**Abstract.** – OBJECTIVE: Intestinal acute graft-versus-host disease (aGVHD) is a serious complication of allogeneic hematopoietic stem cell transplantation (allo-HSCT). Abnormal autophagy levels in intestinal aGVHD have been confirmed in many studies. LncRNAs exert coregulatory functions and participate in a variety of intracellular regulatory processes. In this study, we investigated how lnc-AC145676.2.1-6-3 regulates dysregulated STX3-related autophagy in aGVHD.

MATERIALS AND METHODS: First, we established a mouse model of aGVHD by transplanting a mononuclear cell suspension from Balb/c donor mice treated with 60Co X-rays into CB6F1 recipient mice. STX3-related indicators were analyzed by Western blotting (WB) and immunohistochemistry which confirmed that STX3 plays an important role in dysregulating autophagy in intestinal aGVHD. TNF-α induced Caco-2 cells, which is an in vitro model of intestinal barrier dysfunction, were established to verify the effect of STX3. The direct interaction between the partners of Inc-AC145676.2.1-6-3-mediated hsa-miR-1292-3p and STX3 axis was evaluated by the Dual-Luciferase activity assay. We performed PCR, WB, and immunofluorescence in Caco-2 cells to determine whether the abnormal autophagy levels were influenced by Inc-AC145676.2.1-6-3.

RESULTS: The results showed that Inc-AC145676.2.1-6-3 could significantly suppress the number of autophagic vacuoles, the LC3-II/I ratio, and beclin1 levels by increasing STX3 levels.

CONCLUSIONS: Lnc-AC145676.2.1-6-3 may play an important role in intestinal aGVHD by targeting STX3.

**Introduction**

Intestinal acute graft-versus-host disease (aGVHD) is one of the most fatal complications in the early stage after allogeneic hematopoietic stem cell transplantation (allo-HSCT). The main manifestations are intractable diarrhea, severe colitis, hematochezia and intestinal obstruction, which can be life-threatening. Autophagy is the process by which a cell phagocytoses its own cytoplasmic proteins or organelles via encapsulation into vesicles, which fuse with lysosomes to form autophagic lysosomes that degrade the encapsulated contents. Our previous work suggests that autophagy protects the intestinal mucosa by degrading damaged organelles and other substances, which slow aGVHD progression. Some studies have shown that synaptexin 3 (STX3) affects autophagy by regulating the PTEN-Pi3K-AKT-mTOR signaling pathway. In addition, STX3 is associated with congenital diarrhea disorders and intestinal epithelial differentiation diseases and plays a role in the protection of the intestinal mucosa. LncRNAs can act as miRNA sponges, whereby they regulate the target genes of miRNAs by binding the miRNAs...
and reducing their “silencing effect” on the target genes. These IncRNAs are also referred to as competitive endogenous RNAs (ceRNAs). Some studies have reported that the ceRNAs play an important role in hematological diseases. 11. In recent years, IncRNAs have been demonstrated to affect aGVHD. Therefore, we investigated whether IncRNAs can influence aGVHD pathology by regulating STX3-mediated autophagy.

Materials and Methods

Reagents and Antibodies

The IncRNA expression plasmid and negative control (NC) plasmid, the IncRNA wild type and IncRNA mutant Dual-Luciferase reporter plasmids, the STX3 wild type and STX3 mutant Dual-Luciferase reporter plasmids, the IncRNA-specific RT-PCR and quantitative RT-PCR (qRT-PCR) primers and the STX3 RT-PCR primers were obtained from Jierui Bioengineering Company, Limited (Shanghai, China). AKT1 rabbit monoclonal antibody (mAb) (A17909), phospho-AKT1-S473 rabbit mAb (AP0637), mTOR rabbit polyclonal antibody (pAb) (A11355), phospho-mTOR-S2448 rabbit pAb (AP0094), mouse anti-Bcl-2 antibody (bsm-33047M), STX3 pAb (15556-1-AP) and PTEN rabbit pAb (A2113) were purchased from Seville Biotechnology Company, Limited (Wuhan, Hubei, China). All-in-one miRNA qRT-PCR detection kits were purchased from GeneCopoeia (Rockville, MD, USA). MAbs against LC3-II, LC3-I, Beclin-1, P62, p-AMPK, AMPK, mTOR and GAPDH were purchased from Affinity Biosciences (Cincinnati, OH, USA). RIPA lysis buffer, avidin-biotinylated peroxidase, DAB, bovine serum albumin, polycarbonate lucifer yellow (dextran), the reverse transcription kit, the Millipore system and fluorescence plate reader were provided by Sparkjade (Jinan, Shandong, China). Rabbit anti-LC3B antibody was purchased from Abcam (Cambridge, UK). Alexa Fluor 488-conjugated AffiniPure goat anti-rabbit antibody and Cy3-conjugated goat anti-rabbit secondary antibody (BOSTER Biological, OH, USA) was used.

Induction of the Mouse Model

The mice were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. (animal production license number was SCXK (Beijing) 2012-0001). Ten healthy Balb/c H-2d male mice were used as donors (weighing 18-22 g and aged 8-10 weeks), and 56 CB6F1 female mice were used as transplant recipients (weighing 18-22 g and aged 8-10 weeks); all mice were housed in SPF conditions. Inbred Balb/c H-2d mice were sacrificed by cervical dislocation, and bone marrow and spleen mononuclear cells were aseptically collected. Next, CB6F1 mice were irradiated once with 60Co X-rays, and then subjected to HSCT by infusion via tail vein the mononuclear cell suspension (1×10⁷ bone marrow cells + 1×10⁷ spleen cells) obtained from donor Balb/c mice (above). The mice were randomly divided into a control group and a model group.

After the model was established, the clinical score for aGVHD was determined by observing weight loss, posture, activity, hair, skin integrity, and stool on the 15th day after transplantation using criteria established by Cooke et al13. Small intestinal tissues were collected from mice for histological analysis of the mucosa and scoring of pathological inflammation.

Immunohistochemical Staining for STX3, PTEN, mTOR and LC3

Tissues of the small intestine (2-3 cm) were fixed with 70% ethanol overnight and then embedded in paraffin. The tissues were sliced into sections at a thickness of 4-6μm before they were rinsed three times in PBS for 5 min and treated with 3% hydrogen peroxide in PBS for 15 min at 37°C to block endogenous peroxidase activity. The samples were then blocked with 5% bovine serum albumin and incubated with primary antibody in a humidified chamber at 4°C overnight. After several washes, the sections were incubated with secondary antibody for 60 min at 37°C, rehydrated in PBS and incubated with avidin-biotinylated peroxidase complex for 45 min at 37°C. After washing with PBS, peroxidase activity was evaluated by treating the sections with the chromogen DAB.

Luciferase Reporter Constructs and Luciferase Activity Assay

The direct interactions between the constituents of the Inc-AC145676.2.1-6-3-mediated ceRNA network were evaluated by the Dual-Luciferase activity assay. A luciferase reporter vector (pmirGLO Dual-Luciferase miRNA Target Expression Vector; Promega) was used to generate the luciferase constructs. Lnc-AC145676.2.1-6-3 and the 3’ UTR of STX3 were cloned by RT-PCR. Plasmids containing Lnc-AC145676.2.1-6-3-WT, Lnc-AC145676.2.1-6-3-mutant and the WT or mutant STX3-3’ UTR were constructed. 293T cells were seeded onto 24-well plates and allowed to grow for ~24 h in antibiotic-free medium before
they were transfected. The constructed reporter vectors (300 ng) were cotransfected into cells with the miRNA (hsa-miR-1292-3p) mimics or NC mimics (100 nM) using Lipofectamine 2000 (2 μl). The cells were lysed ~24 h after transfection, and the luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to that of Renilla Luciferase. All experiments were performed independently in triplicate.

**Cell Lines and Culture**

The human colon carcinoma cell line Caco-2 is used as an intestinal barrier dysfunction cell model when treated with TNF-α. Cells were grown in Dulbecco’s modified Eagle’s minimum essential medium (DMEM, pH 7.4) (Invitrogen, Carlsbad, CA, USA) supplemented with 25 mM glucose, 10% inactivated fetal bovine serum (FBS) (Lonza), 1% penicillin/streptomycin (PS) and 1% nonessential amino acid solution (Invitrogen, Carlsbad, CA, USA). After the cells were stimulated with TNF-α (100 ng/ml) for 21 h at 37°C, their viability was measured. Caco-2 cells were randomly divided into the following groups: control, model, lncRNA siRNA, siRNA-NC and lncRNA siRNA+miRNA inhibitor. The lncRNA siRNA+miRNA inhibitor group was treated with TNF-α after transfection.

**Cell Transfection**

Caco-2 cells were assigned to the control (normal Caco-2 cells), model (Caco-2 cells stimulated with TNF-α (100 ng/ml) for 21 h at 37°C), siRNA (Caco-2 cells transfected with siRNA), siRNA-NC (Caco-2 cells transfected with NC siRNA), miR-1292-3p inhibitor (Caco-2 cells transfected with the miR-1292-3p inhibitor and then stimulated with TNF-α (100 ng/ml) for 21 h at 37°C), and miR-1292-3p inhibitor combined with siRNA (Caco-2 cells cotransfected with the miR-1292-3p inhibitor and siRNA) groups. The Inc-AC145676.2.1-6-3 mimic, Inc-AC145676.2.1-6-3 siRNA (si-IncRNA) and hsa-miR-1292-3p inhibitor were constructed by Gene Pharma (Shanghai, China).

**Cell Viability Assay**

Caco-2 cells in the logarithmic growth phase were conventionally trypsinized, washed with PBS, pelleted by centrifugation, resuspended, and counted. Then, the cells were seeded into 96-well plates at 2×10^3 cells/well in a volume of 100 μl per well. After 24, 48, and 72 h of incubation, 10 μl of CCK-8 solution was added to each well and incubated for an additional 2 h after which the absorbance at a wavelength of 450 nm of each well was measured with a microplate reader and the mean was calculated for each group.

**Western Blotting (WB) Analysis**

Intestinal tissue was homogenized in tissue protein extraction reagent (T-Per) solution supplemented with HALT protease inhibitors at 10 ml/g wet weight. Caco-2 cells were lysed using radio immunoprecipitation (RIPA) lysis buffer, and total protein was collected. Next, 20 μg of protein from each sample was loaded onto 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, concentrated at 80 V for 20 min and separated at 120 V for ~1 h (as soon as the bromophenol blue color marker ran off the end of the gel). The samples were then transferred to polyvinylidene difluoride (PVDF) membranes at 110 V for 100 min at 4°C using the wet transfer method. Each membrane was blocked in 5% nonfat milk Tris Buffered Saline and Tween-20 (TBST) for 2 h prior to being incubated overnight at 4°C with primary antibodies against microtubule-associated protein 1 light chain 3 (LC3), P62, Beclin-1, STX3, PTEN, mTOR or AKT. Then, the membranes were washed 3 times with TBST for 10 min before they were incubated with diluted secondary antibodies in blocking solution for 2 h at room temperature. After the secondary antibody was fully washed away, a PTG ECL chemiluminescence detection kit was used to develop the blot, which was transferred to an imaging machine for exposure and analysis.

**Immunofluorescence Staining of LC3 in Cells**

To identify autophagosome puncta in the cytoplasm, cells were fixed with polyformaldehyde for 15 min, permeabilized with 0.1% Triton X-100, washed three times with 0.5% Triton X-100 in PBS and finally stained with anti-LC3B antibody. Then, the cells were incubated with Alexa Fluor 488-conjugated AffiniPure goat anti-rabbit antibody in the dark for 2 h. Both the primary and secondary antibodies were diluted with 0.5% Triton X-100 in phosphate-buffered saline (PBS). After the glass slides were stained with DAPI and mounted with an anti-fluorescence sealant, images were captured directly by fluorescence microscopy.

**Transmission Electron Microscopy (TEM)**

Autophagic vesicles were observed using TEM (Hitachi, HT7700). Treated cells were fixed with
2.5% glutaraldehyde and sodium pyruvate for 24 h, postfixed with 2% OsO₄ for 2 h, and then dehydrated. After the samples were washed and dried, TEM images were obtained.

**Statistical Analysis**

The data are presented as the means ± standard deviation (SD) of three independent experiments. SPSS Statistics 26.0 software (IBM, Armonk, NY, USA) was used for the analyses. Student's t-test was used for comparisons between two groups.

**Results**

**Detection of STX3-Related Autophagy In Vivo**

The model group had more pronounced damage to the small intestinal mucosal epithelium structure with a higher level of inflammatory cell infiltration (Figure 1A). To examine autophagy in vivo, we collected small intestine tissues and blotted for autophagy-related proteins. The WB results showed that autophagy was decreased in the model group, as indicated by the decreased LC3II/I ratio, Beclin1 expression and PTEN expression induced by higher STX3 expression and the increased levels of mTOR and P62 (p<0.01) (Figure 1B). The immunohistochemistry results also showed that the protein levels of PTEN and LC3 were decreased and those of STX3 were increased in tissues of the model group compared with the control group. Additionally, the mTOR pathway was activated (p<0.01) (Figure 1C). Taken together, these results suggest that autophagy was inhibited in the model group.

**Construction and Verification of the LncRNA-miRNA-mRNA Interaction Network**

LncRNAs most commonly interact with miRNAs to regulate downstream molecules. To further explore the mechanism of action of Inc-AC145676.2.1-6-3 in aGVHD, the target miRNAs and mRNAs of Inc-AC145676.2.1-6-3 were predicted by miRanda and TargetScan, which revealed 5 miRNAs: hsa-miR-1292-3p, hsa-miR-665, hsa-miR-4458, hsa-miR-3064-5p and hsa-miR-6504-5p. According to the cumulative weighted context++ score, we selected the top 1700 target genes of these miRNAs with TargetScan. KEGG analysis indicated that the most enriched term, “MAPK signaling pathway”, was related to autophagy, and 19 target genes were identified as participating in this process. Cytoscape was used to visualize the ceRNA network of Inc-AC145676.2.1-6-3, hsa-miR-1292-3p and STX3 (Figure 2A). Among the identified downstream miRNAs, STX3 encodes a member of the STX family and is relevant to our research. TargetScan analysis predicted 3 target binding sites between STX3 and Inc-AC145676.2.1-6-3-interacting miRNAs (Table I). These results show that Inc-AC145676.2.1-6-3 might regulate the downstream hsa-miR-1292-3p/STX3 axis, which induces aGVHD. The binding sites of hsa-miR-1292-3p on STX3 were identified with the assistance of a bioinformatics prediction website. The results of the Dual-Luciferase reporter assay showed that hsa-miR-1292-3p mimics had no significant effect on the luciferase activity of the Mut-lnc-AC145676.2.1-6-3/STX3 plasmid group but led to a clear decrease in the luciferase activity of the WT-lnc-AC145676.2.1-6-3/STX3 plasmid group (p<0.01) (Figure 2A). The changes in the luciferase activities of lncRNAs were the same as those mentioned above. All these data confirmed the existence of the lnc-AC145676.2.1-6-3/hsa-miR-1292-3p/STX3 axis.

**The Mechanism of Action of Lnc-AC145676.2.1-6-3/hsa-miR-1292-3p/STX3 In Vitro**

After Caco-2 cells were induced by TNF-α, the downstream mechanism effects of the lnc-AC145676.2.1-6-3/hsa-miR-1292-3p/STX3 axis were assessed with transepithelial electrical resistance (TER), WB and immunofluorescence staining. Compared with the IncRNA siRNA + miRNA inhibitor and siRNA-NC groups, the IncRNA siRNA group had an increased TER (p<0.05).

To confirm the relationship of the Inc-AC145676.2.1-6-3/hsa-miR-1292-3p/STX3 axis, the levels of hsa-miR-1292-3p and STX3 were evaluated in Caco-2 cells. In the IncRNA siRNA group, miRNA expression was significantly increased compared with that in the model and siRNA-NC groups (p<0.01) (Figure 3B). Upon IncRNA interference, the level of miRNA binding to the IncRNA was significantly increased, while the level of STX3 was decreased (Figure 3C).

The levels of the autophagy-related proteins LC3II/I, Beclin1, P62, PTEN, Akt and mTOR as well as of STX3 were measured by WB. The results were similar to those observed in vivo, but in the IncRNA siRNA group, STX3 expression was
Figure 1. Detection of autophagy-related indexes in the small intestine of mice. A, Analysis of the mouse small intestine pathological score (magnification, 200×). B, Detection of the autophagy-related proteins LC3II/I, Beclin1, AKT, PTEN, mTOR and STX3 by Western blotting. C, Immunohistochemical analysis (magnification, 200×). D, Detection of autophagic vesicles. (**p<0.01, *p<0.05).
Table I. The binding sites between STX3 and hsa-miR-665, hsa-miR-4458 and hsa-miR-3064-5p as predicted by TargetScan.

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<th>Position</th>
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<th>Context++ score percentile</th>
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<tr>
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<tr>
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significantly decreased, whereas the expression of autophagy-related proteins was significantly increased, indicating that when inhibition of lnc-AC145676.2.1-6-3 increased the level of autophagy ($p<0.01$) (Figure 3D).

**Autophagy Level Test in Caco-2 Cells**

In the lncRNA siRNA group, the relative number of LC3 puncta per cell was significantly increased compared with that in the model, siRNA-NC, and lncRNA siRNA + mRNA inhibitor groups, as observed by immunofluorescence microscopy (Figure 4A). To examine autophagy *in vitro*, we used Caco-2 cells and detected autophagy by TEM. There were more biphasic membrane autophagosomes with normal mitochondrial structure in the lncRNA siRNA group than in the siRNA-NC and lncRNA siRNA + mRNA inhibi-
itor groups ($p<0.01$) (Figure 4B). These results confirm that when lnc-AC145676.2.1-6-3 was silenced, the level of STX3 was decreased leading to increased levels of autophagy and consequent inhibition of aGVHD progression.

**Discussion**

aGVHD is the primary complication of allo-HSCT and the main cause of death after HSCT. STX3 has been shown to be involved in autophagy and can regulate the PTEN-PI3K-AKT-mTOR pathway. aGVHD manifests in the gastrointestinal tract, skin, and liver. There have been many studies on its pathogenesis, which may be affected by the intestinal flora, inflammatory reactions and so on. The most common type of aGVHD is intestinal aGVHD, the symptoms of which are refractory diarrhea, severe colitis, hemorrhage, and intestinal obstruction that will affect the implantation and endanger the life of the patient.

Figure 3. Detection of autophagic protein levels in Caco-2 cells. A, TER test in Caco-2 cells. B, The level of miRNA after lncRNA was knocked down. C, The level of STX3 after lncRNA was knocked down. D, Detection of the autophagy-related proteins LC3II/I, Beclin1, AKT, PTEN, mTOR and STX3 by Western blotting ($**p<0.01$, $*p<0.05$).
signaling pathway. Several studies have reported that the PTEN-PI3K-AKT-mTOR pathway and many members of the STX family are closely related to autophagy. For example, STX2 affects autophagy through the NF-κB pathway, STX7 has been associated with autophagy signaling, and STX6 has been shown to promote autophagy. Furthermore, STX3 is associated with congenital diarrhea, intestinal epithelial differentiation diseases, and intestinal microvilli inclusion body diseases and participates in the protection of intestinal mucosa. Inhibition of PTEN can enhance Akt phosphorylation and accelerate GVHD progression, and excessive activation of Akt/mTOR signaling may be related to the occurrence of aGVHD. In a mouse model of aGVHD (Figure 1A), the level of PTEN was decreased, the levels of Akt and STX3 were increased, and the mTOR pathway was activated (Figure 1B,C). A TNF-α-induced Caco-2 in vitro cell model was used to mimic the damage in the intestinal mucosal barrier and elicited similar results as those of the in vivo experiments (Figure 3D).

Autophagy, as an intracellular degradation pathway necessary for cellular homeostasis, can reduce inflammasome activation by removing inflammasome components or cytokines associated with intracellular pathogenic molecules. A previous study found that after ectopic expression of inhibitors of apoptosis proteins (IAPs), the level of LC3 (an autophagic gene and marker of disease) was decreased, which suggests that aGVHD...
not only activates apoptosis but also is accompanied by the inhibition of autophagy, which was also observed in our results (Figure 4A). Some studies have suggested that autophagy activation attenuates intestinal mucosal barrier dysfunction by preventing and reducing oxidative stress. At the same time, inflammasome activation can upregulate autophagy to reduce the damage to host tissues caused by an exacerbated inflammatory response. Our experiments showed that the expression levels of the autophagy-related factors LC3, p62, and Beclin1 were changed and that mTOR was activated in aGVHD (Figure 1B, C and Figure 3D). Combined with the TEM and immunohistochemistry results, these findings indicate that the autophagy level was changed. Therefore, the decrease in mitochondrial autophagy may be involved in the pathogenesis of intestinal aGVHD, and the specific mechanism may be abnormal mitochondrial autophagy, which results in a decreased ability to scavenge damaged mitochondria and to inhibit oxidative damage and inflammatory damage to the intestinal mucosal barrier in aGVHD, resulting in the occurrence and aggravation of intestinal rejection. We found that the mitochondria in cells subjected to aGVHD were seriously damaged, which may be one of drivers of the occurrence of aGVHD (Figure 1D and Figure 4B).

Previous studies have shown that IncRNAs play an important role in the occurrence and development of aGVHD, but research on the specific mechanism involved is still rare and with little to no evidence on the role of IncRNAs with respect to STX3 mRNA in aGVHD. An increased level of Inc-AC145676.2.1-6-3 was observed in aGVHD in our previous study. We used miRanda and TargetScan to predict the target miRNAs and mRNAs of Inc-AC145676.2.1-6-3; the top miRNA was hsa-miR-1292-3p. Among the downstream mRNAs, STX3 was relevant to our research. These results suggested that Inc-AC145676.2.1-6-3 might regulate the downstream hsa-miR-1292-3p/STX3 axis (Figure 2A). After the relationship of Inc-AC145676.2.1-6-3/hsa-miR-1292-3p/STX3 axis was verified by a luciferase reporter assay (Figure 2B), the in vivo data confirmed that Inc-AC145676.2.1-6-3 can influence STX3 function by sponging hsa-miR-1292-3p. The IncRNA siRNA group data showed that when the level of autophagy was increased and the level of STX3 was decreased, the aGVHD progression was ameliorated (Figure 3 and Figure 4).

We conducted a preliminary experimental study on the mechanism by which Inc-ac145676.2.1-6-3 affects the process of intestinal aGVHD, which provides a new approach for the clinical treatment of intestinal GVHD. However, the studies on successfully treating this disease lack sufficient data regarding its molecular targets, and detailed experiments on how to utilize this lncRNA and its target genes to treat aGVHD in vivo, will be part of our future directions.

Conclusions

STX3-induced increases in autophagy levels via the PTEN/AKT/mTOR pathway in aGVHD were confirmed in animal models and Caco-2 cells. Then, from our previous study, upregulated Inc-AC145676.2.1-6-3 was verified to regulate the hsa-miR-1292-3p/STX3 axis by bioinformatic analysis and luciferase reporters. After conducting tests in vivo, we concluded that Inc-AC145676.2.1-6-3 plays an important role in STX3-induced increases in autophagy levels in aGVHD.

Funding

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Conflicts of Interest

The authors declare no conflicts of interest.

Availability of Data and Material

We would like support with depositing and managing their data of individual users as well as submit datasets to the Editorial Manager. All data are available from the corresponding author upon reasonable request.

Authors’ Contributions

Dr. Xing Cui designed and performed the experiments, analyzed the data, and wrote the manuscript; Master Xiaoqi Sun wrote the manuscript; Dr. Guoqing Tan provided the experimental site and some equipment; Dr. Zong Gao obtained and tested mouse tissue samples; Dr. Xiujuan Liu, Master Yanyu Zhang, Runjie Sun and Mengtong Xia provided vital new reagents and performed experiments. All authors have read and approved the manuscript and ensure the accuracy of the information presented.
Lnc-AC145676.2.1-6-3 in intestinal aGVHD


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