

# Inhibition of Connexin 43 reverses ox-LDL-mediated inhibition of autophagy in VSMC by inhibiting the PI3K/Akt/mTOR signaling pathway

Xuqing Qin<sup>1,2,3,\*</sup>, Wenjun He<sup>2,3,4,\*</sup>, Rui Yang<sup>1,2,3</sup>, Luqian Liu<sup>2,3,4</sup>, Yingying Zhang<sup>1,2,3</sup>, Li Li<sup>2</sup>, Junqiang Si<sup>1,2,3</sup>, Xinzhi Li<sup>2,3,4</sup> and Ketao Ma<sup>1,2,3</sup>

<sup>1</sup> Shihezi University School of Medicine, Department of Physiology, Shihezi, Xinjiang, China

<sup>2</sup> Ministry of Education, Shihezi University School of Medicine, Key Laboratory of Xinjiang Endemic and Ethnic Diseases, Shihezi, Xinjiang, China

<sup>3</sup> First Affiliated Hospital, Shihezi University School of Medicine, NHC Key Laboratory of Prevention and Treatment of Central Asia High Incidence Diseases, Shihezi, Xinjiang, China

<sup>4</sup> Shihezi University School of Medicine, Department of Pathophysiology, Shihezi, Xinjiang, China

\* These authors contributed equally to this work.

## ABSTRACT

**Background:** Oxidized low-density lipoproteins (ox-LDL) may induce foam cell formation from the vascular smooth muscle cell (VSMC) by inhibiting VSMC autophagy. This process accelerates the formation of atherosclerosis (AS). Connexin 43 (Cx43), which is the most widely distributed connexin in VSMC is associated with autophagy. However, the mechanism of action and the involvement of Cx43 in ox-LDL-inhibited VSMC autophagy remain unclear.

**Methods:** The primary VSMC were obtained and identified, before primary VSMC were pretreated with an inhibitor (Cx43-specific inhibitor Gap26 and PI3K inhibitor LY294002) and stimulated with ox-LDL.

**Results:** Ox-LDL not only inhibited autophagy in VSMC *via* downregulation of autophagy-related proteins (such as Beclin 1, LC3B, p62), but also increased Cx43 protein levels. Then we added Gap26 to VSMC in the ox-LDL+Gap26 group, in which autophagy-related proteins were increased and the accumulation of lipid droplets was reduced. These result suggested that an enhanced level of autophagy and an alleviation of lipid accumulation might be caused by inhibiting Cx43 in VSMC. The phosphorylation levels of PI3K, AKT, mTOR were increased by ox-LDL, thus down-regulating autophagy-related proteins. However, this situation was partially reversed by the Gap26. Moreover, Cx43 expression were decreased by LY294002 in ox-LDL-induced VSMCs.

**Conclusion:** Inhibiting Cx43 may activate VSMC autophagy to inhibit foam cell formation by inhibiting the PI3K/AKT/mTOR signaling pathway.

Submitted 30 July 2021  
Accepted 30 January 2022  
Published 16 March 2022

Corresponding authors  
Xinzhi Li, [lixinzhi@shzu.edu.cn](mailto:lixinzhi@shzu.edu.cn)  
Ketao Ma, [maketao@hotmail.com](mailto:maketao@hotmail.com)

Academic editor  
Joanna Moraczewska

Additional Information and  
Declarations can be found on  
page 11

DOI [10.7717/peerj.12969](https://doi.org/10.7717/peerj.12969)

© Copyright  
2022 Qin et al.

Distributed under  
Creative Commons CC-BY 4.0

OPEN ACCESS

**Subjects** Biochemistry, Cell Biology, Molecular Biology

**Keywords** Atherosclerosis, Vascular smooth muscle cells, Autophagy

## INTRODUCTION

Atherosclerotic cardiovascular disease is the main contributor to the increase in global morbidity and mortality (Arnett *et al.*, 2019). Atherosclerotic plaques are formed in the vessel wall due to the accumulation of lipids and other substances in the process of atherosclerosis (AS) (Poznyak *et al.*, 2020). These lipid-rich cells in atherosclerotic plaques are called foam cells (FCs). Many past studies suggested that the majority of FCs originated from macrophages. However, recently it has been shown that most of the FCs in mouse and human atherosclerotic plaques originate from smooth muscle cell (SMC) (Wang *et al.*, 2019a; Allahverdian *et al.*, 2014). Accordingly, understanding the mechanism of SMC-derived FCs will contribute to the prevention and treatment of atherosclerotic diseases.

Autophagy is a physiological process in which eukaryotic cells maintain internal homeostasis (Qin, 2019). It is also involved in various physiological and pathological processes, including hypertension and the formation of coronary atherosclerotic plaques (Hughes, Beyer & Gutterman, 2020). In human atherosclerotic plaques, autophagy may be induced in VSMC, macrophages, and endothelial cells stimulated by lipids, metabolic stress, cytokines and reactive oxygen species (Grootaert *et al.*, 2018). Over time, almost all types of cells have a certain degree of autophagy dysfunction, which negatively impacts plaque progression, but the underlying mechanisms are different (Grootaert *et al.*, 2018). In addition, more studies have proven that regulation of autophagy may improve the occurrence and development of AS (Kumar *et al.*, 2020; Shi *et al.*, 2020). Rosuvastatin has been shown to enhance autophagic activity in macrophages by inhibiting the activation of the PI3K/Akt/mTOR signaling pathway and increasing autophagic flux, thus achieving the anti-AS effect (Zhang *et al.*, 2021). The PI3K/Akt/mTOR signaling pathway is a major regulatory pathway that inhibits autophagy initiation. The PI3K/AKT/mTOR signaling pathway is a classical autophagic pathway. Meanwhile, it was also found that Danlou tablets (DLTs) can promote autophagy in macrophage by inhibiting this signaling pathway, thereby reducing FCs formation and improving AS (Liu *et al.*, 2021). Despite lots of studies on the role of autophagy in anti-AS, those on the specific mechanism of autophagy is limited.

Gap junction channel (GJC), which is composed of connexin (Cx) as the basic unit, is a special channel to mediate direct communication at the intercellular (Beyer & Berthoud, 2018). Cx43 is the major Cx forming GJ in SMCs (Morel & Kwak, 2012). Gap26 is a specific Cx43 mimic peptide (Qing *et al.*, 2021) that may inhibit Cx43 expression (Li *et al.*, 2015, 2017) when used as a Cx43-GJ inhibitor. The increased expression of connexin 43 in intimal VSMC was observed in the early stages of human and mouse atherosclerotic lesions (Leybaert *et al.*, 2017). This effect may be associated with various risk factors for AS, such as ox-LDL (Wang *et al.*, 2019b) or angiotensin II (Lei *et al.*, 2019), which may upregulate the expression of Cx43. Recently, studies have demonstrated that there is an important interaction between Cx and autophagy, that is Cx could be used not only as an autophagy substrate, but also an autophagy regulator (Iyyathurai *et al.*, 2016). Besides that, it is reported that the regulation of Cx43 has impacts on the phosphorylation level of

proteins in some signaling pathways, such as PI3K/Akt signaling pathway and related downstream pathways (Ji *et al.*, 2019; Wang *et al.*, 2020). This finding suggests that Cx43 and PI3K/Akt-related downstream pathways may be interrelated. Nevertheless, the connection between Cx43 in VSMC and autophagy remains elusive in atherosclerotic lesions. Therefore, it is worth exploring whether Cx43 takes part in regulating the PI3K/AKT/mTOR signaling pathway to have an impact on autophagy and understanding the role of Cx43 in autophagy may reveal its importance as a regulatory mechanism of disease development, making it a potential therapeutic target.

## MATERIALS AND METHODS

### Culture of thoracic aortic VSMC and study design

Male Sprague-Dawley (SD) rats, 6–8 weeks of age (210–250 g), were selected from the Animal Experimental Center of Xinjiang Medical University. The license number was SCXK Xin (2018-001). All animals were treated humanely. Experimental research strictly conformed to the regulations of the Medical Ethics Committee of Shihezi University.

High-purity VSMC were then obtained from the thoracic aortas of the rats (drug anaesthesia, 2% pentobarbital sodium (40 mg/kg), intraperitoneal injection) by *in vitro* culture, as described previously (Owens *et al.*, 1986). The rats were euthanized *via* intravenous overdose of pentobarbital sodium. Third- to sixth-passage (P3–P6) VSMC were used in all experiments and cultured with DMEM/F12 (10% FBS).

Ox-LDL (80  $\mu\text{g/ml}$ ) was treated to primary VSMC for periods of 0, 3, 6, 12, 24 and 48 h and different concentrations of ox-LDL (0, 20, 40, 80, 160  $\mu\text{g/ml}$ ) were incubated for 24 h. The experiment was then divided into four groups, including the Control group, ox-LDL (Li *et al.*, 2014) (80  $\mu\text{g/ml}$ , 24 h) group, ox-LDL + Gap 26 (Wang *et al.*, 2019b) (Cx43 specific inhibitor, 100  $\mu\text{M}$  pretreatment for 45 min, and then ox-LDL for 24 h,) group, ox-LDL + LY294002 (Kim *et al.*, 2020) (20  $\mu\text{M}$  pretreatment for 30 min and then ox-LDL for 24 h) group, and the Gap 26 group.

### Reagents and antibodies

ox-LDL (Cat. No. YB-002, Yiyuan Biotechnology, Guangzhou, China), TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), Gap26 (Cat. No. A1044; APExBIO Technology LLC, Houston, TX, USA), LY294002 (Cat. No. A8250, APExBIO Technology LLC, Houston, TX, USA), anti- $\alpha$ -SMA (ab124964), anti-Desmin (ab32362), anti-LC3B (ab192890), anti-p62 (ab109012), the secondary antibody of western blotting were obtained from ZSGB-BIO (Beijing, China), the secondary antibody of immunofluorescence were obtained from ZSGB-BIO (Beijing, China), polyclonal rabbit anti-Beclin 1 (ab62557), and anti-Cx43 (ab11370) were obtained from Abcam (Cambridge, UK). Anti-PI3K p85 (#4257), anti-AKT (#4060), anti-mTOR (#2983), anti-phospho-PI3K p85 (Tyr458)/p55 (Tyr199)-actin (#17366), anti-phospho-AKT (#13038), and anti-phospho-mTOR (#5536) were obtained from CST (Inc).

### Western blot analysis

The proteins from each VSMC samples were lysed after their respective drug treatments. The protein concentration was measured with BCA assays. The proteins were separated on 10% or 12% SDS-PAGE, electroblotted onto PVDF membranes, and blocked with 5% BSA, followed by immunoblotting. The primary (1:1,000) and secondary (1:10,000) antibodies were incubated in order. Finally, image analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### Immunofluorescence analysis

After the VSMC samples were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 3 min and blocked by 5% BSA for 30 min, they were then incubated with primary (1:100) and secondary (1:100) antibodies. Subsequently, DAPI (1:1,000, Sigma-Aldrich, MerckKGaA, Burlington, MA, USA) was counterstained for 10 min to display where the nucleus is. Images were acquired using laser confocal microscopy (Zeiss LSM 510 META, Jena, Germany).

### Quantitative real-time PCR

The total RNA from VSMC sample was isolated using TRIzol reagent. Next, cDNA was synthesized using a kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The gene primers used were:

Cx43	Forward : TCACGTCCCACGGAGAAAAC	Reverse : ATCCGCAGTCTTTTGATGGG
GAPDH	Forward : GACATGCCGCTGGAGAAAAC	Reverse : AGCCCAGGATGCCCTTTAGT

SYBR Green Real-time PCR master mix (Toyota Corporation, Toyota City, Japan) was used to amplify the cDNA in the reaction system, which consisted of 40 cycles of 50, 95, and 60 °C. The  $2^{-\Delta\Delta Ct}$  method was used to analyze the data ([Livak & Schmittgen, 2001](#)).

### Transmission electron microscopy (TEM)

After the cells terminated digestion, the bottom cells were left to add 2.5% glutaraldehyde (special for electron microscopy) at 4 °C overnight. After that, autophagosomes were observed by transmission electron microscopy (JEOL, Tokyo, Japan).

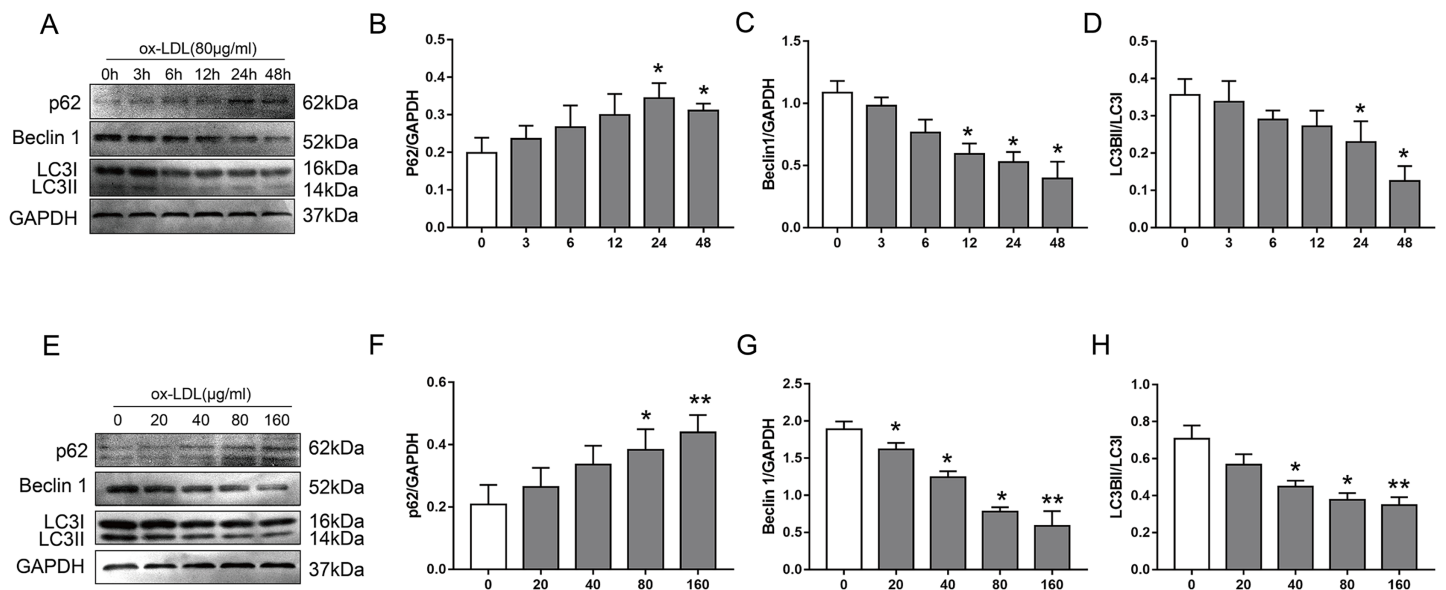
### Oil red O staining

The accumulation of lipid droplets was determined by referring to the instructions of the oil red staining kit (Cat. No. D027-1, NJJC BIO, Nanjing, China).

### Statistical analysis

GraphPad 6.01 software was used for statistical analysis and chart preparation and the results are expressed as mean  $\pm$  SD. One-way analysis of variance (ANOVA) was used to compare the differences among groups, and the Student's test was used for pairwise comparisons between groups. A value of  $P < 0.05$  was considered to be statistically significant.





**Figure 1** The effect of various concentrations of ox-LDL for different lengths of time on autophagy marker proteins in VSMCs. (A–H) Immunoblot analysis of p62, Beclin 1 and LC3B, after ox-LDL (80 µg/ml) was treated at various time (A–D) points (0, 3, 6, 12, 24 and 48 h) and different doses (E–H) of ox-LDL (0, 20, 40, 80, 160 µg/ml) were incubated for 24 h. (\* $P < 0.05$  vs. Control, \*\* $P < 0.01$  vs. Control,  $n = 3$ , data shown as the mean  $\pm$  SD). [Full-size !\[\]\(fcc3264021d438d9732560e78099f674\_img.jpg\) DOI: 10.7717/peerj.12969/fig-1](https://doi.org/10.7717/peerj.12969/fig-1)

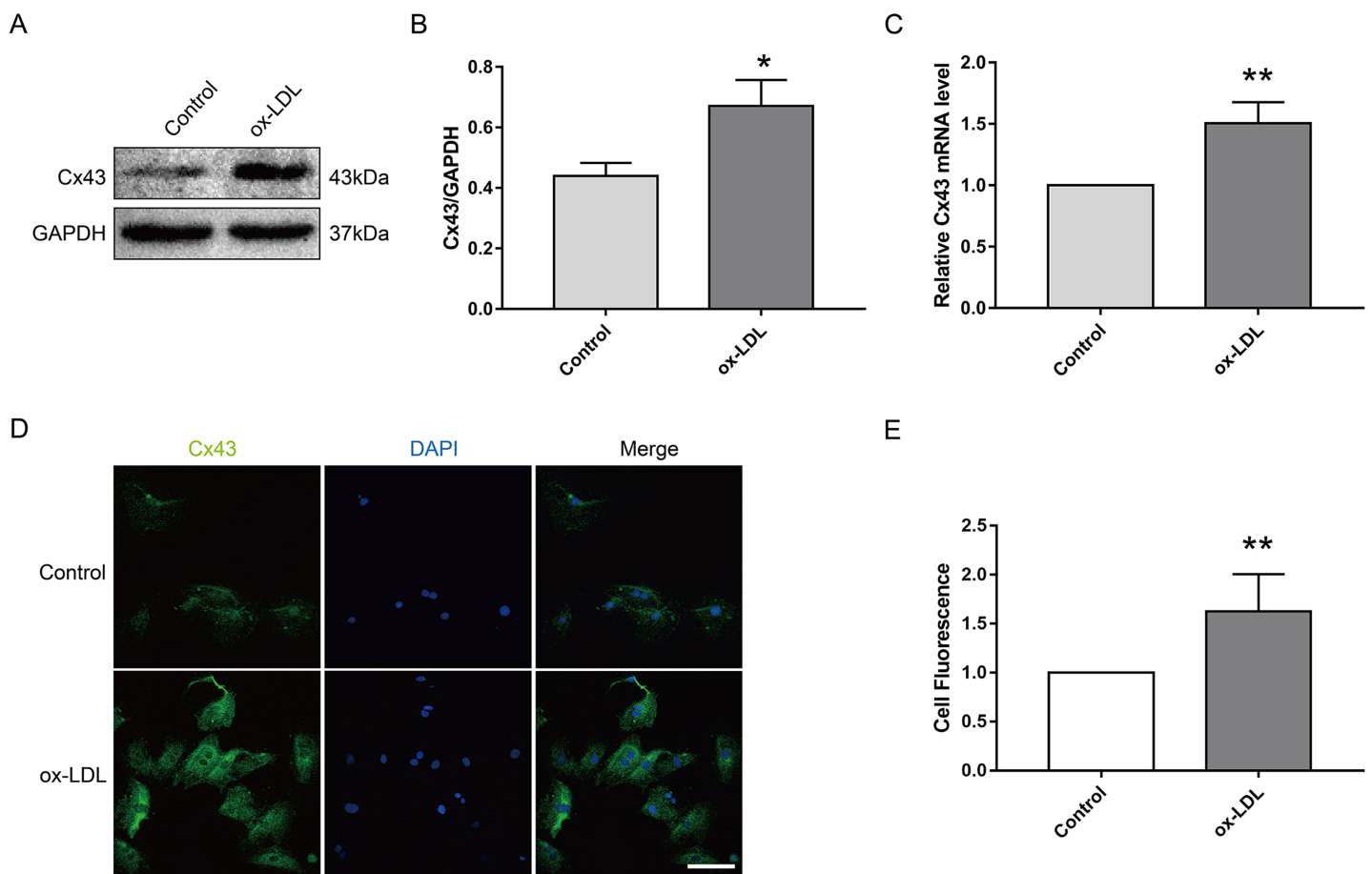
## RESULTS

### Ox-LDL inhibited autophagy in VSMC *via* downregulation of autophagy-related protein

To examine the effect of ox-LDL on autophagy in VSMC, we monitored changes in the autophagy marker proteins p62, Beclin1, and LC3B, after they were treated with various concentrations of ox-LDL, and ox-LDL was used at different lengths of time. In our study, ox-LDL decreased Beclin-1 protein levels and the ratio of LC3II to LC3I at various time points in VSMC while the p62 protein levels were increased (Figs. 1A–1D). The p62 levels were increased with the elevation of ox-LDL concentrations, which was contrary to the results of Beclin 1 and LC3B (Figs. 1E–1H). These data indicated that ox-LDL inhibited autophagy in VSMC in a time- and concentration-dependent manner.

### Ox-LDL increased Cx43 protein levels in VSMC

Next, we investigated the effect of ox-LDL on Cx43 in VSMC. The results appeared that ox-LDL treatment (80 µg/ml, 24 h) obviously elevated the protein (Figs. 2A and 2B) and mRNA (Fig. 2C) levels of Cx43 in VSMC. Moreover, the semi-quantitative results of immunofluorescence appeared that the relative fluorescence intensity of Cx43 were evidently enhanced after ox-LDL treatment, and Cx43 was mainly distributed in the cytoplasm and cell membrane (Figs. 2D and 2E). Thus, the above results prompted us that ox-LDL-inhibited autophagy in VSMC was accompanied by the upregulation of Cx43 protein levels.

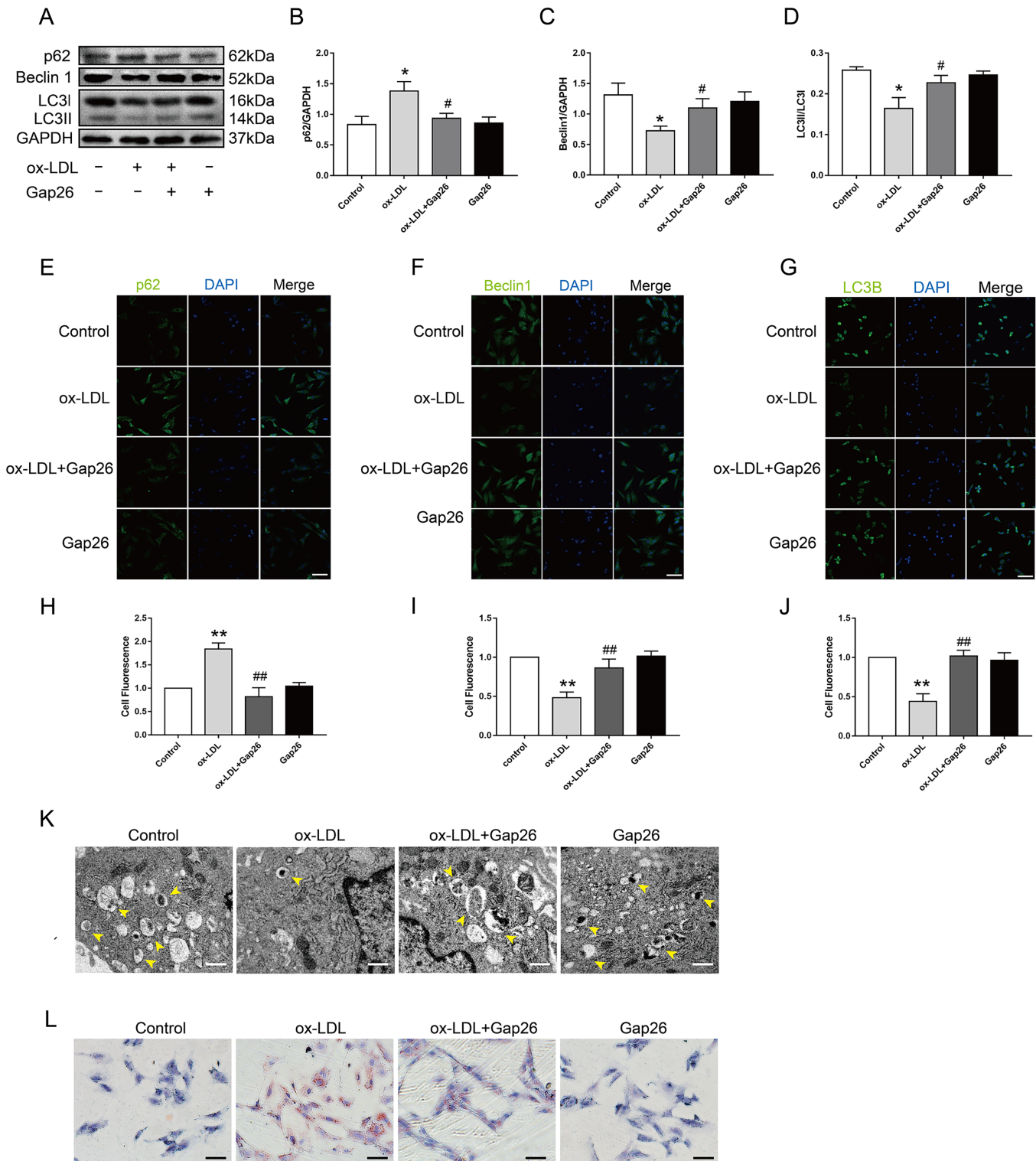


**Figure 2** The effect of ox-LDL on the expression and localization of Cx43 in VSMCs. (A and B) Immunoblot analysis of Cx43 on ox-LDL-treated (80  $\mu\text{g/ml}$ , 24 h) VSMC. (C) qRT-PCR analysis for Cx43 mRNA levels in VSMC. (D and E) Immunofluorescence analysis of Cx43 (green), DAPI (blue) staining of the nucleus. Scale bar = 50  $\mu\text{m}$ . (\* $P < 0.05$  vs. Control, \*\* $P < 0.01$  vs. Control,  $n = 3$ , data shown as the mean  $\pm$  SD).

Full-size DOI: 10.7717/peerj.12969/fig-2

## Involvement of Cx43 in VSMC autophagy and FCs formation mediated by Ox-LDL

To identify whether Cx43 was involved in ox-LDL-inhibited autophagy in VSMC, we assessed the role of Gap26, an Cx43-specific inhibitor, on autophagy. The results demonstrated that Gap26 treatment increased Beclin 1 protein levels and the induction of LC3II in VSMC incubated with ox-LDL, which was the opposite of p62 protein levels (Figs. 3A–3D). And the immunofluorescence results were consistent with those of the Western blot (Figs. 3E–3J). At the ultrastructural level, autophagosomes presents a double-membraned structure, which contains undigested cytoplasmic contents. The TEM studies indicated that Gap26 treatment increased the number of autophagosomes compared with that in the ox-LDL group (Fig. 3K). These results revealed that inhibiting Cx43 could improve the autophagy level of VSMC. To clarify whether alternation of autophagy levels affected transformation of VSMC into foam cells, we evaluated the changes in lipid droplets (Fig. 3L). The findings suggested that inhibition of Cx43



**Figure 3** The function of Cx43 in ox-LDL-inhibited autophagy in VSMCs. (A–D) Immunoblot analysis of p62 (B), Beclin 1 (C) and LC3B (D) in ox-LDL-mediated VSMCs after Gap26 pretreatment. (E–J) Immunofluorescence analysis of p62 (E and H), Beclin 1 (F and I) and LC3B (G and J) in VSMCs. DAPI (blue) staining of the nucleus. Scale bar = 50  $\mu$ m. (K) The changes in autophagosome were detected by TEM. The yellow arrow indicated autophagosomes. Scale bar = 2  $\mu$ m. (L) The accumulation of lipid droplets in VSMCs was examined by oil red O staining. Scale bar = 100  $\mu$ m. (\* $P$  < 0.05 vs. Control, \*\* $P$  < 0.01 vs. Control, # $P$  < 0.05 vs. ox-LDL, ## $P$  < 0.01 vs. ox-LDL,  $n$  = 3, data shown as the mean  $\pm$  SD).

Full-size DOI: 10.7717/peerj.12969/fig-3

prevented the ox-LDL-induced lipid droplet accumulation in VSMC, thereby suppressing FCs formation.

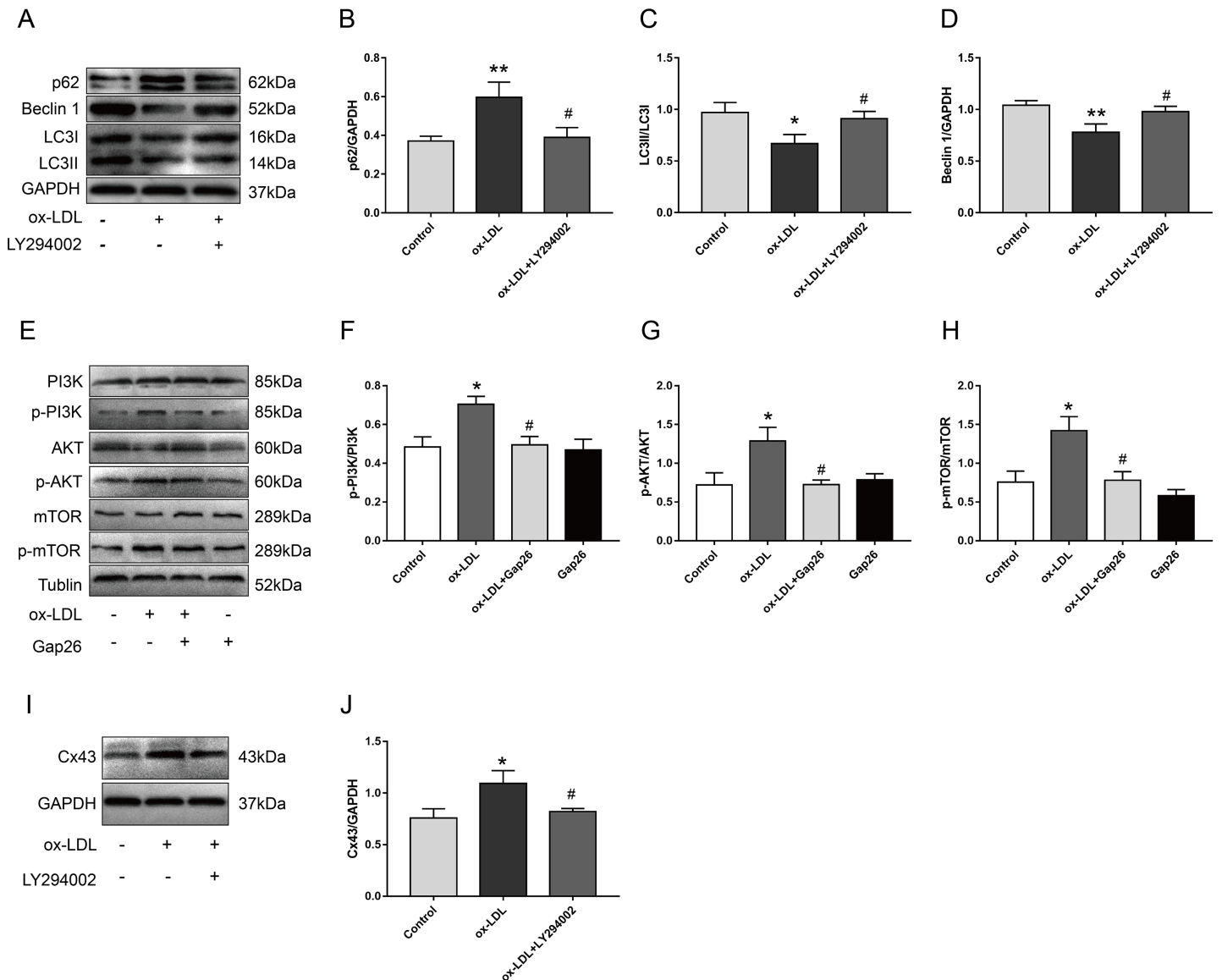
### **Inhibition of Cx43 up-regulated the autophagy levels of VSMC after ox-LDL treatment *via* inhibiting the PI3K/AKT/mTOR signaling pathway**

We pretreated VSMC with LY294002 and Gap26 to explore the potential mechanism by which Cx43 is involved in ox-LDL-inhibited autophagy in VSMC. As compared with ox-LDL-treated VSMC, LY294002 markedly inhibited p62 protein levels, increased Beclin-1 protein levels, and promoted the conversion of LC3-I to LC3-II (Figs. 4A–4D), indicating that PI3K/AKT/mTOR signaling pathway might be involved in ox-LDL-mediated autophagy of VSMC. Next, ox-LDL up-regulated the protein levels of p-PI3K, p-AKT, and p-mTOR, which was partially reversed by Gap26 (Figs. 4E–4H), suggesting that Cx43 inhibition prevented the activation of this pathway. To further analyse the relationship between Cx43 and this signaling pathway, LY294002 treatment was used to examine changes in Cx43 protein levels in ox-LDL-treated VSMC. Cx43 protein levels was decreased as VSMC was exposed to LY294002 (Figs. 4I and 4J).

## **DISCUSSION**

VSMC are an important source of FCs in atherosclerotic lesions and have received increased attention in recent years. Many studies have reported that FCs formation may be inhibited by regulating autophagy in atherosclerotic lesions (Kumar *et al.*, 2020; Shi *et al.*, 2020; Hui *et al.*, 2021). We demonstrated that ox-LDL-inhibited autophagy induction in VSMC *via* the downregulation of autophagy-related protein. Cx43 protein levels were also found to increase after ox-LDL treatment. The inhibition of Cx43 could activate autophagy and reduced ox-LDL-mediated lipid droplet accumulation by inhibiting PI3K/Akt/mTOR signaling pathway.

There are many risk factors for the formation of AS, and one of which is ox-LDL (Khatana *et al.*, 2020). It is closely associated with the formation of FCs. Consequently, we used the atherogenic factor ox-LDL (Zhao *et al.*, 2020) to induce the formation of VSMC-derived FCs. There is increasing *in vitro* evidence that autophagy is present in atherosclerotic plaques (Martinet & De Meyer, 2009). However, autophagy has dual effects on AS (Martinet & De Meyer, 2008, 2009) and may be either protective or harmful. Therefore, we examined the effect of ox-LDL on VSMC autophagy and found that it regulated autophagy in a time-dependent manner (Li *et al.*, 2014). Additionally, the effects of different doses of ox-LDL on autophagy in SMC were also different (Ding *et al.*, 2013). In the early stage of AS, a low concentration of ox-LDL increased the protective autophagy in SMCs, while a high concentration of ox-LDL weakened the protective effect of autophagy and enhanced autophagy-induced cell death (Hassanpour *et al.*, 2019). ox-LDL downregulated the accumulation of LC3-II and Beclin1 protein levels in macrophage transformation into the foam cell (Cao *et al.*, 2019). Nevertheless, ox-LDL was found to increase the LC3II/LC3I ratio and Beclin1 protein levels in vascular endothelial cells in a cell injury model of vascular endothelial cells induced by ox-LDL



**Figure 4** The relationship between Cx43 and PI3K/Akt/mTOR. (A–D) Immunoblot analysis of p62 (B), Beclin 1 (C) and LC3B (D) in VSMCs treated with ox-LDL after LY294002 pretreatment. (E–H) Immunoblot analysis of the marker proteins of PI3K/Akt/mTOR signaling pathway in VSMCs after Gap pretreatment. (I and J) Immunoblot analysis of Cx43 in ox-LDL-treated VSMCs after LY294002 pretreatment. (\* $P < 0.05$  vs. Control, \*\* $P < 0.01$  vs. Control, # $P < 0.05$  vs. ox-LDL,  $n = 3$ , data shown as the mean  $\pm$  SD). [Full-size !\[\]\(b345a1c4255362eec3746050dd71ccac\_img.jpg\) DOI: 10.7717/peerj.12969/fig-4](https://doi.org/10.7717/peerj.12969/fig-4)

(Li et al., 2018). This finding implied that there were differences in the influence of ox-LDL on autophagy in different cell models.

We also determined that ox-LDL increased Cx43 expression on VSMC. However, few studies have shown a relationship between Cx43 and autophagy in VSMC and the roles of these factors. It has been demonstrated that Cxs could regulate autophagy (Iyyathurai et al., 2016). Cx43 located on the plasma membrane may directly interact with autophagy-related gene 16 (Atg16), which is the initial step in the formation of autophagosomes. Therefore, Cxs may act as negative regulators of basal autophagy by binding to the components of the initiation complex. Other Cx subtypes (Cx26 or Cx32) may also have



negative effects on autophagic flux, suggesting that some members of the Cx family may regulate autophagy. Pharmaceuticals including tamoxifen and lindane have been shown to increase autophagy in Cx43-expressing cells but not in Cx43-knockout cells (Iyyathurai *et al.*, 2016), which suggests that Cx43 may take part in the regulation of autophagy. Other studies have found the mRNA level of LC3B in Cx43-knockout group was significantly increased compared with untreated porcine early embryos (Shin *et al.*, 2020). However, there have been few reports on the interaction between Cx43 and autophagy in AS (Iyyathurai *et al.*, 2016). Our results showed that the autophagic flux in VSMC was impaired after ox-LDL incubation, and this effect was partly reversed by the specific Cx43 inhibitor, Gap26.

Furthermore, studies have demonstrated that autophagy takes part in lipid metabolism. Autophagy may be effective in the decomposition of lipid droplets to provide free fatty acids for cells (Martinez-Lopez & Singh, 2015). The role of autophagy in lipid metabolism is associated with triglycerides breakdown and cholesterol stored in lipid droplets by selective lysosome-dependent macrophages (Liu & Czaja, 2013). Many important metabolic disorders, such as fatty liver, obesity, and AS, may be associated with impaired lipid autophagy (Robichaud *et al.*, 2021; Khawar, Gao & Li, 2019). Our results show that ox-LDL-induced FC formation may be inhibited by activating autophagy (Ko *et al.*, 2020) and that autophagy levels increased and intracellular lipid droplets decreased significantly after the inhibition of Cx43 in VSMC. This effect may be associated with the activation of autophagy and the increase of autophagosomes, which can engulf more lipid droplets and bind to lysosomes to degrade lipids.

The PI3K/Akt/mTOR signaling pathway has been demonstrated that it plays an important role in regulating autophagy in AS (Jiang *et al.*, 2017) and our study has shown that ox-LDL could activate this pathway (Zhuo *et al.*, 2019). Propofol reduces cell adhesion by inhibiting Cx43 and the downstream PI3K/Akt/NF- $\kappa$ B signaling pathway. Cx43 was overexpressed (or knocked down) on monocytes, resulting in a decrease of the related protein p-AKT with the downregulation of Cx43, and *vice versa* (Ji *et al.*, 2019). Other studies have shown that resveratrol inhibits Akt and its downstream pathway by upregulating Cx43 in colorectal cancer cell lines (Wang *et al.*, 2020). This finding suggests that there is a relationship between Cx43 and the PI3K/AKT pathway. Therefore, we used the PI3K-specific inhibitor LY294002 (Fang *et al.*, 2020) and Gap26 to confirm whether there is a connection between Cx43 and the PI3K/AKT/mTOR signaling pathway. We found that the promoting effect of Cx43 on autophagy may be produced by inhibiting the activation of this pathway, thus preventing FCs formation. Furthermore, we found that LY294002 decreased Cx43 protein levels. This effect may be associated with the fact that when autophagy is induced (such as during hunger), internalized Cx43 will be degraded by autophagy (Catarino *et al.*, 2020). Autophagy has been identified as an important pathway in the degradation of different types of Cxs, and autophagy induced by different factors will directly affect the expression level of Cxs (Iyyathurai *et al.*, 2016; D'hondt *et al.*, 2016). We hypothesized that VSMC autophagy was activated by LY294002, which resulted in the degradation of internalized Cx43, thereby reducing Cx43 expression



(*Bi et al., 2017*). These findings suggest that Cxs can be used as autophagy substrates and as autophagy regulators.

Based on the above results, this study still has some limitations. Although some studies have reported that in podocytes (MPC5 cell line), silencing of Cx43 expression improved autophagy flow experimentally impaired by exposure of the cells to high glucose levels (*Ji et al., 2019*), the role of Cx43 in ox-LDL-mediated autophagy of primary VSMC still needs to be verified by more experimental techniques, such as knockdown Cx43 by transfection technology. In addition, we can also explore the role of gap junction intercellular communication (GJIC) function in VSMC autophagy. It has been found that GJIC negatively regulated autophagy and inhibited autophagy flux (*Zou et al., 2020*). In summary, ox-LDL prevented VSMC autophagy. The inhibition of Cx43 significantly reversed this effect. The underlying mechanism may be mediated by inhibiting the PI3K/Akt/mTOR signaling pathway. These findings may become an important regulatory mechanism for disease development, making it a potential target for the treatment of AS.

## ACKNOWLEDGEMENTS

We would like to thank the Key Laboratory of Xinjiang Endemic and Ethnic Diseases, the NHC Key Laboratory of Prevention and Treatment of Central Asia High Incidence Diseases, and the Department of Physiology and Pathophysiology of Shihezi University School of Medicine for their experimental platform and technology support.

## ADDITIONAL INFORMATION AND DECLARATIONS

### Funding

This work was supported by the National Natural Science Foundation of China (Nos. 81860085, 81860286), Corps Science and Technology Cooperation Project of China (No. 2020BC004), Central Research Institute Fund of Chinese Academy of Medical Sciences (2020-PT330-003). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Grant Disclosures

The following grant information was disclosed by the authors:

National Natural Science Foundation of China: 81860085, 81860286.

Corps Science and Technology Cooperation Project of China: 2020BC004.

Central Research Institute Fund of Chinese Academy of Medical Sciences: 2020-PT330-003.

### Competing Interests

The authors declare that they have no competing interests.

### Author Contributions

- Xuqing Qin conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

- Wenjun He performed the experiments, prepared figures and/or tables, and approved the final draft.
- Rui Yang analyzed the data, prepared figures and/or tables, and approved the final draft.
- Luqian Liu performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Yingying Zhang performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Li Li analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Junqiang Si analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Xinzhi Li conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Ketao Ma conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

### Animal Ethics

The following information was supplied relating to ethical approvals (*i.e.*, approving body and any reference numbers):

The Medical Ethics Committee of Shihezi University approved the study (SCXK Xin (2018-001)).

### Data Availability

The following information was supplied regarding data availability:

The raw data is available in the [Supplemental File](#).

### Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.12969#supplemental-information>.

## REFERENCES

- Allahverdian S, Chehroudi A, Mcmanus B, Abraham T, Francis GA. 2014. Contribution of intimal smooth muscle cells to cholesterol accumulation and macrophage-like cells in human atherosclerosis. *Circulation* **129**(15):1551–1559  
DOI [10.1161/CIRCULATIONAHA.113.005015](https://doi.org/10.1161/CIRCULATIONAHA.113.005015).
- Arnett DK, Blumenthal RS, Albert MA, Buroker AB, Goldberger ZD, Hahn EJ, Himmelfarb CD, Khera A, Lloyd-Jones D, McEvoy JW, Michos ED, Virani SS, Williams KA, Yeboah J, Ziaeian B. 2019. ACC/AHA guideline on the primary prevention of cardiovascular disease: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Circulation* **140**(11):e596–e646  
DOI [10.1161/CIR.0000000000000678](https://doi.org/10.1161/CIR.0000000000000678).
- Beyer E, Berthoud V. 2018. Gap junction gene and protein families: connexins, innexins, and pannexins. *Biochimica et Biophysica acta Biomembranes* **1860**(1):5–8  
DOI [10.1016/j.bbmem.2017.05.016](https://doi.org/10.1016/j.bbmem.2017.05.016).

- Bi Y, Wang G, Liu X, Wei M, Zhang Q. 2017. Low-after-high glucose down-regulated Cx43 in H9c2 cells by autophagy activation via cross-regulation by the PI3K/Akt/mTOR and MEK/ERK1/2 signal pathways. *Endocrine* 56(2):336–345 DOI 10.1007/s12020-017-1251-3.
- Cao H, Jia Q, Yan L, Chen C, Xing S, Shen D. 2019. Quercetin suppresses the progression of atherosclerosis by regulating MST1-mediated autophagy in ox-LDL-induced RAW264.7 macrophage foam cells. *International Journal of Molecular Sciences* 20(23):6093 DOI 10.3390/ijms20236093.
- Catarino S, Ribeiro-Rodrigues TM, Sá Ferreira R, Jé R, Abert C, Martens S, Girão H. 2020. A conserved LIR motif in connexins mediates ubiquitin-independent binding to LC3/GABARAP proteins. *Cells* 9(4):902 DOI 10.3390/cells9040902.
- D'hondt C, Iyyathurai J, Welkenhuyzen K, Himpens B, Leybaert L, Bultynck G. 2016. Nutrient starvation decreases Cx43 levels and limits intercellular communication in primary bovine corneal endothelial cells. *Journal of Membrane Biology* 249(3):363–373 DOI 10.1007/s00232-016-9874-5.
- Ding Z, Wang X, Schnackenberg L, Khaidakov M, Liu S, Singla S, Dai Y, Mehta JL. 2013. Regulation of autophagy and apoptosis in response to ox-LDL in vascular smooth muscle cells, and the modulatory effects of the microRNA hsa-let-7g. *International Journal of Cardiology* 168(2):1378–1385 DOI 10.1016/j.ijcard.2012.12.045.
- Fang Y, Ou S, Wu T, Zhou L, Tang H, Jiang M, Xu J, Guo K. 2020. Lycopene alleviates oxidative stress via the PI3K/Akt/Nrf2 pathway in a cell model of Alzheimer's disease. *PeerJ* 8:e9308 DOI 10.7717/peerj.9308.
- Grootaert MOJ, Moulis M, Roth L, Martinet W, Cécile V, Bennett MR, De Meyer GRY. 2018. Vascular smooth muscle cell death, autophagy and senescence in atherosclerosis. *Cardiovascular Research* 114(4):622–634 DOI 10.1155/2018/7687083.
- Grootaert MOJ, Roth L, Schrijvers DM, De Meyer GRY, Martinet W. 2018. Defective autophagy in atherosclerosis: to die or to senesce? *Oxidative Medicine Cellular Longevity* 2018:1–12 DOI 10.1155/2018/7687083.
- Hassanpour M, Rahbarghazi R, Nouri M, Aghamohammadzadeh N, Safaei N, Ahmadi M. 2019. Role of autophagy in atherosclerosis: foe or friend? *Journal of Inflammation* 16(1):e146 DOI 10.1186/s12950-019-0212-4.
- Hughes W, Beyer A, Guterman D. 2020. Vascular autophagy in health and disease. *Basic Research in Cardiology* 115(4):41 DOI 10.1007/s00395-020-0802-6.
- Hui B, Hou X, Liu R, Liu X-H, Hu Z. 2021. Gypenoside inhibits ox-LDL uptake and foam cell formation through enhancing Sirt1-FOXO1 mediated autophagy flux restoration. *Life Sciences* 264:118721 DOI 10.1016/j.lfs.2020.118721.
- Iyyathurai J, Decuyper J-P, Leybaert L, D'hondt C, Bultynck G. 2016. Connexins: substrates and regulators of autophagy. *BMC Cell Biology* 17:1273 DOI 10.1186/s12860-016-0093-9.
- Ji H, Qiu R, Gao X, Zhang R, Li X, Hei Z, Yuan D. 2019. Propofol attenuates monocyte-endothelial adhesion via modulating connexin43 expression in monocytes. *Life Sciences* 232:116624 DOI 10.1016/j.lfs.2019.116624.
- Ji J, Zhao Y, Na C, Yang M, Zhu X, Shi H, Gan W, Zhang A. 2019. Connexin 43-autophagy loop in the podocyte injury of diabetic nephropathy. *International Journal of Molecular Medicine* 44(5):1781–1788 DOI 10.3892/ijmm.
- Jiang Y, Kou J, Han X, Li X, Zhong Z, Liu Z, Zheng Y, Tian Y, Yang L. 2017. ROS-dependent activation of autophagy through the PI3K/Akt/mTOR pathway is induced by hydroxysafflor yellow a-sonodynamic therapy in THP-1 macrophages. *Oxidative Medicine Cellular Longevity* 2017:8519169 DOI 10.1155/2017/8519169.

- Khatana C, Saini NK, Chakrabarti S, Saini V, Sharma A, Saini RV, Saini AK. 2020.** Mechanistic insights into the oxidized low-density lipoprotein-induced atherosclerosis. *Oxidative Medicine and Cellular Longevity* **2020**(4):1–14 DOI [10.1155/2020/5245308](https://doi.org/10.1155/2020/5245308).
- Khawar M, Gao H, Li W. 2019.** Autophagy and lipid metabolism. *Advances in Experimental Medicine Biology* **2019**:359–374 DOI [10.1007/978-981-15-0602-4\\_17](https://doi.org/10.1007/978-981-15-0602-4_17).
- Kim J, Lee KP, Kim BS, Lee SJ, Moon BS, Baek S. 2020.** Heat shock protein 90 inhibitor AUY922 attenuates platelet-derived growth factor-BB-induced migration and proliferation of vascular smooth muscle cells. *Korean Journal of Physiology & Pharmacology* **24**(3):241–248 DOI [10.4196/kjpp.2020.24.3.241](https://doi.org/10.4196/kjpp.2020.24.3.241).
- Ko M, Oh G, Park J, Kwon HJ. 2020.** Extract of high hydrostatic pressure-treated danshen (*Salvia miltiorrhiza*) ameliorates atherosclerosis via autophagy induction. *BMB Reports* **53**(12):652–657 DOI [10.5483/BMBRep.2020.53.12.184](https://doi.org/10.5483/BMBRep.2020.53.12.184).
- Kumar S, Nanduri R, Bhagyaraj E, Kumar S, Nanduri R, Bhagyaraj E, Kalra R, Ahuja N, Chacko AP, Tiwari D, Sethi K, Saini A, Chandra V, Jain M. 2020.** Vitamin D3-VDR-PTPN6 axis mediated autophagy contributes to the inhibition of macrophage foam cell formation. *Autophagy* **17**(9):2273–2289 DOI [10.1080/15548627.2020.1822088](https://doi.org/10.1080/15548627.2020.1822088).
- Lei Y, Peng X, Li T, Yang G. 2019.** ERK and miRNA-1 target Cx43 expression and phosphorylation to modulate the vascular protective effect of angiotensin II. *Life Sciences* **216**:59–66 DOI [10.1016/j.lfs.2018.11.019](https://doi.org/10.1016/j.lfs.2018.11.019).
- Leybaert L, Lampe PD, Dhein S, Kwak BR, Ferdinandy P, Beyer EC, Laird DW, Naus CC, Green CR, Schulz R, Levy FO. 2017.** Connexins in cardiovascular and neurovascular health and disease: pharmacological implications. *Pharmacological Reviews* **69**(4):396–478 DOI [10.1124/pr.115.012062](https://doi.org/10.1124/pr.115.012062).
- Li X, Jiang S, Yang H, Liao Q, Cao S, Yan X, Huang D. 2017.** Breakthrough cancer pain is associated with spinal gap junction activation via regulation of connexin 43 in a mouse model. *Frontiers in Cellular Neuroscience* **11**:2045 DOI [10.3389/fncel.2017.00207](https://doi.org/10.3389/fncel.2017.00207).
- Li C, Yang L, Wu H, Dai M. 2018.** Paeonol inhibits oxidized low-density lipoprotein-induced vascular endothelial cells autophagy by upregulating the expression of miRNA-30a. *Frontiers in Pharmacology* **9**:95 DOI [10.3389/fphar.2018.00095](https://doi.org/10.3389/fphar.2018.00095).
- Li B, Yin Y-W, Liu Y, Pi Y, Guo L, Cao X-J, Gao C-Y, Zhang L-L, Li J-C. 2014.** TRPV1 activation impedes foam cell formation by inducing autophagy in oxLDL-treated vascular smooth muscle cells. *Cell Death & Disease* **5**:e1182 DOI [10.1038/cddis.2014.146](https://doi.org/10.1038/cddis.2014.146).
- Li X, Zhao H, Tan X, Kostrzewa RM, Du G, Chen Y, Zhu J, Miao Z, Yu H, Kong J, Xu X. 2015.** Inhibition of connexin43 improves functional recovery after ischemic brain injury in neonatal rats. *Glia* **63**(9):1553–1567 DOI [10.1002/glia.22826](https://doi.org/10.1002/glia.22826).
- Liu C, Chen G, Chen Y, Dang Y, Nie G, Wu D, Li J, Chen Z, Yang H, Li He D, Sun Z, Lu J, Wang J, L. 2021.** Danlou tablets inhibit atherosclerosis in Apolipoprotein E-deficient mice by inducing macrophage autophagy: The role of the PI3K-Akt-mTOR pathway. *Frontiers in Pharmacology* **12**:724670 DOI [10.3389/fphar.2021.724670](https://doi.org/10.3389/fphar.2021.724670).
- Liu K, Czaja M. 2013.** Regulation of lipid stores and metabolism by lipophagy. *Cell Death & Differentiation* **20**(1):3–11 DOI [10.1038/cdd.2012.63](https://doi.org/10.1038/cdd.2012.63).
- Livak K, Schmittgen T. 2001.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**(4):402–408 DOI [10.1006/meth.2001.1262](https://doi.org/10.1006/meth.2001.1262).
- Martinet W, De Meyer G. 2008.** Autophagy in atherosclerosis. *Current Atherosclerosis Reports* **10**(3):216–223 DOI [10.1007/s11883-008-0034-y](https://doi.org/10.1007/s11883-008-0034-y).

- Martinet W, De Meyer G. 2009.** Autophagy in atherosclerosis: a cell survival and death phenomenon with therapeutic potential. *Circulation Research* **104**(3):304–317 DOI [10.1161/CIRCRESAHA.108.188318](https://doi.org/10.1161/CIRCRESAHA.108.188318).
- Martinez-Lopez N, Singh R. 2015.** Autophagy and lipid droplets in the liver. *Annual Review of Nutrition* **2015**(35):215–237 DOI [10.1146/annurev-nutr-071813-105336](https://doi.org/10.1146/annurev-nutr-071813-105336).
- Morel S, Kwak B. 2012.** Roles of connexins in atherosclerosis and ischemia-reperfusion injury. *Current Pharmaceutical Biotechnology* **13**(1):17–26 DOI [10.2174/138920112798868638](https://doi.org/10.2174/138920112798868638).
- Owens GK, Loeb A, Gordon D, Thompson MM. 1986.** Expression of smooth muscle-specific alpha-isoactin in cultured vascular smooth muscle cells: relationship between growth and cytodifferentiation. *Journal of Cell Biology* **102**(2):343–352 DOI [10.1083/jcb.102.2.343](https://doi.org/10.1083/jcb.102.2.343).
- Poznyak AV, Wu W-K, Melnichenko AA, Wetzker R, Sukhorukov V, Markin AM, Khotina VA, Orekhov AN. 2020.** Signaling pathways and key genes involved in regulation of foam cell formation in atherosclerosis. *Cells* **9**(3):584 DOI [10.3390/cells9030584](https://doi.org/10.3390/cells9030584).
- Qin ZH. 2019.** *Autophagy: biology and diseases*. Singapore: Springer.
- Qing C, Xinyi Z, Xuefei Y, Xindong X, Jianhua F. 2021.** The specific connexin 43-inhibiting peptide Gap26 improved alveolar development of neonatal rats with hyperoxia exposure. *Frontiers in Pharmacology* **12**:1053 DOI [10.3389/fphar.2021.587267](https://doi.org/10.3389/fphar.2021.587267).
- Robichaud S, Robichaud S, Fairman G, Vijithakumar V, Mak E, Cook DP, Pelletier AR, Huard S, Vanderhyden BC, Figeys D, Lavallée-Adam M, Baetz K. 2021.** Identification of novel lipid droplet factors that regulate lipophagy and cholesterol efflux in macrophage foam cells. *Autophagy* **17**(11):3671–3689 DOI [10.1080/15548627.2021.1886839](https://doi.org/10.1080/15548627.2021.1886839).
- Shi Y, Jiang S, Zhao T, Gong Y, Liao D, Qin L. 2020.** Celastrol suppresses lipid accumulation through LXR $\alpha$ /ABCA1 signaling pathway and autophagy in vascular smooth muscle cells. *Biochemical and Biophysical Research Communications* **532**(3):466–474 DOI [10.1016/j.bbrc.2020.08.076](https://doi.org/10.1016/j.bbrc.2020.08.076).
- Shin K-T, Nie Z-W, Zhou W, Zhou D, Kim J-Y, Ock SA, Niu Y-J, Cui X-S. 2020.** Connexin 43 Knockdown induces mitochondrial dysfunction and affects early developmental competence in porcine embryos. *Microscopy and Microanalysis* **26**(2):287–296 DOI [10.1017/S1431927620000033](https://doi.org/10.1017/S1431927620000033).
- Wang Y, Dubland JA, Allahverdian S, Asonye E, Sahin B, Jaw JE, Sin DD, Seidman MA, Leeper NJ, Francis GA. 2019a.** Smooth muscle cells contribute the majority of foam cells in ApoE (Apolipoprotein E)-deficient mouse atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* **39**(5):876–887 DOI [10.1161/ATVBAHA.119.312434](https://doi.org/10.1161/ATVBAHA.119.312434).
- Wang Y, Wang W, Wu X, Li C, Huang Y, Zhou H, Cui Y. 2020.** Resveratrol sensitizes colorectal cancer cells to cetuximab by connexin 43 upregulation-induced Akt inhibition. *Frontiers in Oncology* **10**:93 DOI [10.3389/fonc.2020.00383](https://doi.org/10.3389/fonc.2020.00383).
- Wang M, Wu Y, Yu Y, Fu Y, Yan H, Wang X, Li T, Peng W, Luo D. 2019b.** Rutaecarpine prevented ox-LDL-induced VSMCs dysfunction through inhibiting overexpression of connexin 43. *European Journal of Pharmacology* **853**:84–92 DOI [10.1016/j.ejphar.2019.03.028](https://doi.org/10.1016/j.ejphar.2019.03.028).
- Zhang X, Qin Y, Wan X, Liu H, Lv C, Ruan W, He L, Lu L, Guo X. 2021.** Rosuvastatin exerts anti-atherosclerotic effects by improving macrophage-related foam cell formation and polarization conversion via mediating autophagic activities. *Journal of Translational Medicine* **19**(1):62 DOI [10.1186/s12967-021-02727-3](https://doi.org/10.1186/s12967-021-02727-3).
- Zhao J, Niu X, Yu J, Xiao X, Li W, Zang L, Hu Z, Siu-Po IP, Li P, W. 2020.** Poria cocos polysaccharides attenuated ox-LDL-induced inflammation and oxidative stress via ERK activated Nrf2/HO-1 signaling pathway and inhibited foam cell formation in VSMCs. *International Immunopharmacology* **80**:106173 DOI [10.1016/j.intimp.2019.106173](https://doi.org/10.1016/j.intimp.2019.106173).

- Zhuo X, Wu Y, Yang Y, Qiao X, Chen T. 2019.** Knockdown of LSD1 meliorates Ox-LDL-stimulated NLRP3 activation and inflammation by promoting autophagy *via* SESN2-mediated PI3K/Akt/mTOR signaling pathway. *Life Sciences* **233**:116696 DOI [10.1016/j.lfs.2019.116696](https://doi.org/10.1016/j.lfs.2019.116696).
- Zou H, Yuan J, Zhang Y, Wang T, Chen Y, Yuan Y, Bian J, Liu Z. 2020.** Gap junction intercellular communication negatively regulates cadmium-induced autophagy and inhibition of autophagic flux in buffalo rat liver 3A cells. *Frontiers in Pharmacology* **11**:596046 DOI [10.3389/fphar.2020.596046](https://doi.org/10.3389/fphar.2020.596046).