qingdao University, and were cared for in accordance with the guidelines established by the China Council on Animal Care.

p65 and PUMA expressed siRNA vector constructs

p65 (RelA) siRNAs and negative control siRNA were chemically synthesized by Ambio (Austin, TX, USA). Negative control siRNA by Ambion (Austin, TX). PUMA siRNA was purchased from Cell Signaling Technology (Shanghai, China).

Donor organ preservation and cardiac transplantation procedure

Heterotypic cardiac transplantation was performed according to the report as previously described [14]. The C57BL/6 recipient mouse was anesthetized with isoflurane. A segment of descending aorta and vena cava below the renal vessels was dissected. The heart was immediately removed from the donor and placed in chilled Eurocollins solution on ice. Heart grafts were harvested and perfused with siRNA solution according to the report as previously described [15]. The BALB. donor heart was then placed in the abdominal cavity of the recipient, and the donor aorta and pulmonary artery were anastomosed in an end-to-side manner to the recipient abdominal aorta and vena cava using 10-0 nylon suture.

Graft examination

The primary end point of the study was the graft survival, which was assessed by daily examination of the graft function by palpation and transabdominal echocardiography under isoflurane. The graft function was graduated in good contraction (+++), mild (++) or severe dysfunction (+), and no contraction (-), which led to termination of each experiment [16].

Quantitative RT-PCR

The changes in tissue target gene expression on mRNA level were detected using quantitative RT-PCR. Total RNA was isolated using Tri reagent (Molecular Research Center) according to the protocol recommended by the manufacturer. One μg of RNA was reverse transcribed using random primers supplied in the High-Capacity cDNA Archieve Kit (Applied Biosystems). To quantify gene expression, cDNA of *p65, PUMA,* TNF-a, IL-1β and IL-6 was amplified using corresponding pair of primers synthesized by Operon, Power SYBR Green PCR Master Mix and ABI 7700HT PCR Machine (both from Applied Biosystems). The mRNA levels of *GAPDH* were used for normalization. All reactions were performed in triplicate. The amount of total proteins was determined with the BCA Protein Assay Kit (Pierce).

Western blot

The amount of total proteins was determined with the BCA Protein Assay Kit (Pierce). 100 μg of total protein were electrophoresed in 4-20% SDS-polyacrylamide gel and transferred onto PVDF membrane (Invitrogen). The membrane was blocked by incubating with 5% milk/Tris-buffered saline plus Tween20 (TBST), and incubated with primary antibodies against p65, PUMA, TNF, IL-1β, IL-6. (all from Santa Cruz

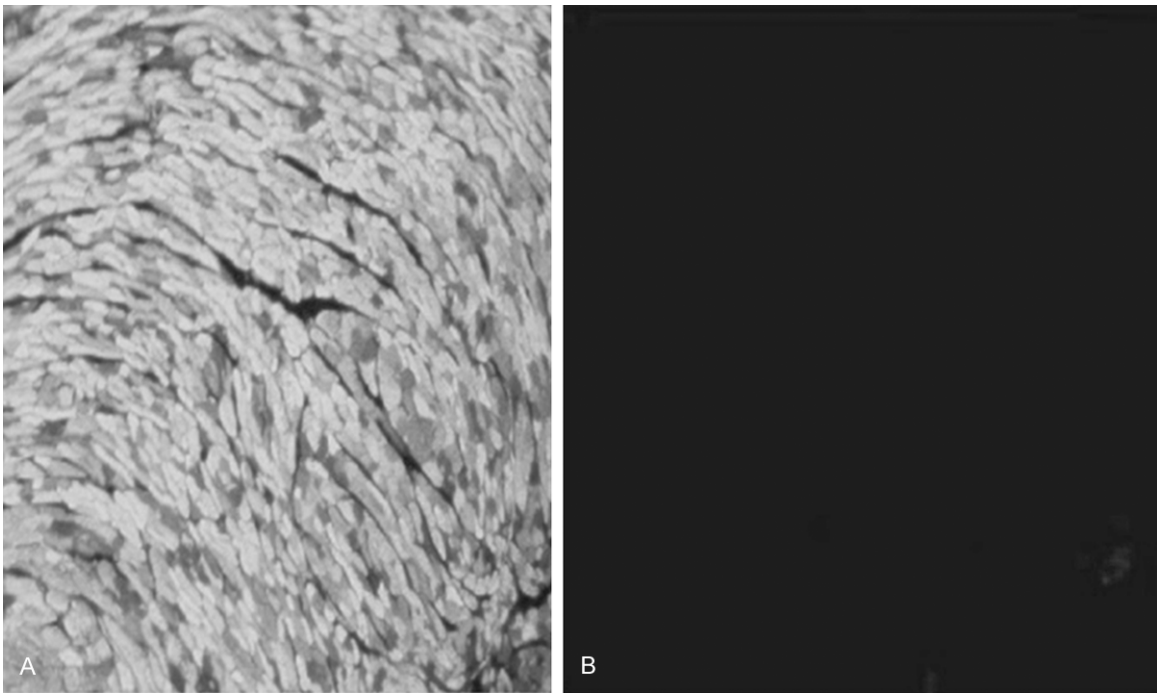

Figure 1. Distribution of siRNA in hearts. Donor hearts were taken from the mice and preserved in a UW solution containing 100 ug GFP-labeled siRNA (A) or UW solution alone (B). Forty-eight hours after preservation, hearts were harvested and sectioned. The distribution of siRNA was tracked by fluorescence microscopy.

Biotechnology). The secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Pierce) or anti-goat IgG (Santa Cruz). Immunoreactive bands were visualized using ECL Plus Western Blotting Detection System (GE Healthcare). The equal protein loading was assessed by probing the same membrane with ACTIN antibody (NeoMarker). All reactions were performed in triplicate.

Electrophoretic mobility shift assay (EMSA)

^{32}P-labeled double-stranded oligonucleotides of 3.0×10^4 cpm spanning the NF-kB sites (5'-GTTCTGGGATTTCCCCCGAT-3') was incubated with 5 μg of nuclear extract at room temperature for 20 min with 2 μg of poly (dI-dC) in 50 mM NaCl, 10 mM Tris pH 7.5, 1 mM DTT and 20% glycerol. Competition experiments were performed by co-incubating the probe with 100-fold molar excess of unlabeled double-stranded oligonucleotide or unlabeled double-stranded oligonucleotide mutated in NF-kB sites (5'-GTTCTG*AACGGGAC*CCCCGAT-3'). Supershift assays were performed by incubating nuclear extracts with specific antibodies against p65 for 30 min at room temperature before the addition of the probe. Band shifts were resolved on non-denaturing 4% polyacrylamide gels.

Histology detection

On day 7 after transplantation, heart grafts were dissected from mice. The tissue, which was taken from the middle part of the left ventricle and stained with hematoxylin and eosin, was embedded in paraffin and sectioned at 3 μm thickness. Bright red-stained neutrophils infiltrated into the cardiac muscle cells and were counted by several people on the research team. The percentage of histology changes in grafts was scored with a semiquantitative scale designed to evaluate the degree of infarction, ischemia, and cast formation on a 5-point scale based on injury area of involvement as follows: 0: 10%; 1: 10% to 25%; 2: 25% to 50%; 3: 50% to 75%; and 4: 75% to 100% [15]. Histopathological neutrophil counting analysis was executed with the samples' identities masked.

Terminal transferase-mediated dUTP nick end labeling (TUNEL) assay

TUNEL assay was performed on paraffin sections of explanted tissues using the Apo-Alert DNA Fragmentation Assay Kit (Clontech) following manufacturer's instructions. TUNEL positive nuclei were then counted using Image J software, and expressed as a percentage of total nuclei counted from DAPI staining.

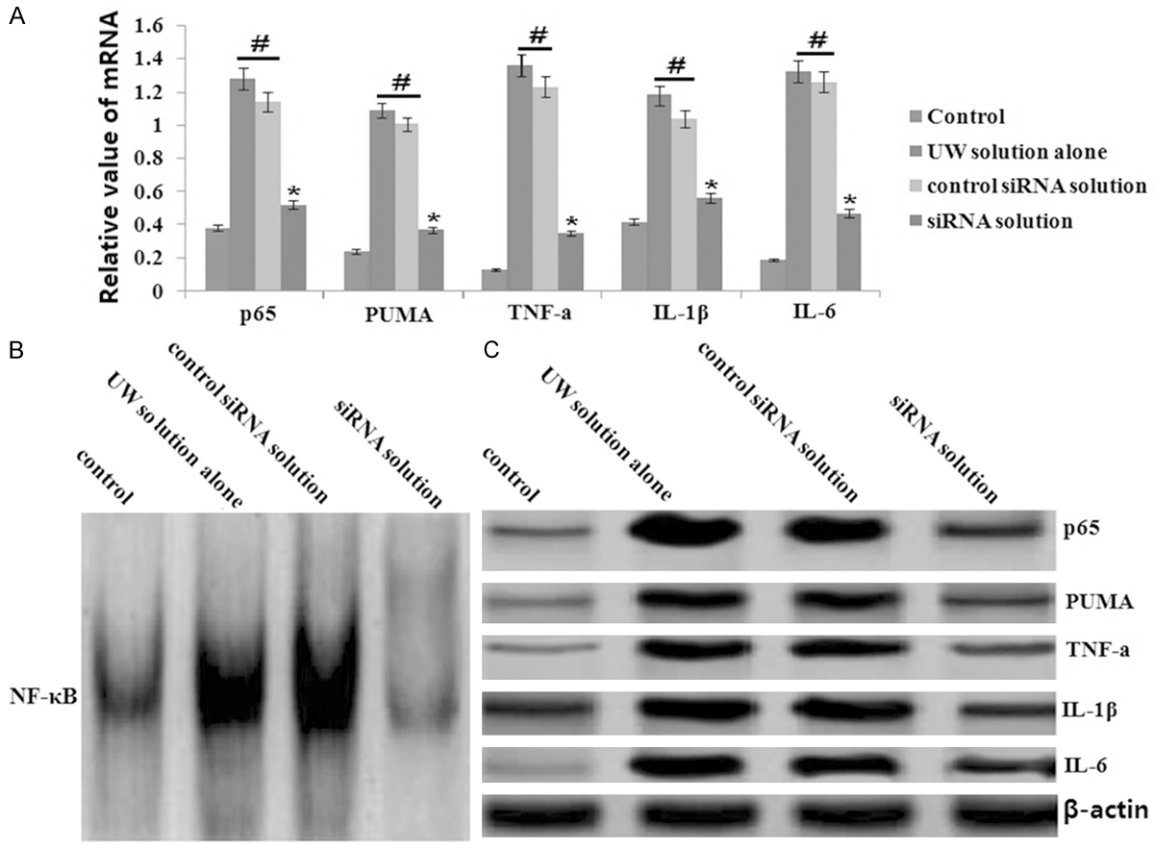

Figure 2. Gene silencing in cardiac grafts. Donor hearts were preserved in siRNA solution, control siRNA solution, or UW solution for 48 hours. The preserved hearts were implanted into syngeneic mice. Forty-eight hours after transplantation, grafts were harvested and total RNAs and protein were extracted. A. p65, PUMA, TNF-a, IL-1β and IL-6 mRNA was detected by qRT-PCR; B. NF-kB activity was detected by EMSA assay; C. Protein of p65, PUMA, TNF-a, IL-1β and IL-6 mRNA was detected by western blot assay. vs control, $^{\#}P < 0.05$; vs UW solution alone or control siRNA solution, vs $^{\#,*}P < 0.05$.

Statistical analysis

For all experiments, Values were expressed as mean ± SEM. Data were assessed by using a Student's t-test. All statistical analysis was performed using Statview software (SAS Institute Inc., Cary, NC) with a value of $P \leq 0.05$ considered to be statistically significant.

Results

Uptake of siRNA in heart tissues

To deliver siRNA into cardiac tissues effectively, we perfused hearts with UW solution containing 100 ug/mL GFP-labeled siRNA for 48 hs. The hearts were harvested and sectioned. The fluorescence distribution in the heart tissues was detected. As shown in **Figure 1A**, the green fluorescence signal was detected in GFP-labeled siRNA heart tissue, and no green fluorescence was detected in the heart tissue preserved in the UW solution alone (**Figure 1B**), suggesting that siRNA could be delivered to cardiac tissue through perfusion and preservation in siRNA solution.

p65 and PUMA gene silencing in vivo

Accumulating studies have shown that I/R could upregulate pro-inflammatory cytokines and pro-apoptotic genes, resulting in cell and tissue ingury [4-7]. Given the ability of the organ storage solution to allow intracellular entry of siRNA, we next assessed functionality of siRNA introduced via this method. After the hearts were taken from BALB/c mice and preserved in siRNA solution containing siRNAs specific to p65 and PUMA genes for 48 hours, and gene expression was examined at the messenger RNA by qRT-PCR. As shown in **Figure 2A**, treatment with p65 and PUMA siRNA vectors significantly decreased p65 and PUMA gene expression compared to the control grafts preserved

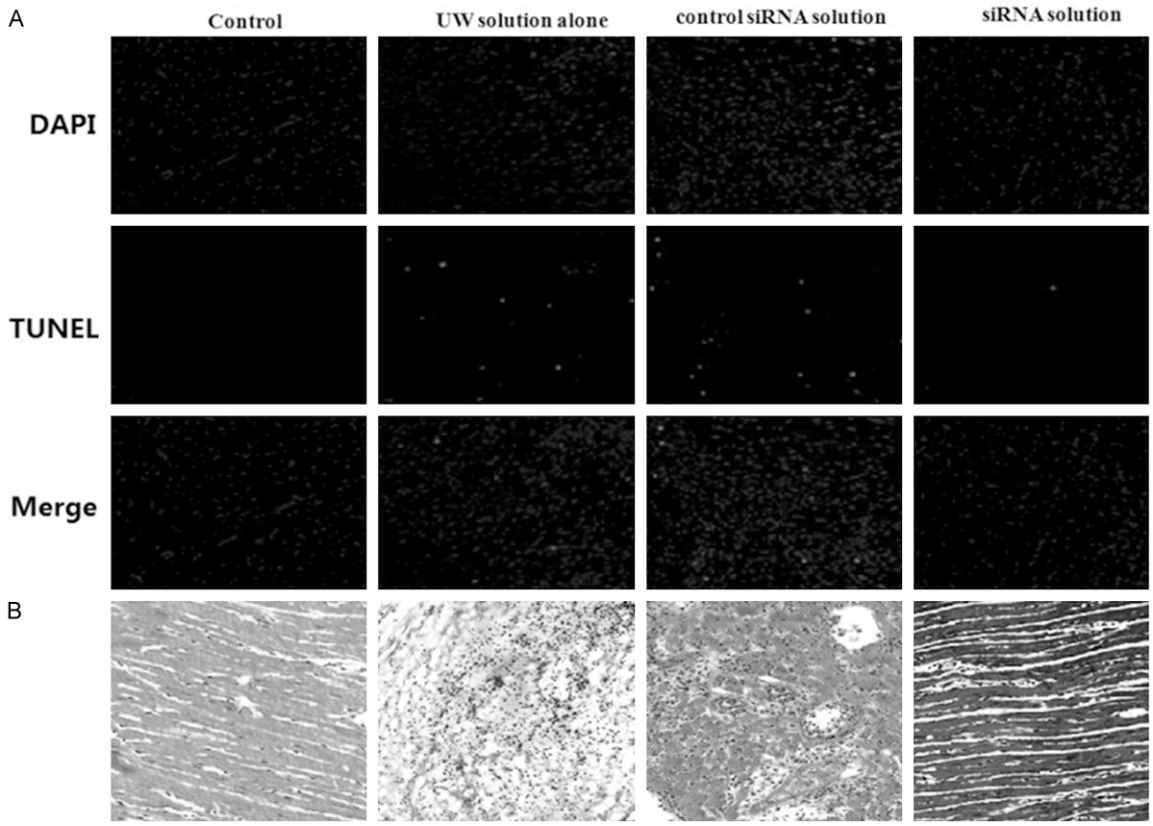

Figure 3. Histopathology of heart grafts. A. TUNEL staining of representative heart allografts harvested at 7 d post transplantation. B. Hematoxylin and eosin staining of representative heart allografts harvested at 7 d post transplantation.

in UW solution alone or control siRNA solution. In addition, NF-κB activity was also significantly decreased by EMSA assay (**Figure 2B**). These data demonstrate that the p65 and PUMA siRNA vectors were capable of knocking down p65 and PUMA gene expression in vivo. We also detected the downstream gene TNF-a, IL-1β and IL-6 of p65, only to find the expression of TNF-a, IL-1β, IL-6 was significantly decreased with targeting p65 and PUMA (**Figure 2A**). Western blot has the same results as qRT-PCR (**Figure 2C**).

Protect cardiac allografts by targeting p65 and PUMA using siRNA

To study the cellular changes associated with protection from I/R injury mediated by the siRNA solution, grafts were harvested on day 7 after transplantation for histological analysis. The sections of hearts were analyzed for the presence of apoptotic cells using the terminal deoxynucleotide transferase-mediated 2'-deoxyuridine-5'-triphosphate nick-end labeling (TUNEL) assay. The numbers of apoptotic cells was (0.2±0.00)% in the heart tissues of the siRNA solution containing siRNAs specific to p65 and PUMA genes compared to that in UW solution alone (1.02±0.06)% or control siRNA solution (0.97±0.04)% (**Figure 3A**, $P < 0.01$, respectively). Histologic examinations of cardiac allografts on day 7 after transplantation was found that grafts preserved in UW solution alone or control siRNA solution exhibited severe myocyte damage, edema, inflammatory cell infiltration, hemorrhage, perivasculitis, and endotheliitis; Allografts from siRNA solution showed some cell infiltration, preserved graft structure, and mild myocardial injuries (**Figure 3B**).

Effect of siRNA on allograft survival

The mean heart graft survival times of the control siRNA solution or UW solution groups was 8 day and 9 days, respectively. However, the heart graft mean survival times in siRNA solution groups was 56 days ($P < 0.01$).

Discussion

I/R injury is known as the primary factor leading to graft dysfunction [17] and graft failure [18, 19]. Understanding the molecular mechanisms of I/R injury is important to for the development of therapies against I/R injury. It is well common, that fundamental features of the response to I/R includes the release of ROS, cytokines, and chemokines from activated endothelium and tissue-resident macrophages and mast cells, recruitment, activation, and endothelial adhesion/emigration of neutrophils, and other formed elements in the blood, endothelial dysfunction, and parenchymal injury [17]. All these changes are contributed to the activation of pro-inflammatory genes and proapoptotic genes, such as NF-kB (p65) and BH3 familiy genes.

Given that I/R injury is fundamentally associated with a key set of genes being upregulated, we decided to target p65 and PUMA that have been well defined as culprits of this inflammatory and proapoptotic process produced locally and systemically after cardiac I/R. RNA interference (RNAi)-based therapeutics have emerged for the treatment of cancer, infectious diseases, and other diseases associated with specific gene disorders. A kidney-targeted siRNA could temporarily inhibit expression of the pro-apoptotic p53 in kidney [20]. Zheng et al. has reported that incorporation of siRNA into organ storage solution is a feasible and effective method of attenuating I/R injury, protecting cardiac function, and prolonging graft survival [21].

In our study, we found that by implementing siRNA technology, we were able to downregulate the expression of p65 and PUMA genes involved in I/R injury and leukocyte emediated tissue injury. We targeted p65 and PUMA for their involvement with the apoptosis, complement, and inflammatory response pathways. By comparing heart organs preserved in control solution and siRNA solution, we demonstrated the potential implications of siRNA technology to organ preservation. The prolongation of cardiac grafts after they were preserved in siRNA-containing solution supports the protective effect of the siRNA solution. The siRNA organ solution that we developed in this study consists of p65 and PUMA targeting 2 different pathways that are crucial to I/R injury. The siRNA solution efficiently and specifically attenuates complement activation, apoptosis of cells, and inflammation of grafts.

Conclusion

The study indicates a novel siRNA solution which could decrease cardiac I/R injury and prolong graft survival in heart transplantation, suggesting potentially significance in clinical application.

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Disclosure of conflict of interest

None.

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