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Upstream stimulating factor1 (USF1) enhances the proliferation of glioblastoma stem cells mainly by activating the transcription of mucin13 (MUC13)

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Glioblastoma is one of the most aggressive and deadly tumors among the adults. MUC13 is a membrane bound mucin and abnormal expression of MUC13 has been identified in various tumors. However, its specific role in glioblastoma has never been explored. Firstly, the expression of MUC13 was explored in glioblastoma stem cells (GSCs) and we found that MUC13 was significantly enhanced in GSCs. Overexpression of MUC13 significantly enhanced GSCs invasion and migration capacities. Further study showed that overexpression of MUC13 significantly increased the phosphorylation levels of AKT and P38. Dual luciferase reporter and ChIP assay demonstrated that USF1 could bind the promoter region of MUC13, thereby enhancing the activation of MUC13. More importantly, when MUC13 was silenced, the phosphorylation levels of AKT and P38 were suppressed even in cells transfected with ad-USF1. These data showed that USF1 prompted glioblastoma progression mainly by activating MUC13. In summary, our study first demonstrated that USF1 could activate the transcription of MUC13, thereby enhancing the proliferation and self-renewal of GSCs.

1. Introduction

Glioblastoma is the most common primary brain tumor in adults, which ranks among one of the most aggressive and deadly tumors (Rousseau et al. 2008; Son et al. 2009). At present, there is no effective treatment method toward glioblastoma since both radiotherapy and chemotherapy exert high toxicity (Eyler et al. 2008; Yao et al. 2008). Cancer stem cells have been isolated from several primary brain tumors, which are characterized by rapid expanding capacity (Rich and Eyler 2008; Zhu et al. 2010). Studies have indicated that cancer stem cells are resistant to tumor therapy (Zhou et al. 2009). Thus, it is of great importance to determine the potential therapeutic targets in cancer stem cells.

Mucins belong to the family of high molecular weight glycoproteins, which are normally expressed at mucosal surfaces and protect cells from the external environment (Hollingsworth et al. 1994). However, upregulation of mucins has been widely identified in many tumor (Devine et al. 1992). Among them, MUC13 is a membrane bound mucin and is frequently expressed at a relative low level in the epithelial surface of gastrointestinal, respiratory and reproductive tracts (Chauhan et al. 2012; Gupta et al. 2012). In various tumors, abnormal expression of MUC13 has been identified (Williams et al. 2001; Chauhan et al. 2012). For instance, MUC13 is found to be upregulated in both ovarian and pancreatic cancers thereby enhancing tumorigenesis (Chauhan et al. 2009; Chauhan et al. 2012). However, the specific expression of MUC13 in glioblastoma has never been studied.

The upstream transcription factor 1 (USF1) is a member of the basic helix-loop-helix (bHLH) leucine zipper family and it is involved in the transcription of many genes (Pajukanta et al. 2004; Plaisier et al. 2009). Through binding the E-box motifs in the promoter region, USF1 activates the transcription of target gens in the form of homodimers or a heterodimer (USF1/2) (Pajukanta et al. 2004). For instance, many genes related to lipid and glucose metabolism has been reported to be modulated by USF1 (Pajukanta et al. 2004; Meex et al. 2008; Plaisier et al. 2009). To our knowledge, the expression and potential functional role of USF1 in glioblastoma has not been reported previously.

In the current study, we investigated the expression of USF1 and MUC13 in glioblastoma and found both of them were significantly upregulated. Further study showed that two E-box motifs were located at the promoter region of MUC13. ChIP assay demonstrated USF1 could activate the transcription of MUC13, thereby enhancing tumorigenesis in glioma cancer stem cells.

2. Investigations and results

2.1. Overexpression of MUC13 in glioblastoma

Firstly, we explored the expression of MUC13 in glioblastoma. As shown in Fig. 1A, the expression of MUC13 was significantly upregulated in glioblastoma. Real time PCR analysis also showed the mRNA level of MUC13 was significant in GBMs compared with control (Fig. 1B).

2.2. MUC13 enhanced GSCs invasion and migration

To further explore the role of MUC13 on glioblastoma progression, MUC13 was overexpressed in GSCs through adenovirus vectors. We found that overexpression of MUC13 significantly enhanced cell invasion and migration capacities of GSCs (Fig. 2A). Furthermore, we explored the activation of AKT and P38 signaling, which play key roles in cancer cell proliferation. Here, we found that overexpression of MUC13 significantly increased the phosphorylation levels of AKT and P38 (Fig. 2B).

2.3. USF1 could activate the expression of MUC13 through USF1

Then, we try to identify the potential transcriptional factors that regulate the expression of MUC13. Interestingly, two E-box

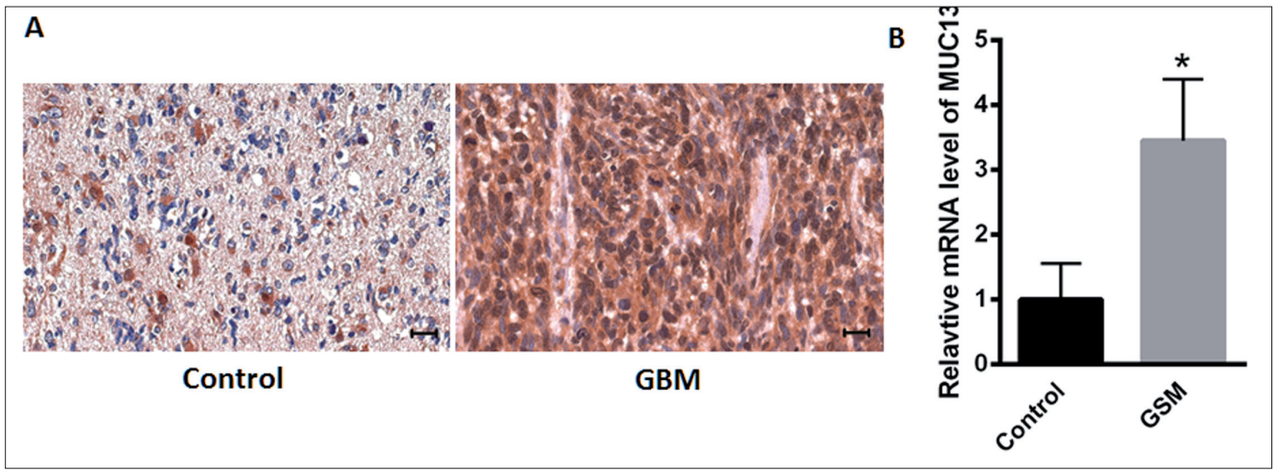

Fig. 1: The expression of MUC13 was significantly enhanced in GBMs. (A) IHC analysis of MUC13 expression in glioblastoma. (B) The mRNA level of MUC13 was significantly in GBMs (n=12) compared with control (n=12). *P<0.05 *vs* control.

elements were identified in the promoter region of MUC13 (Fig. 3A). Then, we analyzed the expression of USF1 in the glioblastoma. Compared with normal control, the expression of USF1 was significantly enhanced in the glioblastoma (Fig. 3B). Next, the promoter region of MUC13 was cloned into the pGL3 promoter reporter vector. Dual luciferase reporter assay showed that overexpression of USF1 could significantly enhance the relative luciferase reporter activity of pGL3-MUC13 compared with blank vector (Fig. 3C). Furthermore, we performed ChIP assay and found that the knockdown of USF1 expression in GSCs reduced the interaction between USF1 and the MUC13 promoter (Fig. 3D), while the over-expression of USF1 enhanced its interaction with MUC13 promoter (Fig. 3E). These data suggested that USF1 could activate the expression of MUC13.

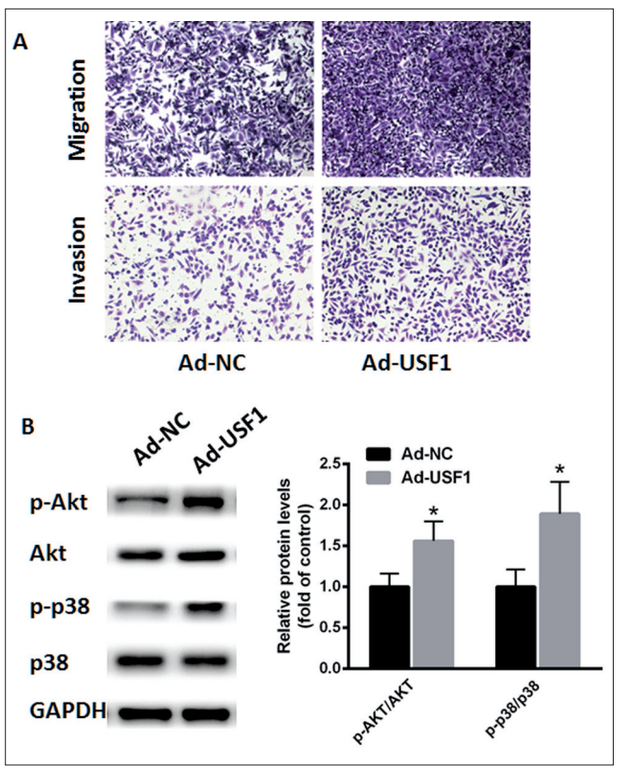

Fig. 2: MUC13 significantly enhanced cell invasion and migration capacities of GSCs. (A) Invasion and motility assays. (B) Western blot analysis of AKT and P38 activation when MUC13 was overexpressed. n=3 independent experiments, *P<0.05, **P<0.01 vs control.

2.4. Overexpression of USF1 enhanced GSCs invasion and migration

We further explored the role of USF1 on the progression of glioblastoma. As shown in Fig. 4A, cell migration and invasion capacity was significantly enhanced when USF1 was overexpressed in GSCs. Furthermore, western blot analysis showed that overexpression of USF1 markedly enhanced the protein level of MUC13 (Fig. 4B). Meanwhile, the phosphorylation levels of AKT and P38 were also increased. In comparison, when MUC13 was silenced, the phosphorylation levels of AKT and P38 were suppressed even in cells transfected with ad-USF1 (Fig. 4C). These data showed that USF1 prompted glioblastoma progression mainly by activating MUC13.

3. Discussion

As the most common malignant brain tumor, glioblastoma multiforme (GBM) incidence continued to increase in recent years. Due the poor prognosis technique, a median survival of affected patients is one year (Kang and Kang 2007). Thus, it is important to elucidate the underlying mechanism of GBM. Cancer stem cells are isolated from primary brain tumors, which are characterized by rapid expanding and self-renewal capacity. GSCs and other cancer stem cells are found to be responsible for tumor maintenance and recurrence after therapy (Reya et al. 2001; Park and Rich 2009; Rosen and Jordan 2009). To identify molecular targets in cancer, exploring novel signaling pathways in cancer stem cell biology are of great importance.

Mucins play key roles in carcinogenesis and metastasis (Kufe 2009). For instance, MUC13 is reported to be enhanced in colon cancer and is closely related to poor prognosis (Gupta et al. 2014). However, the specific role of MUC13 in glioblastoma has never been studied. Cancer stem cells are functionally defined through their capacity for sustained self-renewal. Thus, in the current study, we explored the role of MUC13 on GSCs invasion and migration capacity. Interestingly, upregulation of MUC13 markedly enhanced cancer cell invasion and migration. Further study demonstrated that overexpression of MUC13 significantly enhanced the activation of AKT and P38, which were widely reported to prompt cancer cell proliferation. These data suggested an oncogenic role of MUC13 in GSC growth, survival, and self-renewal in vitro.

USF1 is an ubiquitous transcription factor of the bHLH-LZ family that is widely involved in lipid and glucose metabolism (Landa et al. 2009; Yuan et al. 2016). In the current study, we first explored the expression of USF1 in glioblastoma and found it significantly upregulated. More importantly, two conserved E-box elements were identi-

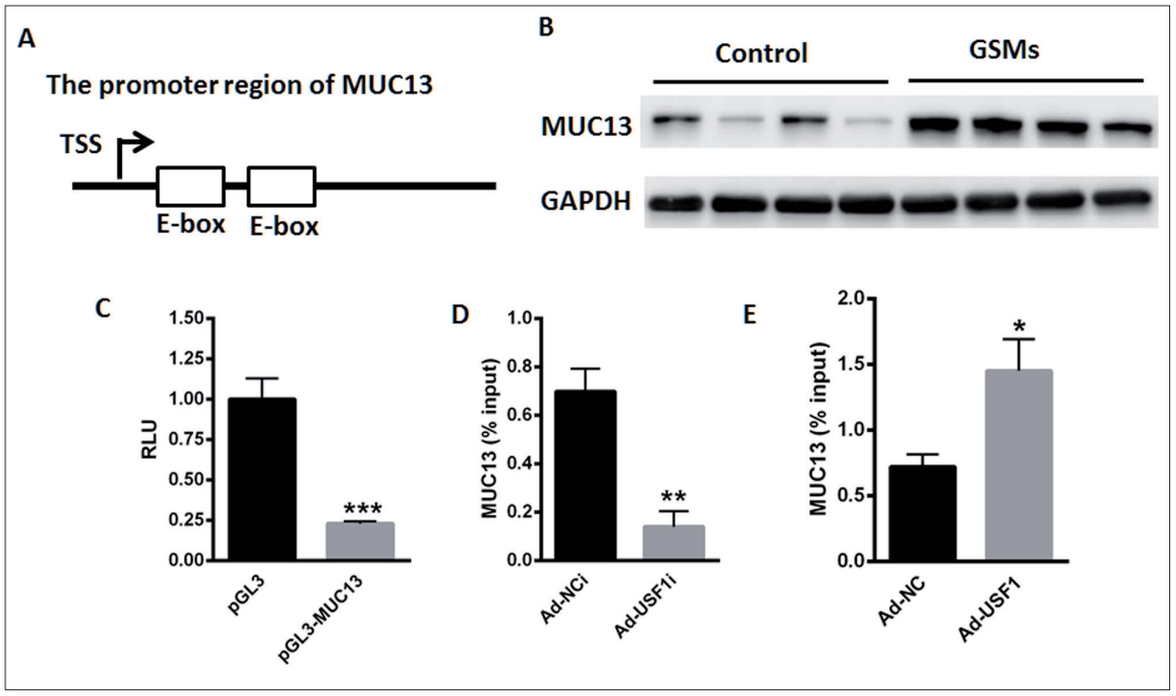

Fig. 3: USF1 could activate the expression of MUC13 through USF1. (A) Two E-boxes were identified in the promoter region of MUC13. (B) The expression of USF1 was significantly enhanced in the glioblastoma. (C) Dual luciferase reporter assay showed that overexpression of USF1 could significantly enhance the relative luciferase reporter activity of pGL3-MUC13 compared with blank vector. (D) ChIP assay showed that the knockdown of USF1 expression in GSCs reduced the interaction between USF1 and the MUC13 promoter. (D) ChIP assay showed that the over-expression of USF1 enhanced its interaction with MUC13 promoter. n=3 independent experiments, *P<0.05, **P<0.01 *vs* control.

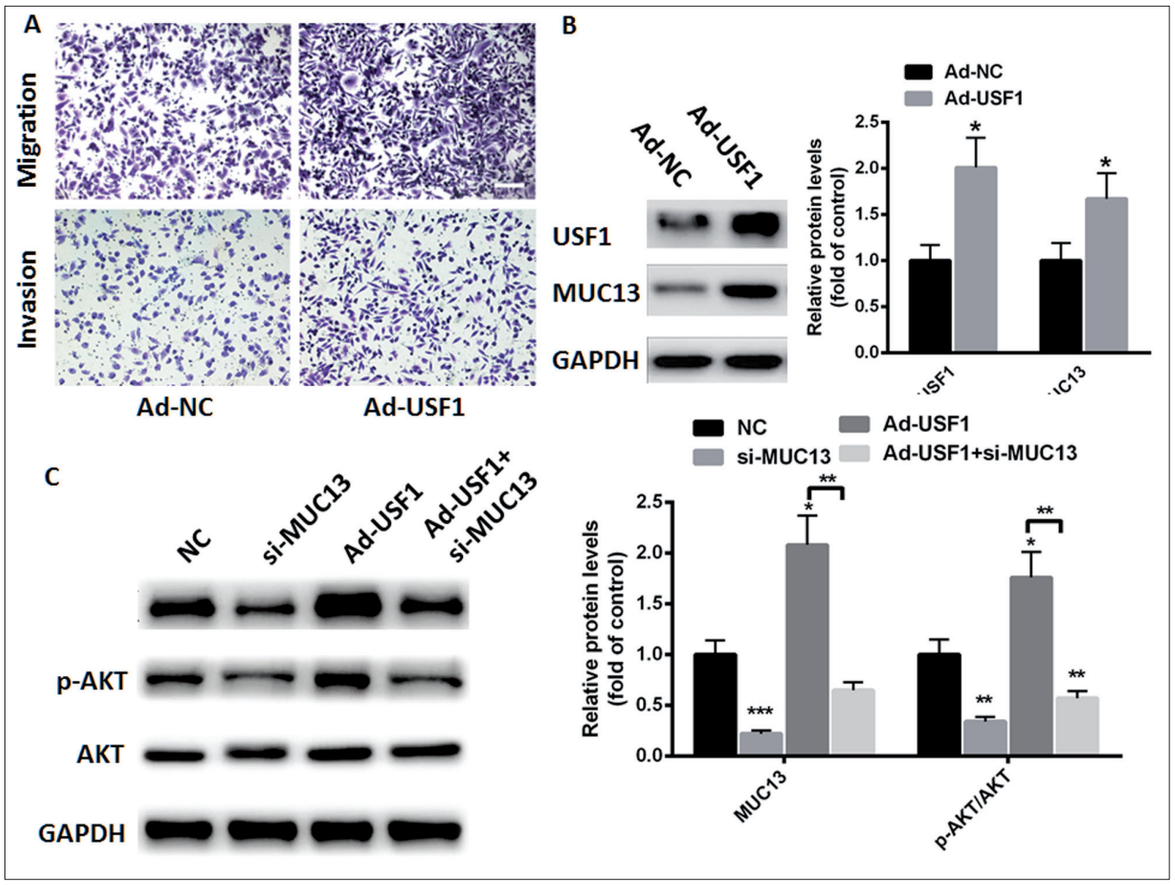

Fig. 4: Overexpression of USF1 enhanced GSCs invasion and migration. (A) Cell migration and invasion capacity was significantly enhanced when USF1 was overexpressed in GSCs. (B) Silence of USF1 markedly reduced GSCs migration and invasion. (C) Western blot analysis of AKT and P38 activation in GSCs transfected with ad-USF1 and / or ad-MUC13i. n=3 independent experiments, *P<0.05, **P<0.01 vs control.

fied in the promoter region of MUC13. ChIP assay and luciferase reporter assay demonstrated that USF1 could significantly activate the transcription of MUC13 in GSCs. Further study showed that USF1 could also enhance GSCs migration through AKT and P38 activation. More importantly, we determined that USF1 associates with MUC13 and activates AKT/P38-mediated GSCs proliferation.

In summary, we first reported that USF1 could activate the transcription of MUC13, thereby enhancing the proliferation and self-renewal of GSCs.

4. Experimental

4.1. Ethics statement

Primary human brain tumor patient specimens were obtained from patients and the informed consent were obtained under protocols approved by Review Boards of Tianjin Medical University. Bioptic samples from high-grade glioma patients and healthy control were frozen in liquid nitrogen and stored at -80 °C. Twenty patients 24 to 70 years of age with newly diagnosed and histologically confirmed GBM (World Health Organization [WHO] grade IV astrocytoma) were eligible for the study.

4.2. Isolation and culture of matched GSCs and non-stem glioma cells

To isolate GSCs, a Papain Dissociation System (Worthington Biochemical) was appplied to dissociate tumors according to the manufacturer's instructions (http://www.worthington-biochem.com/PDS/default.html). Then, to recover surface antigens, the cells were cultured in Neurobasal medium supplemented with B27 without vitamin A, l-glutamine, sodium pyruvate (Invitrogen), 10 ng/ml basic fibroblast growth factor (bFGF), and 10 ng/ml epidermal growth factor (EGF) (R&D Systems) for at least 6 h. To isolate GSCs, the cells were labeled with an allophycocyanin (APC)-conjugated CD133 antibody (Miltenyi Biotec), and sorted by fluorescence-activated cell sorting (FACS). And the cells stained with CD133 were considered as GSCs while those without CD133 were collected as non-stem glioma cells. GSCs were cultured GSCs were cultured in the earlier-defined medium. And the matched non-stem glioma cells were cultured in DMEM containing 10% serum for 24 h and then neurobasal medium was supplemented for at least 12 h before experiments were performed in identical medium.

4.3. Construction of adenovirus vectors

And the adenovirus vectors overexpressing and inhibiting MUC13 or USF1 were constructed by Genechem (Shanghai, China).

4.4. Chromatin immunoprecipitation (ChIP)

ChIP assay was performed using the Chromatin Immunoprecipitation Assay Kit (Millipore, Boston, Massachusetts, USA). Briefly, the nuclei DNA was extracted from cells and sonicated into 200-1000 bp. Precleared chromatin was immunoprecipitated with anti-USF1 and normal IgG antibodies according to the manufacturer's instructions. Immunocomplexes were added into 50 µl of protein A/G-Sepharose beads and purified with Qiaquick (QIAGEN, Duesseldorf, German) PCR purification columns. The precipitated DNA was amplified with SREBP1-specific primers. The primers specific to the USF1 binding sites on the MUC13 promoter were 5'-GTACCCTCAG-GTCATACTGC-3' and 5'-AAGCTCTCTGGATTGCCTT-3'.

4.5. RNA extraction and real-time PCR

The total RNA from cultured cells was isolated with TriZol (Invitrogen) according to the manufacturer's instructions. The total RNA was reverse transcribed into complementarty DNA (cDNA) with TaqMan RNA Reverse Transcription Kit (Applied Biosystems). A quantitative real-time PCR assay was performed using SYBR Green Supermix (Bio-Rad) in a BIO-RAD iCycleriQ real-time PCR detection system as previously described (Guo et al. 2014).

4.6. Protein extraction and Western blot analysis

Proteins samples were extracted in RIPA buffer (1% TritonX-100, 15 mmol/L NaCl, 5 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.0) (Solarbio, China) supplemented with a protease and phosphatase inhibitor cocktail (Sigma) and then separated by 10% SDS-PAGE, followed by electrophoretical transfer to a PVDF membrane. After soaking with 8% milk in PBST (pH 7.5) for 2 h at room temperature, the membranes were incubated with the following primary antibodies: anti-USF1, anti-MUC13, anti-phospho-AKT (Thr308), anti-AKT, anti-phospho-p44/42 MAPK (P381/2), anti- MAPK (P381/2) and anti-GAPDH (Cell signaling). Immunodetection was performed by enhanced chemiluminscence detection system (Millipore) according to the manufacturer's instructions. The house-keeping gene GAPDH was used as the internal control.

4.7. Invasion and motility assays

Firstly, cells were seeded in the top chamber of each insert at 1.0×10^5 cells/well (BD Biosciences, San Jose, CA, USA) with 8.0-mm pores for motility assay. And for the invasion assays, 2.0×10^5 cells were cultured in a chamber (BD Biosciences) pre-coated with 0.2% Matrigel (Collaborative Research, Boston MA, USA) at 37 °C. As a chemoattractant, 10% fetal bovine serum was added to the culture medium in the lower chamber. After 24 hours, the cells retained in the upper compartment were removed by cotton swabs, and those invaded through the membrane were stained with a dye solution containing 20% methanol and 0.1% crystal violet. The cells were then imaged under a light microscope (Olympus) and ten individual fields were counted per insert. The results are presented as an average of three separate experiments.

4.8. Promoter reporter analysis

The promoter region of MUC13 was amplified from the genomic DNA of GSC cells. The PGL3 promoter vector and the amplified fragments were digested with *Xho*I/KpnI and purified by gel electrophoresis. The digested fragment was then inserted into the PGL3 vector up-stream of the SV40 promoter. HEK293T cells were co-transfected with the PGL3 plasmids and the PRL-TK vector using the VigoFect Transfection Reagent (Beijing, China). The cells were harvested and lysed 48 h post-transfection. The relative light units (RLU) were determined using the dual-luciferase reporter assay system (Promega, Madison, Wisconsin, USA) according to the standard protocols. Normalized luciferase data (firefly/renilla) was compared with the empty pGL3-promoter vector. The RLU were determined using the dual-luciferase reporter assay system (Promega, Madison, Wisconsin, USA) according to the standard protocol. The primers for amplification were as follows: MUC13-F: 5'-GGGTACCCTTCTCT-GCTCAACGAGGTCA-3', MUC13-R: 5'-CCTCGAGGTCTTTACCCTGTGC-GGAA-3, The restriction sites for KpI and XhoI are underlined.

4.9. Statistical analysis

Data were presented as mean±SD from 3 independent experiments or 5 mice. Statistical analysis was carried out with Student's t test. $P < 0.05$ was considered as statistically significant difference.

Conflicts of interest: None declared.

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