Oxidized low-density lipoprotein enhances Orai3 expression levels to increase proliferation of human lens epithelial cells

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Research article

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Abstract

Background

Serum lipid levels, especially of oxidized low-density lipoprotein (oxLDL), are related to the development of cataracts, but the mechanisms underlying the role of oxLDL in cataract development in human lens epithelial cells (HLEpiCs) remain unclear. Calcium (Ca^{2+}) overload is also known to be involved in lens turbidity. Store-operated Ca^{2+} entry (SOCE) is an important pathway mediating Ca^{2+} influx in lens epithelial cells. The Orai family proteins, which form a type of SOCE channel, localize at the plasma membrane and control Ca^{2+} influx in response to the depletion of Ca^{2+} stores in the endoplasmic reticulum.

Methods

In the present study, we used RNA sequencing, western blot analyses, cell proliferation assays, Ca^{2+} measurements, and small interfering (si)RNA transfections to investigate the effects of oxLDL on SOCE and proliferation of human lens epithelial cells (HLEpiCs).

Results

The key findings of our study that suggest this conclusion are that (1) oxLDL enhances the expression levels of Orai3, but not Orai1 or Orai2, in HLEpiCs in vitro; (2) oxLDL significantly increases the proliferation of HLEpiCs in a concentration-dependent manner; (3) oxLDL significantly increases ATP-induced SOCE without affecting Ca^{2+} release in HLEpiCs; and (4) knockdown of Orai3 significantly reduces cell proliferation and ATP-induced SOCE in HLEpiCs.

Conclusions

These findings suggest that Ca^{2+} signaling altered by overexpression of Orai3 may be a mechanism whereby oxLDL increases HLEpiC proliferation to contribute to the development of cataract. Thus, Orai3 may be a target warranting development for the treatment of cataract associated with obesity or hyperlipidemia.

1. Introduction

Cataract is one of the leading causes of visual impairment and blindness in the world [1]. The transparency of the lens can only be maintained by coordinated proliferation, migration, and elongation of the lens epithelial cells [2]. Myriad factors increase the risk of cataract, including diabetes, obesity, and hyperlipidemia [3]. Obesity is associated with dyslipidemia, and low-density lipoprotein cholesterol levels

are higher in the serum of patients with obesity [4]. Hyperlipidemia is an imbalance of cholesterol levels, which is mainly attributable to increases in serum low-density lipoprotein cholesterol levels and in oxidized low-density lipoprotein (oxLDL) levels [5, 6]. High levels of serum oxLDL are involved in various physiological and pathological mechanisms, from monocyte adhesion to smooth muscle cell proliferation and endothelial cell dysfunction [7]. Accumulating evidence indicates that cataract is associated with high levels of oxLDL in the serum [8]. However, the molecular mechanisms that underpin this association in the human lens epithelial cell has been rarely reported.

Calcium (Ca^{2+}) participates in regulating many physiological and pathological processes [9]. Previous reports have suggested that homeostasis of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is involved in maintaining the homeostasis of the lens and in preventing opacity of the lens [10, 11]. Store-operated Ca^{2+} entry (SOCE) mediated by the interaction of the highly Ca^{2+}-selective Ca^{2+} release–activated calcium modulator Orai channel with stromal interaction molecule 1 is an crucial Ca^{2+} signaling regulation mechanism in non-excitatory cells [12]. The depletion of Ca^{2+} from the endoplasmic reticulum leads to the accumulation of stromal interaction molecule 1 protein in the endoplasmic reticulum membrane, which eventually evokes Ca^{2+} influx by activating Orai channels located in the cell membrane [13]. SOCE participates in various physiological and pathological activities, such as cell proliferation and migration regulation, immune response, and cancer development [14–16]. However, the regulatory effect of SOCE in human lens epithelial cells has been rarely investigated.

Although the continuous improvement of surgical methods and the development of intraocular lens implants have greatly improved the cure rate of cataract, the surgery is accompanied by potential complications, such as retinal detachment, iris prolapse, and even blindness [17]. Thus, it is beneficial to explore the underlying molecular mechanisms associated with cataract and find new therapeutic targets for this condition. Therefore, in the present study, we used RNA sequencing, western blot analyses, cell proliferation assays, Ca^{2+} measurements, and small interfering (si)RNA transfections to investigate the effects of oxLDL on SOCE and proliferation of human lens epithelial cells (HLEpiCs).

2. Materials And Methods

2.1. Cell culture and transfection

The HLEpiCs were purchased from the American Type Culture Collection (HB-8065, Manassas, VA, USA) and then cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS;Gibco) at 37 °C in an incubator with 5% carbon dioxide. The culture media in the oxLDL groups contained various concentrations of oxLDL(Guangzhou Yiyuan biotech.Co.,Ltd.), whereas media for the control groups contained equal volumes of DMEM. The HLEpiCs were transfected with siRNA specific to human Orai3 (5'-GGGUCAAGUUUGUGCCCAU-3') or with scrambled siRNA (5'-ACGCGUAACGCGGGAAUUU-3') using Lipofectamine 2000 (Invitrogen) by following the manufacturer's instructions. The siRNAs were designed and purchased from Biomics Biotechnologies. The final siRNA

concentration was 200 nm/L. The HLEpiCs were cultured in an incubator with 5% carbon dioxide at 37 °C for 24 h. Western blotting was conducted to determine the transfection efficiency.

2.2. RNA sequencing

RNA was extracted from cell lysates using an RNA extraction kit (Hefei Nuower Biotechnology Co., Ltd, China). A high-throughput Illumina HiSeq 2500 system was used to sequence the samples. We obtained RNA-Seq FastQ raw data and used the read trimming tool Trimmomatic to remove adaptors and lower mass readings [18]. The quality of the clean data was assessed using FastQC software [19]. The transcript expression level was determined as fragments per kilobase of transcript per million fragments (FPKM) [20], the fold change (FC) represented the ratio of the expression level FPKM between the two groups of samples. During the detection of differentially expressed genes, $\log_2 FC \geq 1$ was used as a screening criterion for significant differences. The edgeR package was used to detect differences in the number of reads [21].

2.3. Western blot analysis

Western blotting was conducted as previously described [22]. In brief, the cells were lysed using RIPA buffer (1% Nonidet P-40, 150 mmol/L NaCl, and 20 mmol/L Tris-HCl; pH 8.0). The lysate was centrifuged at 12,000 rpm for 20 min in a 4 °C thermostatic centrifuge. The supernatant was mixed with loading buffer (Beyotime Biotechnology.Co., Ltd.) in a ratio of 4:1 by volume, and the mixed solution was placed in a metal water bath for 10 min at 100 °C. Total proteins (30 μg) under denaturing conditions were loaded onto a 10% gel for sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were then transferred onto a polyvinylidene difluoride membrane (Millipore, USA). The membranes with the proteins were immersed in a blocking solution of phosphate-buffered saline (PBS) with Tween 20 that contained 5% nonfat milk for 1 h at room temperature. The membranes were then incubated with one of the following antibodies at 4 °C overnight: β-tubulin (1:1000; cat. no.sc-9104; Santa Cruz), Orai1(1:500; cat. no. sc-68895; Santa Cruz), Orai2（1:500; cat. no. sc-376749; Santa Cruz） or Orai3 (1:500; cat. no.25766-I-AP; affinity). The next day, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody（Promega） for 2 h at room temperature. An enhanced chemiluminescence system (Shanghai Peiqing Science & Technology Co., Ltd, China) was then used to detect the immunosignals. Each protein band was normalized to β-tubulin and was quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, Maryland).

2.4. Cell proliferation assay

Cell proliferation was determined using a Cell Counting Kit-8 (Shanghai Beyotime Biotechnology Co., Ltd, China) according to the manufacturer's instructions. Differently treated cell suspensions (1×10^4/well in 100 μL) were seeded in a 96-well cell plate. After incubation for 24 h in the cell culture incubator, Cell Counting Kit-8 reagent (10 μL) was added to each well. After the cells were incubated for 2 h at 37 °C in a dark cell culture incubator, the absorbance at a wavelength of 450 nm was measured using a microplate reader.

2.5. $[Ca^{2+}]$ measurement

The $[Ca^{2+}]_i$ was determined as previously described [23]. Briefly, HLEpiCs were loaded with the green fluorescent calcium-binding dye Fluo-8/AM (10 μmol/L; abcam) and 0.02% pluronic acid and placed in a dark incubator for 1 h at 37 °C. The Ca^{2+} release was activated by 100 μmol/L ATP (Sigma) in Ca^{2+}-free PBS that contained (in mmol/L) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 0.2 EGTA, and 5 HEPES (pH 7.4). After the baseline stabilized, SOCE was detected by adding 2 mmol/L $CaCl_2$. The $[Ca^{2+}]_i$ was recorded using fluorescence microscopy (Nikon, Japan) because the fluorescence intensity of Fluo-8/AM increases when it binds to Ca^{2+}. Changes in the $[Ca^{2+}]_i$ were calculated as the ratio of fluorescence intensities before (F_0) and after (F_1) adding $CaCl_2$ (F_1/F_0).

2.6. Statistical analysis

Values are expressed as means ± SEM. A two-tailed unpaired Student's *t*-tests was used to compare the significance between two groups. Values of $P < 0.05$ were considered statistically significant. All values were analyzed using SigmaPlot software.

3. Results

3.1. OxLDL enhances Orai3 expression in HLEpiCs

RNA-seq is a powerful tool for detecting transcript changes. Our RNA-seq analysis showed that the expression level of Orai3 transcripts in HLEpiCs pretreated with oxLDL (100 μg/mL) for 24 h significantly increased, but there was no significant difference in the expression levels of Orai1 or Orai2 (Fig. 1a). We also detected the expression levels of Orai proteins using western blot analysis of HLEpiCs treated with oxLDL (100 μg/mL) for 24 h. OxLDL significantly increased the expression levels of Orai3 protein (Fig. 1b and e) in HLEpiCs but had no significant effect on the protein expression levels of Orai1 or Orai2 (Fig. 1b, c and d). The results of western blot analyses were consistent with the transcript changes determined using the RNA-sEq. Therefore, together the data suggested that oxLDL treatment enhanced Orai3 expression in HLEpiCs in vitro. To further investigate the effects of Orai3 on HLEpiCs, we knocked down the expression of Orai3 by a specific Orai3 siRNA. We found that transfection with specific siRNA significantly inhibited Orai3 expression in HLEpiCs (Fig. 2a and b).

3.2. Roles of oxLDL and of Orai3 siRNA in proliferation of HLEpiCs

Previous reports have suggested that oxLDL is associated with cell proliferation [24]. To explore the effect of oxLDL on the proliferation of HLEpiCs, we treated HLEpiCs for 24 h with 25 μg/mL, 50 μg/mL, 100 μg/ml, or 200 μg/mL oxLDL and then detected cell proliferation by using a Cell Counting Kit-8. The results showed that oxLDL increased the proliferation of HLEpiCs in a concentration-dependent manner, with 100 μg/mL causing the greatest proliferation (Fig. 3a). To further investigate the effects of Orai3 on

the proliferation of HLEpiCs, we knocked down the expression of Orai3 using an Orai3-specific siRNA. We found that the proliferation of HLEpiCs was significantly decreased when they were transfected with Orai3 siRNA compared with when they were transfected with scrambled siRNA (Fig. 3b). However, there was no significant difference in cell proliferation between HLEpiCs transfected with Orai3 siRNA and treated with oxLDL and HLEpiCs treated with Orai3 siRNA alone. These results suggested that oxLDL may increase cell proliferation by regulating Orai3 expression in HLEpiCs.

3.3. Roles of oxLDL and of Orai3 siRNA in ATP-induced Ca^{2+} signaling in HLEpiCs

We measured $[Ca^{2+}]_i$ to examine the effect of oxLDL on Ca^{2+} signaling in HLEpiCs. As shown in Fig. 4, ATP-induced SOCE was significantly enhanced in HLEpiCs treated with oxLDL (100 μg/mL) for 24 h (Fig. 4a and c). By contrast, there was no significant difference in Ca^{2+} release (Fig. 4a and b). Thus, these results indicated that ATP-induced SOCE in HLEpiCs was increased by oxLDL. To examine the role of Orai3 in ATP-induced Ca^{2+} signaling in HLEpiCs, we used Orai3 siRNA to knock down the expression levels of Orai3. Transfection of Orai3 siRNA into HLEpiCs significantly attenuated ATP-induced SOCE compared with that in HLEpiCs transfected with scrambled siRNA (Fig. 4d and e). However, no difference was found in ATP-induced SOCE between HLEpiCs transfected with Orai3 siRNA and those transfected with Orai3 siRNA and treated with oxLDL (100 μg/mL). These results suggested that oxLDL may enhance ATP-induced SOCE in HLEpiCs by regulating the expression of Orai3.

4. Discussion

In the present study, we explored the expression levels and function of Orai in HLEpiCs and the molecular mechanisms underlying the development of cataract associated with obesity or with hyperlipidemia. The main findings were as follows: (1) OxLDL enhanced the expression of Orai3 in HLEpiCs without significantly altering the expression of Orai1 or Orai2. (2) OxLDL significantly increased the proliferation of HLEpiCs in a concentration-dependent manner. (3) OxLDL significantly increased ATP-induced SOCE in HLEpiCs although there was no significant effect on Ca^{2+} release. (4) Knockdown of Orai3 significantly decreased cell proliferation and ATP-induced SOCE in HLEpiCs. Together, our results indicated that oxLDL may play an essential role in cell proliferation and ATP-induced SOCE in HLEpiCs and that these effects may be related to an increased expression level of Orai3. Therefore, Orai3 may be a new target for development in the treatment of cataract associated with obesity or hyperlipidemia.

Previous studies have indicated that SOCE participates in maintaining $[Ca^{2+}]_i$ homeostasis in lens epithelial cells [25]. In our study, we cultured HLEpiCs in media containing oxLDL to mimic a high-fat environment in vitro. Our RNA-seq analysis and western blotting results showed that treatment with oxLDL enhanced the expression of Orai3 in HLEpiCs, whereas the expression levels of Orai1 and Orai2 did not significantly change. Our Ca^{2+} measurement results showed that treatment with oxLDL significantly enhanced ATP-mediated SOCE in HLEpiCs. To examine the effect of Orai3 on oxLDL-

mediated Ca^{2+} signaling in HLEpiCs, we knocked down Orai3 expression by transfecting these cells with Orai3-specific siRNA. Our results indicated that knockdown of Orai3 significantly inhibited ATP-induced SOCE in HLEpiCs. Additionally, no difference was found in ATP-induced SOCE in HLEpiCs transfected with Orai3 siRNA and in transfected cells treated with oxLDL. These results strongly suggested that oxLDL affected ATP-induced SOCE by regulating Orai3 expression. Therefore, we speculate that cataract associated with obesity or hyperlipidemia may be related to a change in Ca^{2+} signaling in HLEpiCs.

According to epidemiological statistics, plasma oxLDL levels of patients with cataract are significantly increased compared with matched individuals without cataract [8]. In individuals with age-related cataract, the severity of cataract is related to the excessive proliferation of lens epithelial cells [26, 27]. A mouse model of cataract established using transgenic technology found that increased differentiation and proliferation of lens epithelial cells led to the formation of anterior subcapsular plaque [28]. It is generally believed that oxLDL is involved in regulating cell proliferation in the cardiovascular system [29, 30]. OxLDL induces abnormal proliferation of smooth muscle cells, leading to the formation of atherosclerotic plaques [31]. Therefore, exploring the pathological role of oxLDL in HLEpiCs is useful for understanding the mechanisms of the development of cataract associated with hyperlipidemia or obesity. In the present study, we showed that treatment with increasing concentrations of oxLDL increased the proliferation of HLEpiCs in a concentration-dependent manner. To our knowledge, this is the first evidence showing a pathological role of oxLDL in HLEpiCs. Previous reports have indicated that Orai3 plays vital roles in the development of cancer and of cardiovascular disease [32, 33]. To examine the role of Orai3 in oxLDL-mediated proliferation in HLEpiCs, we knocked down Orai3 expression by transfecting them with Orai3-specific siRNA. We found that Orai3 knockdown significantly inhibited the proliferation of HLEpiCs. Our results also showed no difference in cell proliferation when HLEpiCs were transfected with Orai3 siRNA and treated with oxLDL and when HLEpiCs were transfected with Orai3 siRNA but not treated with oxLDL. These results suggested that oxLDL affects the proliferation of HLEpiCs by regulating Orai3 expression.

5. Conclusion

The present study showed that an increased proliferation of HLEpiCs was associated with an overload of intracellular Ca^{2+} induced by the overexpression of Orai3, and that this process may contribute to the development of cataract. This study provides novel insights into the mechanisms underpinning the effect of oxLDL on the proliferation of HLEpiCs and the potentially critical role of Orai3 in the development of cataract.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Y.W., S.B. designed and performed the experiments. R.Z. wrote the paper. S.B.and Y.W. analyzed the data, and provided financial support. All authors read and approved the final manuscript.

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Not applicable.

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Figures

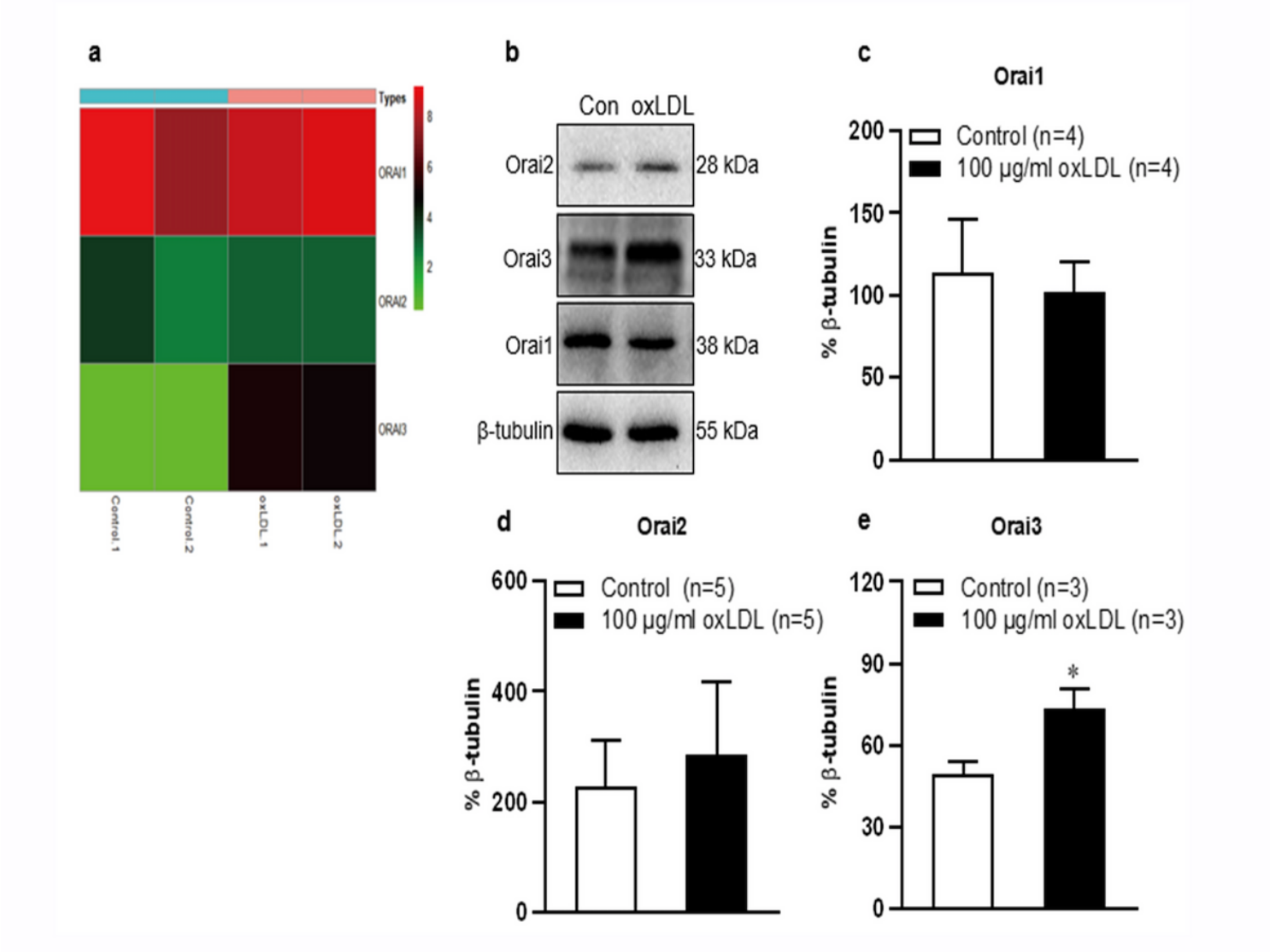

Figure 1

Effects of oxLDL treatment on Orai1, Orai2, and Orai3 expression levels in HLEpiCs. (a) Cluster analysis of RNA-seq data from HLEpiCs pretreated with oxLDL (100 μg/mL) for 24 h. Color scale indicates fragments per kilobase of transcript per million fragments (n = 2). B–E, Representative immunoblots (b) and summary data (c, d, and e) showing Orai protein expression levels in HLEpiCs treated with oxLDL (100 μg/mL) or DMEM (control) for 24 h (n = 3–5; mean ± S.E.M.; *P < 0.05).

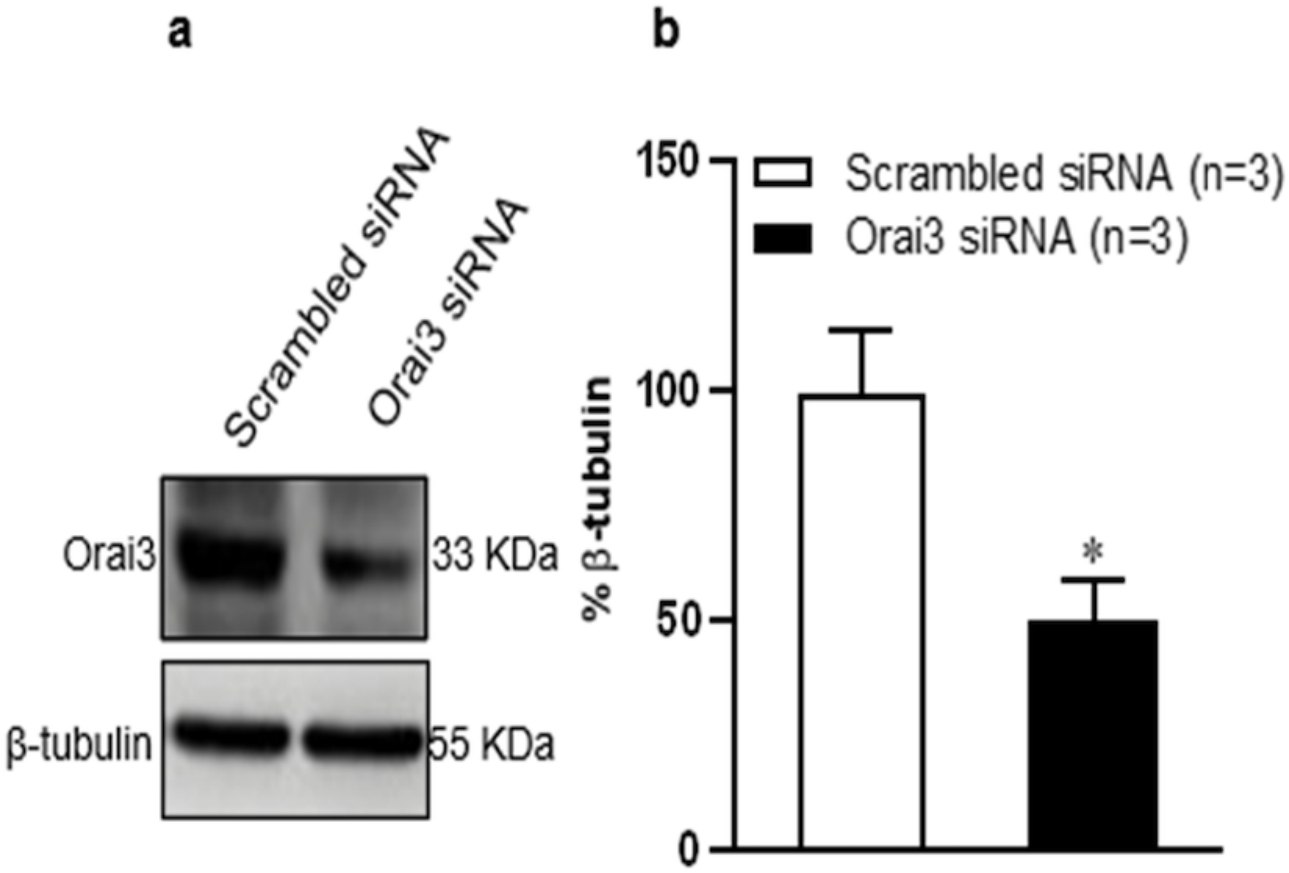

Figure 2

The effects of Orai3 siRNA on the expression of Orai3 in HLEpiCs. Representative immunoblots (a) and summary data (b) showing Orai3 protein expression levels in HLEpiCs transfected with scrambled siRNA or Orai3 siRNA for 24 h. (n = 3; mean ± S.E.M.; *P < 0.05: scrambled siRNA vs. Orai3 siRNA).

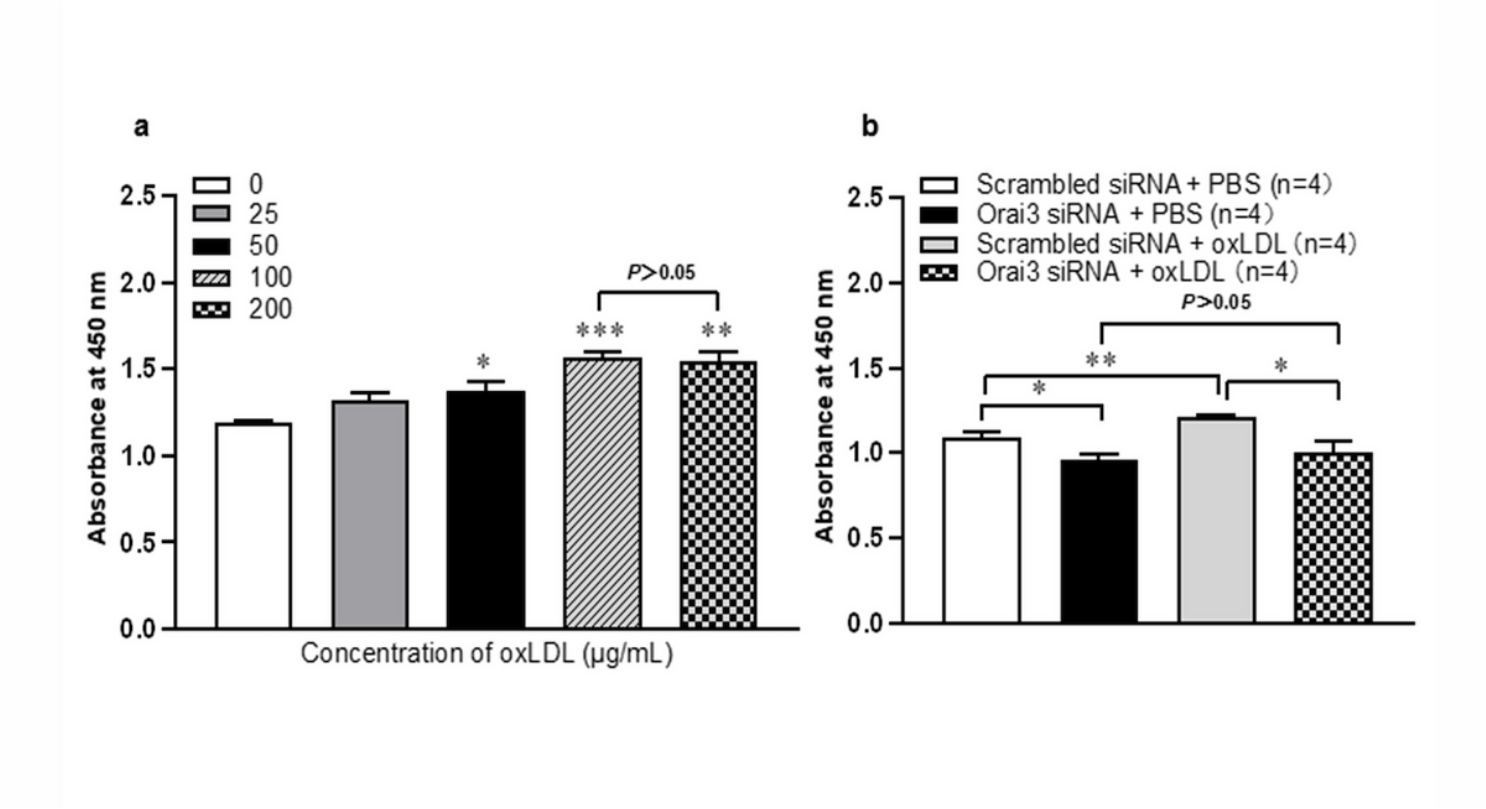

Figure 3

Roles of oxLDL and of Orai3 siRNA in HLEpiC proliferation. Summary data showing the number of HLEpiCs (directly proportional to absorbance levels at 450 nm) following treatment with oxLDL at 25 μg/mL, 50 μg/mL, 100 μg/mL, or 200 μg/mL (a) and transfected with scrambled siRNA or Orai3 siRNA and treated with PBS or oxLDL (100 μg/mL; b) for 24 h (n = 3–4; mean ± S.E.M.; *P < 0.05: control vs. 50 μg/mL oxLDL (a); scrambled siRNA + PBS vs. Orai3 siRNA + PBS (b); scrambled siRNA + oxLDL vs. Orai3 siRNA + oxLDL (b). **P < 0.01, control vs. 200 μg/mL oxLDL (a); scrambled siRNA + PBS vs. scrambled siRNA + oxLDL (b). ***P < 0.001, control vs. 100 μg/mL oxLDL (a). P > 0.05, Orai3 siRNA + PBS vs. Orai3 siRNA + oxLDL (b).

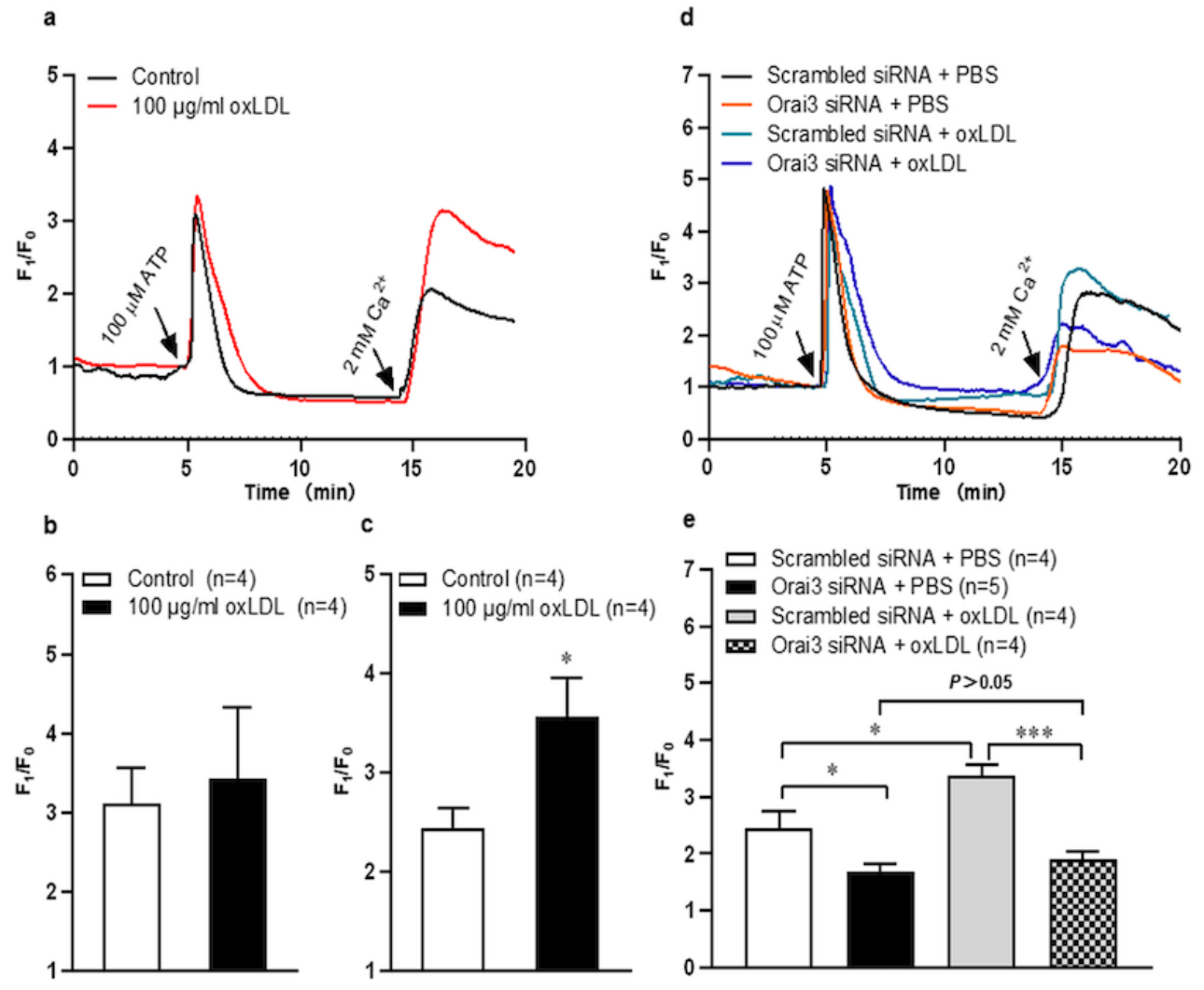

Figure 4

Roles of oxLDL and of Orai3 siRNA in ATP-induced Ca2+ signaling in HLEpiCs. Representative traces (a and d) and summary data (b, c, and e) showing the changes in ATP-induced Ca2+ release (b) and in the following SOCE (c and e) in HLEpiCs treated with oxLDL (100 μg/ml) or in HLEpiCs transfected with scrambled siRNA or Orai3 siRNA and treated with PBS or oxLDL (100 μg/ml) for 24 h. (n = 4–5; mean ± S.E.M.; *P < 0.05: control vs. oxLDL treatment (c); scrambled siRNA + PBS vs. Orai3 siRNA + PBS (e); scrambled siRNA + PBS vs. scrambled siRNA + oxLDL (e). ***P < 0.001, scrambled siRNA + oxLDL vs. Orai3 siRNA + oxLDL (e). P > 0.05, Orai3 siRNA + PBS vs. Orai3 siRNA + oxLDL (e).