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ORIGINAL RESEARCH

# Knockdown of ORM2 improves cerulein-induced acute pancreatitis *in vitro* via the p38 MAPK signaling pathway

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## Abstract

The pathophysiology of acute pancreatitis remains poorly understood, and it is a severe inflammatory pancreatic disease that is becoming more widespread globally. The protein orosomucoid 2 (ORM2) is closely linked with tissue injury and inflammation. The purpose of this study was to investigate ORM2 expression in pancreatic acinar cells and its potential regulatory function in the regulation of inflammation and damage in acute pancreatitis. The model was established by culturing rat pancreatic acinar AR42J cells with 10 nM azuretin. Lentivirus was used to transfect AR42J cells to generate negative control and ORM2 knockdown cell lines. In order to track changes in ORM2 expression, cell proliferation, apoptosis, and the production of inflammatory cytokines in AR42J cells, this study carried out a number of tests. Western blotting showed expression changes in the p38 mitogen-activated protein kinases (MAPK) pathway. The results showed that ORM2 expression is up-regulated in acute pancreatitis *in vitro*, and knocking down ORM2 reversed the decrease in survival rate, the increase in apoptosis and the elevated inflammatory factor levels in AR42J cells induced by cerulein. Knockdown of ORM2 also inhibited the phosphorylation of p38 and extracellular signal-regulated kinase (ERK). In summary, ORM2 may significantly influence damage and inflammation in the *in vitro* rat pancreatic acinar cells model of acute pancreatitis by modulating the phosphorylation levels of p38 MAPK signaling.

## Keywords

Acute pancreatitis; Inflammation; ORM2; Pancreatic acinar cells; p38 MAPK

## 1. Introduction

Acute pancreatitis (AP) is a severe inflammatory pancreatic disease that is becoming more widespread globally. Characterized by inflammatory cell infiltration, acinar cell death, intrapancreatic digestive zymogen and leukocyte activation [1]. The only treatments available to patients with AP are supportive care and fluid resuscitation, despite tremendous advancements in recent years in our understanding of the disease's pathophysiology and heterogeneity of disease symptoms [2]. The development and pathophysiology of the sterile inflammatory response in acute pancreatitis are the subject of an increasing amount of research. The main focus of acute pancreatitis is acinar cells [3]. Early activation of the acinar cell inflammatory pathway can result in pancreatic injury and a local inflammatory response, which is important in the pathophysiology of pancreatitis [4]. As a result, one therapy approach to stop the progression of the disease is to treat early inflammatory processes within acinar cells.

In this study proteomic analysis of plasma proteins from patients with severe acute pancreatitis and healthy volunteers was carried out, and the results indicated that orosomucoid 2 (ORM2) may serve as a potential biomarker for the severity of disease [5]. ORM2 is a glycoprotein with a molecular weight of 41–43 kDa and is one of the two main members of the ORM family, along with ORM [6]. ORM2, an acute-phase protein is recognized for its anti-inflammatory and immunomodulatory properties due to its antineutrophil and anticomplement effects [7]. For example, a deficiency in ORM2 exacerbates intestinal inflammation and dysbiosis, suggesting new insights about the benefits of administering mixed bacterial flora rather than single probiotics for treating obesity [8]. In addition, the acute phase protein ORM2, which regulates the extracellular signal-regulated kinase (ERK) 1/2 pathway has been implicated in alleviating non-alcoholic steatohepatitis [9]. However, more research is needed to fully understand ORM2's function and mechanism in acute pancreatitis.

Within family, one of the most extensively expressed enzymes is the serine/threonine kinase p38 mitogen-activated protein kinase (p38 MAPK) [10]. Activation of p38 MAPK is known to result in the expression of proinflammatory cytokines [11]. Strong evidence links AP to p38 MAPK as indicated by studies showing that inhibition of p38 MAPK pathway in pulmonary microvascular endothelial cells' reduces lung damage associated with acute pancreatitis [12]. Here, we conducted research targeting the p38 MAPK pathway in AP.

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In this study, we utilized the cerulein-induced pancreatic acinar cell line AR42J to mimic AP conditions *in vitro*. Our objectives were to determine the precise role of ORM2 in AP and to explore its potential underlying mechanisms of action.

## 2. Materials and methods

### 2.1 Cell culture

The American Type Culture Collection's rat pancreatic acinar AR42J cells were cultivated at 37 °C and 5% carbon dioxide ($CO_2$). Dulbecco's modified Eagle's medium (Sigma-Aldrich) was used to sustain the cells. It contained antibiotics (100 U/mL penicillin and 100 $\mu$g/mL streptomycin) and 10% fetal calf serum (A5256701, Gibco, New York, NY, USA).

### 2.2 Cell transfection and grouping

Small interfering RNA (siRNA) sequences were obtained from Gene Pharma (Shanghai, China). Using the Lipofectamine 2000 reagent (11668500, Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions, cells were transiently transfected when they reached 50–60% confluence. After 48 h, AR42J cells were co-cultured with cerulein (10 nM, C9026, Sigma-Aldrich, St. Louis, MO, USA) for 24 h to induce the AP cell model. The siRNA sequence targeting *ORM2* is: si-*ORM2*: 5′-GAAACGAGGAGUACAAAUAATT-3′.

### 2.3 Cell proliferation and apoptosis assays

For 72 hours, transfected cells were cultured in 96-well plates after being seeded. In each well, add one Cell Counting Kit-8 (CCK-8, C0037, Beyotime, Shanghai, China) and incubate for two hours to measure the absorbance at 450 nm. Using the Annexin V-fluorescein isothiocyanate/propidium iodide kit (V13242, Sigma-Aldrich, St. Louis, MO, USA), the apoptotic rate of treated AR42J cells was measured. Afterwards, apoptosis rates were assessed and computed via flow cytometry.

### 2.4 Enzyme-linked immunosorbent assay (ELISA)

Cell samples were obtained, and ELISA kits (70-EK2822/2, 70-EK206, 70-EK206, Lianke Biotechnology Co., Ltd., Hangzhou, China) were used to evaluate the amounts of inflammatory factors, such as interleukin-6 (IL-6), transforming growth factor alpha (TGF-$\alpha$), and interleukin-1$\beta$ (IL-1$\beta$).

### 2.5 Real-time reverse transcriptase-polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol reagent (MT0123, Life Technologies, Carlsbad, CA, USA). Superscript II (18064022, Invitrogen, Carlsbad, CA, USA) and Oligo (dT) primers were used for reverse transcription. ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) was utilized for detection, along with a one-step SYBR PrimeScript RT-PCR kit (Perfect Real-Time; 639676, Takara Bio, Dalian, China). As an internal control, employ *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). The primer sequence is: *ORM2*: Forward, 5′-TTG TCA TGG TGA GCC TCC TG-3′, Reverse, 5′-ATG AAG GCC CCA TGC ATC TT-3′; *GAPDH*: Forward, 5′-TGG GCT ACA CTG AGC ACC AG-3′, Reverse, 5′-GGG TGT CGC TGT TGA AGT CA-3′.

### 2.6 Western blot

To extract proteins, cells were lysed with Immunoprecipitation (IP) lysis buffer (Rockford, IL, USA) for 30 minutes on ice. Proteins were separated and transferred to membranes, which were subsequently blocked with 5% nonfat dry milk at 4 °C for 1 h before addition of primary antibodies. After two hours at room temperature incubation with a secondary antibody (goat anti-rabbit), protein levels were detected using chemiluminescence reagent following TBS with Tween-20 (TBST) washes. Images were acquired using Image J software (7.0, National Institutes of Health, Bethesda, Montgomery County, MD, USA). The primary antibodies (abcam, Cambridge, UK) used are as follows: ORM2 (1:1000, ab231906), tumour necrosis factor alpha (TNF-$\alpha$, 1:1000, ab183218), IL-6 (1:1000, ab233706), IL-1$\beta$ (1:1000, ab283818), p38 (1:1000, ab170099), p-p38 (1:1000, ab178867), ERK (1:1000, ab32537), p-ERK (1:1000, ab79483) and GAPDH (1:1000, ab8245).

### 2.7 Statistical analysis

GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA) and SPSS 21.0 (IBM, Chicago, IL, USA) were used to analyze the data. At least three independent duplicates of each experiment were carried out. The information is displayed as mean $\pm$ standard deviation. The means of many groups were compared using a two-way Analysis of Variance (ANOVA), and the difference between two independent groups was ascertained using an unpaired Student's $t$ test. Data with $p$ values $< 0.05$ were defined statistically significant.

## 3. Results

### 3.1 ORM2 expression is upregulated in *in vitro* models of acute pancreatitis

Previous studies have suggested that ORM2 could serve as a potential biomarker for acute pancreatitis (AP) based on proteomic analysis [5]. To validate this *in vitro*, we detected the protein and messenger RNA (mRNA) expression of ORM2 in AR42J cells before and after cerulein treatment. We found that ORM2 protein and mRNA expression increased significantly after cerulein treatment (Fig. 1A,B). These outcomes support earlier research and imply that ORM2 might be involved in the start and development of AP.

### 3.2 Knockdown of ORM2 promotes the proliferation of AR42J cells

To investigate the role of ORM2, transfection was done to decrease ORM2 levels in AR42J cells. The results demonstrated a significant decrease in both ORM2 protein and mRNA expression (Fig. 2A,B). Cerulein treatment markedly decreased AR42J cell survival and increase apoptosis rates; however, these effects were mitigated by knocking down

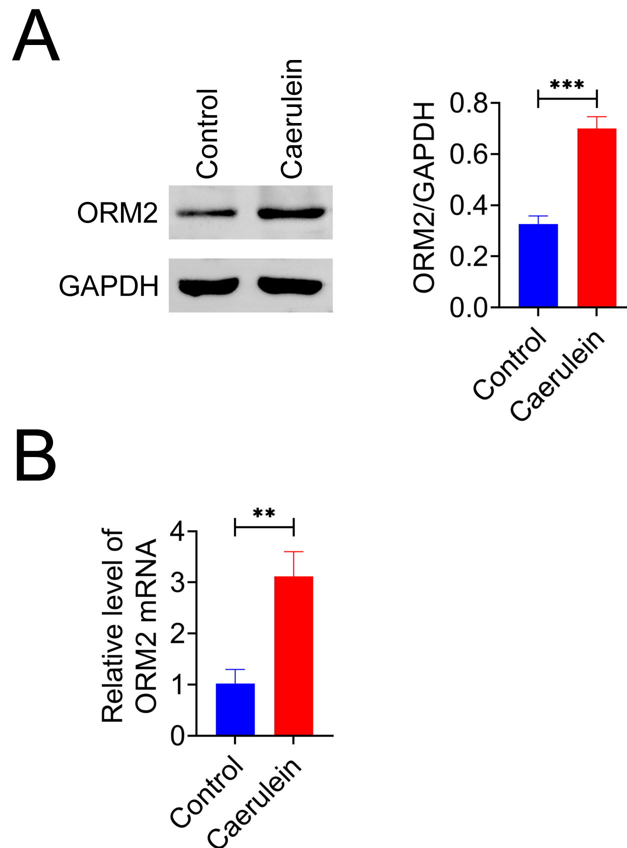


**FIGURE 1. ORM2 expression is upregulated in *in vitro* models of acute pancreatitis.** (A) Expression of ORM2 in cerulein-treated AR42J cells. (B) ORM2 mRNA expression in cerulein-treated AR42J cells. Values are presented as mean ± SD (standard deviation). $^{**}p < 0.01$, $^{***}p < 0.001$ versus control group. n = 3. ORM2: orosomucoid 2; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; mRNA: messenger RNA.

ORM2 (Fig. 2C,D). This indicates that OR GAPDHM2 protein is crucial for AR42J cell survival.

### 3.3 Knockdown of ORM2 suppresses inflammation in AR42J cells

To further explore the impact of ORM2 on inflammation, we performed experiments with ORM2 knockdown in AR42J cells. Analysis of ELISA and Western blot data demonstrated that ORM2 knockdown significantly inhibited the release of pro-inflammatory cytokines TNF-$\alpha$, IL-6 and IL-1$\beta$ (Fig. 3A) and their expression levels in the cells (Fig. 3B). These findings imply that ORM2 has a part in modulating AP inflammation.

### 3.4 Knockdown ORM2 inhibits the p38 MAPK signaling pathway

To determine the effect of ORM2 on p38 MAPK signaling pathway, we employed Western blotting to compare the expression levels of key p38 MAPK signaling components. Our findings indicated that cerulein-induced activation of p38 MAPK pathway in AR42J cells. ORM2 knockdown led to the reduction in the phosphorylation of the p38 and ERK proteins (Fig. 4). This suggests that ORM2 regulates p38 MAPK signaling, which is critical in cerulein-induced AR42J cells.

## 4. Discussion

Acute pancreatitis is among the most prevalent condition. Although mild pancreatitis generally has a favorable prognosis, the condition can rapidly progress to severe pancreatitis, leading to multi-organ failure. The mechanisms by which acute pancreatitis develops into severe pancreatitis remain poorly understood, and the current primary clinical treatment focuses on anti-infection therapy [13]. ORM2 known for its anti-inflammatory and immunomodulatory properties has been shown to promote rheumatoid inflammation [7], regulate neuroinflammatory diseases [14], and alleviate non-alcoholic steatohepatitis [9], *etc*. Consistent with previous results, in our study revealed for the first the involvement of ORM2 in pancreatitis. We discovered that ORM2 can suppress inflammation and damage in AR42J cells induced by cerulein through the p38 MAPK pathway, thereby delaying the progression of pancreatitis. These results provide novel targets and theoretical avenues for the management of pancreatitis in clinical settings.

The primary cell type in the pancreas, pancreatic acinar cells are the primary targets of pancreatic toxins and their damage plays a significant role in the development of pancreatitis [15]. Unfortunately, no particular targets have been identified to prevent inflammation and damage to acinar cells, which could be a crucial step in delaying acute pancreatitis from becoming

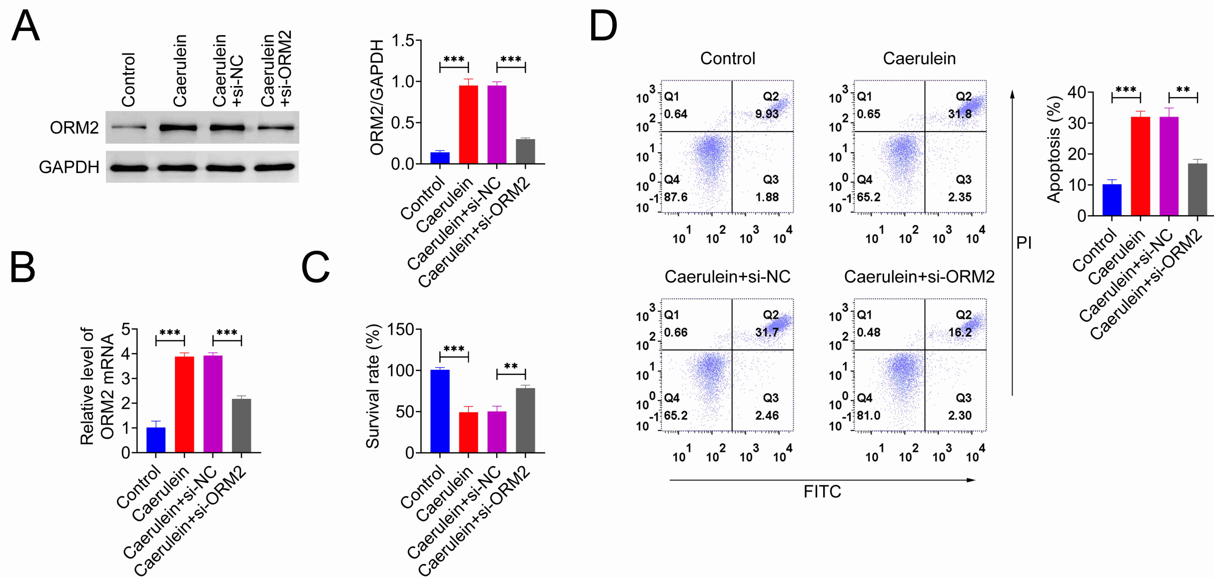


**FIGURE 2. Knockdown of ORM2 promotes the proliferation of AR42J cells.** (A) ORM2 protein expression in AR42J before and after transfection with si-ORM2. (B) ORM2 mRNA expression in AR42J before and after transfection with si-ORM2. (C) CCK8 experiment assessed the survival rate of AR42J cells. (D) Detection of apoptosis rate of AR42J cells by flow cytometry. Values are presented as mean ± SD. $**p < 0.01$, $***p < 0.001$. n = 3. ORM2: orosomucoid 2; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; si-NC: si-control; FTIC: fluorescein isothiocyanate; PI: propidium iodide; mRNA: messenger RNA.

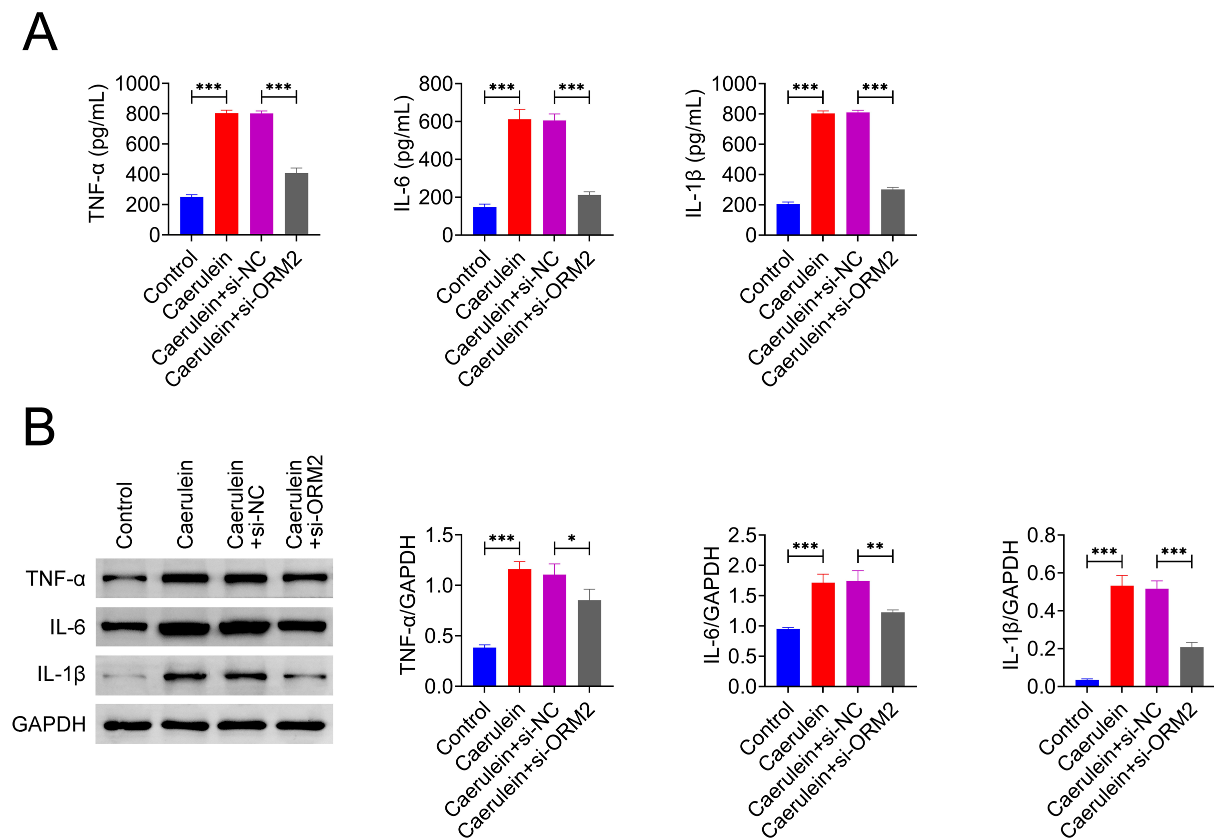


**FIGURE 3. Knockdown of ORM2 suppresses inflammation in AR42J cells.** (A) ELISA detects TNF-$\alpha$, IL-6 and IL-1$\beta$ levels in AR42J cells. (B) Western blotting to detect TNF-$\alpha$, IL-6 and IL-1$\beta$ protein expression in AR42J cells. Values are presented as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. n = 3. ORM2: orosomucoid 2; IL: interleukin; TNF-$\alpha$: tumour necrosis factor alpha; si-NC: si-control; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

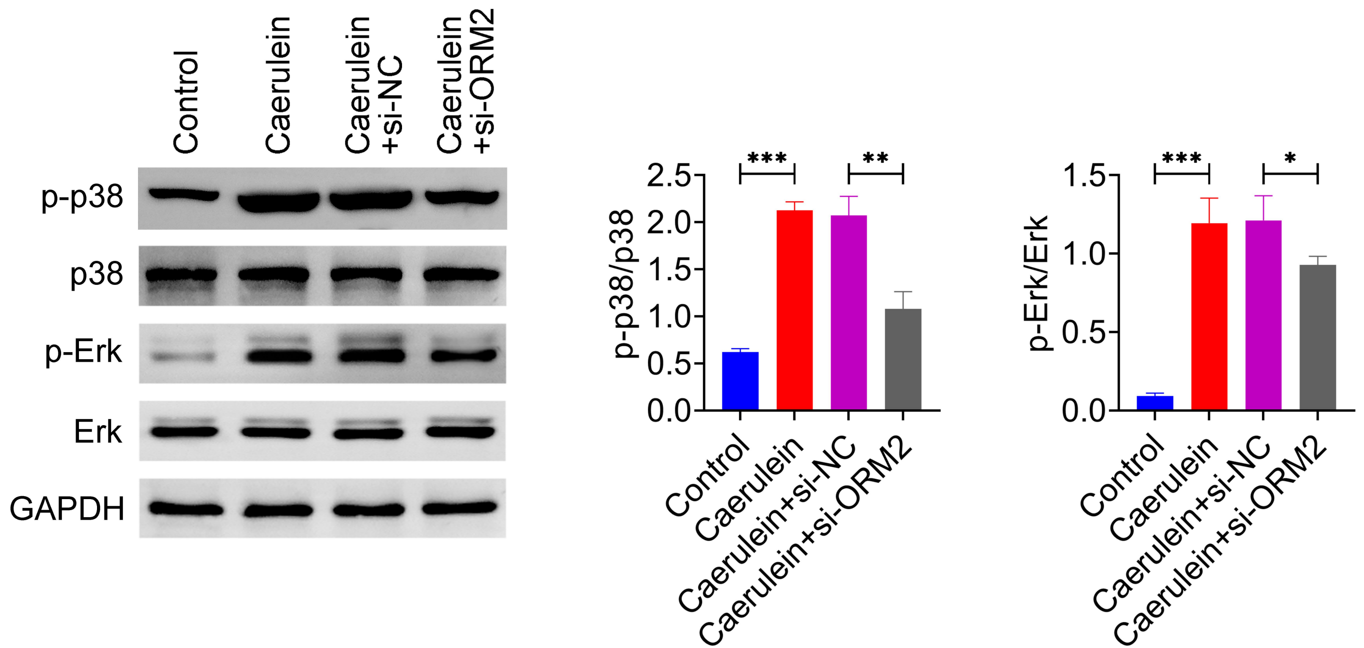


**FIGURE 4. Knockdown of ORM2 inhibits the p38 MAPK signaling pathway.** Western blotting to detect p38, p-p38, ERK and p-ERK protein expression in AR42J cells. Values are presented as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. n = 3. ERK: extracellular signal-regulated kinase; ORM2: orosomucoid 2; si-NC: si-control; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

severe pancreatitis [16]. This study utilized an *in vitro* model of AP to verify the role of ORM2 in the progression of disease. Our findings demonstrated that ORM2 was markedly upregulated in AR42J cells treated with cerulein. Consequently, *in vitro* studies have shown that ORM2 knockdown decreased the expression and secretion of inflammatory factors such as TNF-$\alpha$, IL1-$\beta$ and IL-6 as well as promoted the proliferation and prevented apoptosis of AR42J cells. For the first time, it has been established that ORM2 contributes to pancreatitis, offering new therapeutic options and targets.

Research has indicated that p38 MAPK is crucial in controlling inflammatory reactions since it functions as an upstream regulator of several inflammatory cytokines, including TNF-$\alpha$, IL-1 and IL-6, which itself regulate both intracellular and extracellular inflammation [17, 18]. The formation of the AP inflammatory response involves several different signal transduction pathways. These signaling molecules are hypothesized to control inflammatory responses through the p38 MAPK signaling pathway [19]. Previous studies have Cao *et al.* [20] discovered that the p38 MAPK signaling pathway controls the inflammatory response in AP. Another study Liu discovered that via triggering the p38 MAPK signaling pathway, plakophilin2 overexpression reduced capillary leak syndrome in cases with severe acute pancreatitis [21]. This work also demonstrated that ORM2 knockdown reduces the phosphorylation levels of p38 and ERK in the AP cell model.

Nevertheless, the study's findings are subject to several limitations. There were no animal models used in the research; it was all done *in vitro*. In the future, more thorough *in vivo* study will be required.

## 5. Conclusions

In short, we confirmed the association of ORM2 with AP *in vitro*. Additionally, we found that ORM2 significantly reduced inflammation and damage caused by cerulein in pancreatic acinar cells via activating the p38 MAPK signaling pathway. Future research needs to explore in depth connection between ORM2 and p38 MAPK in AP. These findings suggest potential new avenues for investigation which could result in novel treatments for AP.

## ABBREVIATIONS

ORM2, orosomucoid 2; AP, acute pancreatitis; ERK, extracellular signal-regulated kinase; p38 MAPK, p38 mitogen-activated protein kinase; siRNA, small interfering RNA; CCK-8, Cell Counting Kit-8; ELISA, Enzyme-linked immunosorbent assay; IL-6, interleukin-6; IL-1$\beta$, interleukin-1$\beta$; TGF-$\alpha$, transforming growth factor alpha; RT-qPCR, real-time reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

## AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

## AUTHOR CONTRIBUTIONS

JD—designed the study and carried them out. JD, CMZ, TZZ, SDL, ZJ and ZJW—supervised the data collection.

JD, CMZ, TZZ, SDL and ZJ—analyzed the data. JD, CMZ, TZZ and SDL—interpreted the data. JD and ZJW—prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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