Research Paper

Visfatin increases ICAM-1 expression and monocyte adhesion in human osteoarthritis synovial fibroblasts by reducing miR-320a expression

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ABSTRACT

Pathophysiological events that modulate the progression of structural changes in osteoarthritis (OA) include monocyte adhesion and infiltration, and synovial inflammation. In particular, the adhesion protein intercellular adhesion molecule type 1 (ICAM-1) promotes monocyte recruitment into the synovial tissue. Visfatin is an adipocyte hormone that promotes the release of inflammatory cytokines during OA progression. We report that visfatin enhances ICAM-1 expression in human OA synovial fibroblasts (OASFs) and facilitates the adhesion of monocytes with OASFs. AMPK and p38 inhibitors, as well as their respective siRNAs, attenuated the effects of visfatin upon ICAM-1 synthesis and monocyte adhesion. We also describe how miR-320a negatively regulates visfatin-induced promotion of ICAM-1 expression and monocyte adhesion. We detail how visfatin affects ICAM-1 expression and monocyte adhesion with OASFs by inhibiting miR-320a synthesis via the AMPK and p38 signaling pathways.

INTRODUCTION

Synovial inflammation is an important contributor to the pathogenesis of osteoarthritis (OA) [1], with the synthesis of chondrolytic enzymes and pro-inflammatory mediators by the inflamed synovium eroding cartilage and enhancing the inflammatory process [2, 3]. Halting the excretion of chondrolytic enzymes and inflammatory mediators by OA synovial fibroblasts (OASFs) is expected to mitigate OA disease [2, 4–7].

Infiltration of mononuclear cells to the inflammatory sites and their adhesion to the synovium membrane are

critical steps during OA progression [8]. Several different adhesion molecules regulate monocyte migration and adhesion in the articular micro-environment during OA development [8, 9], including intercellular adhesion molecule-1 (ICAM-1) [10]. ICAM-1 is a critical modulator of monocyte recruitment into the synovial tissue and high levels of ICAM-1 expression have been found in the synovium of OA patients [11, 12]. Downregulation of ICAM-1 expression in synovial fluid is suggested to be an effective method for inhibiting inflammatory activity and ameliorating symptoms in OA [13, 14].

Micro RNAs (miRNAs) control the synthesis of target genes at the post-transcriptional level and inhibit the expression of target genes [15]. Numerous miRNAs regulate OA pathogenesis [16], although it is not clear as to how miRNAs regulate ICAM-1 expression in OA. The proinflammatory adipokine visfatin is produced by visceral white adipose tissue in the bone marrow, skeletal muscles and liver [17]. By stimulating the expression of interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) in OASFs, visfatin contributes to synovial joint damage [18]. It is not known how visfatin affects ICAM-1-dependent monocyte adhesion during OA progression. We describe how visfatin increases monocyte adhesion to OASFs by increasing ICAM-1 expression. The reduction in miR-320a expression via the adenosine monophosphate-activated protein kinase (AMPK) and p38 signaling pathways is mediated by the effects of visfatin, indicating that this adipokine may be an appropriate therapeutic target in OA.

RESULTS

Visfatin and ICAM-1 expression are positively correlated in OA

Our analysis of records from the Gene Expression Omnibus (GEO) database revealed higher levels of visfatin and ICAM-1 expression in inflamed synovial tissue compared with normal synovial tissue (Figure 1A, 1B). A positive correlation was observed for levels of visfatin and ICAM-1 expression (Figure 1C). An ELISA assay confirmed significantly higher serum visfatin and ICAM-1 concentrations in patients with OA compared with healthy controls (Figure 2A, 2B). Serum visfatin and ICAM-1 concentrations were positively correlated (Figure 2C).

Visfatin increases ICAM-1 expression and monocyte adhesion in OASFs

In this study, visfatin (1–30 ng/mL) dose-dependently stimulated transcription of ICAM-1 mRNA and also increased the translation of ICAM-1 at the protein level (Figure 3A, 3B) and also the excretion of ICAM-1 protein by OASFs (Figure 3C). Visfatin markedly increased the adhesiveness between OASFs and monocytes (THP-1 cells) in a concentration-dependent manner (Figure 3D), indicating that visfatin promotes ICAM-1 expression and monocyte adhesion in human OASFs.

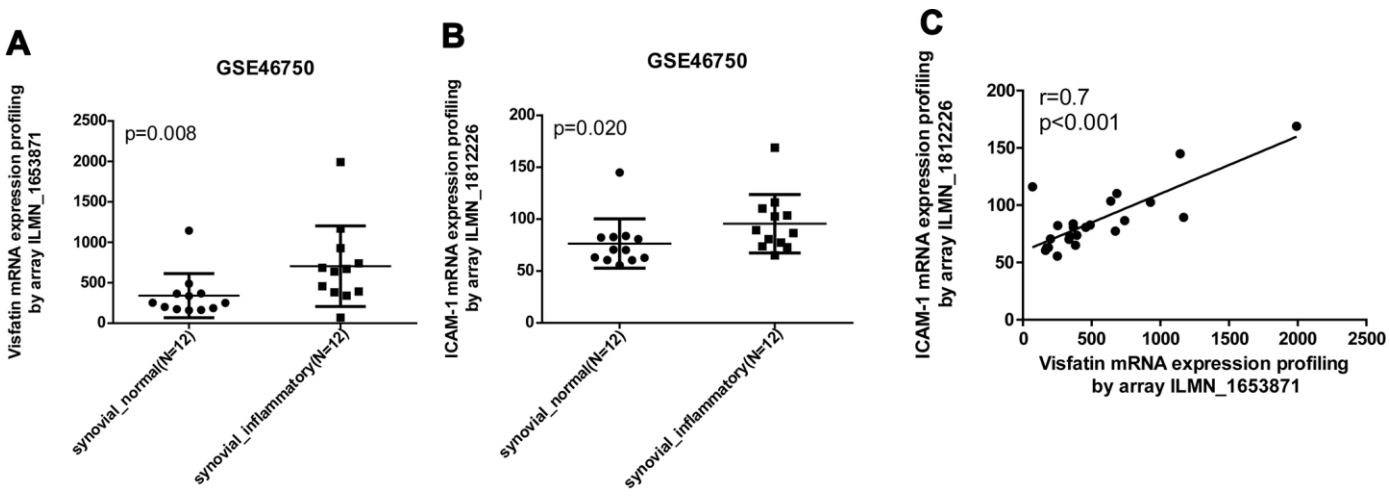

Figure 1. Visfatin expression is positively correlated with ICAM-1 expression in inflammatory synovial tissues. (**A**, **B**) Expression levels of visfatin and ICAM-1 in 12 paired normal and inflammatory synovial tissues retrieved from the GEO dataset (accession code: GSE46750). (**C**) Correlation between levels of visfatin and ICAM-1 expression in inflammatory synovial tissues. Mann-Whitney testing was applied in (**A**, **B**).

Visfatin promotes ICAM-1 expression and monocyte adhesion via the AMPK and p38 signaling pathways

OASFs were pretreated with AMPK inhibitors (Ara A and compound C) or were transfected with AMPKα1 and AMPKα2 small interfering RNAs (siRNAs). RT-qPCR, Western blot and ELISA assays confirmed that the AMPK inhibitors and AMPK siRNAs significantly reduced visfatin-increased ICAM-1 synthesis in OASFs (Figure 4A–4C). These compounds also mitigated monocyte adhesion to OASFs (Figure 4D). In Western blot analysis, visfatin time-dependently promoted AMPK phosphorylation (Figure 4E).

Treatment of OASFs with a p38 inhibitor (SB203580) or transfection of OASFs with p38 siRNA prior to visfatin stimulation markedly diminished visfatin-induced increases in ICAM-1 expression and monocyte adhesion (Figure 5A–5D). In Western blot analysis, the time-dependent promotion of p38 phosphorylation by visfatin (Figure 5E) was reduced by AMPK inhibitors (Figure 5F). These findings suggest that visfatin facilitates ICAM-1 production and monocyte adhesion in human OASFs through the AMPK and p38 signaling pathways.

Visfatin increases ICAM-1 production and monocyte adhesion via the inhibition of miR-320a synthesis

Open-source software (TargetScan, miRMap, RNAhybrid, and miRWalk) suggested that miR-320a interferes with ICAM-1 transcription (Figure 6A).

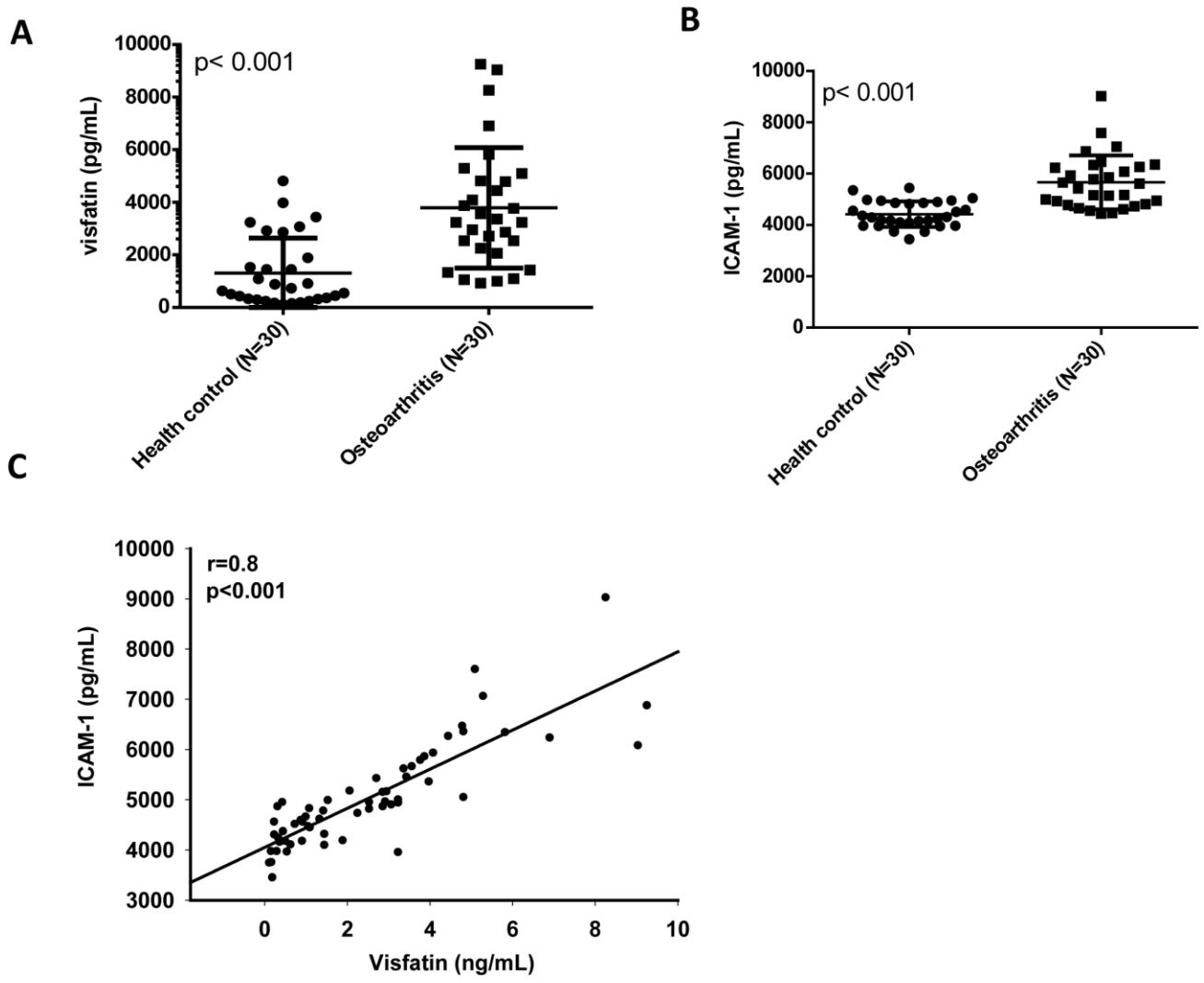

Figure 2. Visfatin expression is positively correlated with ICAM-1 expression in OA patients. (**A**, **B**) ELISA analysis showing higher serum visfatin and ICAM-1 levels among OA patients (n=30) compared with healthy controls (n=30). (**C**) Correlation between levels of visfatin and ICAM-1 expression in serum samples retrieved from OA patients. All ELISA procedures were independently repeated three times. Mann-Whitney testing was applied in (**A**, **B**).

Visfatin treatment of OASFs concentration-dependently reduced miR-320a expression (Figure 6B). ICAM-1 expression and monocyte adhesion in visfatin-treated OASFs was lower after transfection with miR-320a mimic compared with after mimic control (serving as the vehicle control) (Figure 6C–6F).

The luciferase reporter vector with the wild-type 3'UTR of ICAM-1 mRNA (wt-ICAM-1-3'UTR) and a mutated vector harboring mismatches in the predicted miR-320a binding site (mut-ICAM-1-3'UTR) were used to determine whether miR-320a controls transcription of the *ICAM-1* gene (Figure 7A). The miR-320a mimic inhibited visfatin-increased luciferase activity in the wt-ICAM-1-3'UTR plasmid only (Figure 7B). AMPK and p38 inhibitors reversed the effects of visfatin on miR-320a expression (Figure 7C). These results suggest that visfatin inhibits miR-320a expression via the AMPK and p38 signaling pathways. Visfatin shRNA reduced visfatin and ICAM-1 expression in OASFs (Figure 8A, 8B) and also monocyte adhesion (Figure 8C), confirming that visfatin regulates the expression of ICAM-1 and adhesion of monocytes in OASFs.

DISCUSSION

OA pathogenesis remains poorly understood, although it is established that synovium inflammation has a

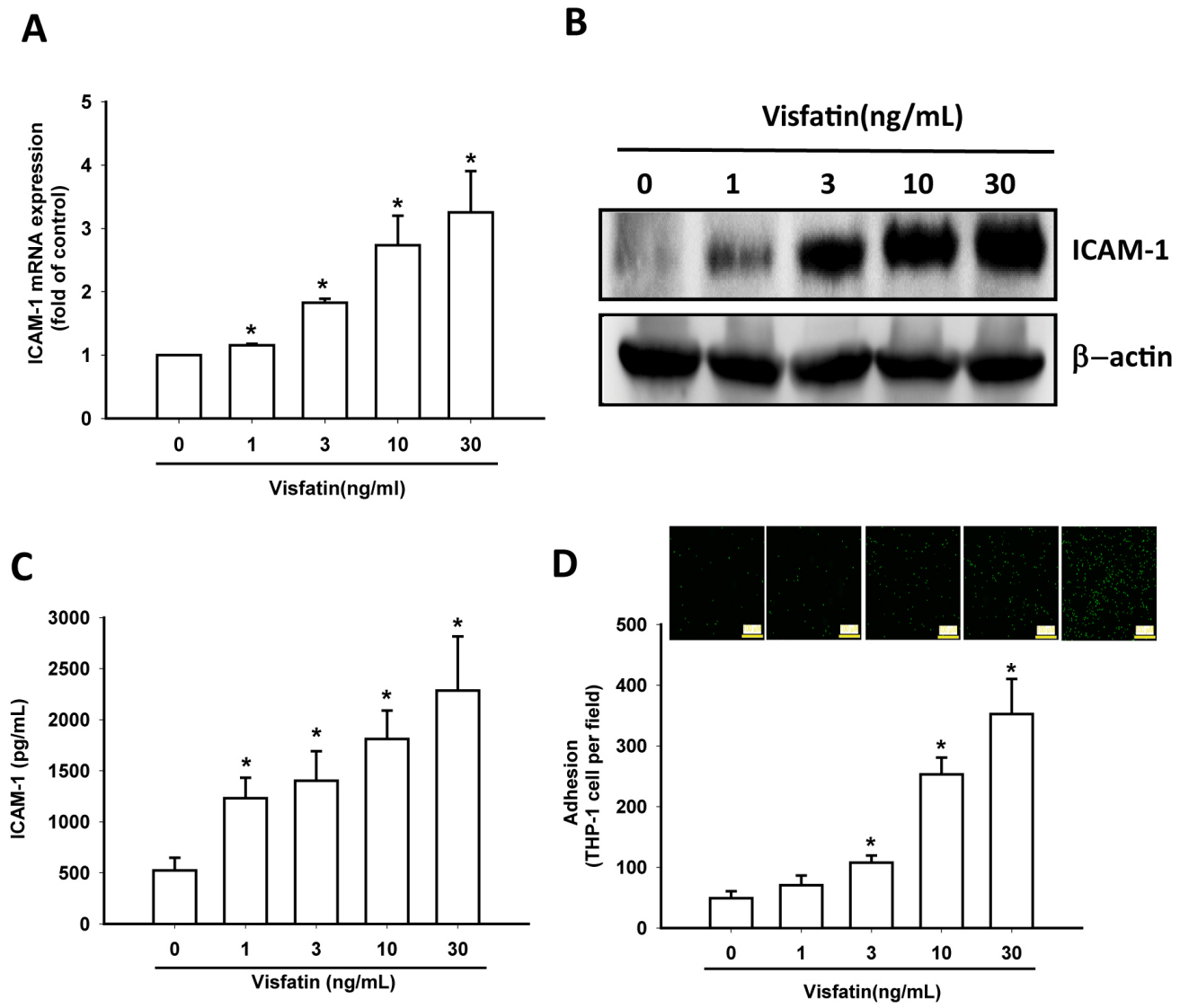

Figure 3. Visfatin stimulates ICAM-1 expression and monocyte adhesion in OASFs. (**A**–**C**) OASFs were incubated with visfatin (1–30 ng/mL) for 24 h, and ICAM-1 expression was examined by RT-qPCR, Western blot and ELISA analysis. (**D**) OASFs were incubated with visfatin (1–30 ng/mL) for 24 h. THP-1 cells loaded with BCECF-AM were added to OASFs for 6 h, then THP-1 cell adherence was measured by fluorescence microscopy. * $p<0.05$ compared with the control group.

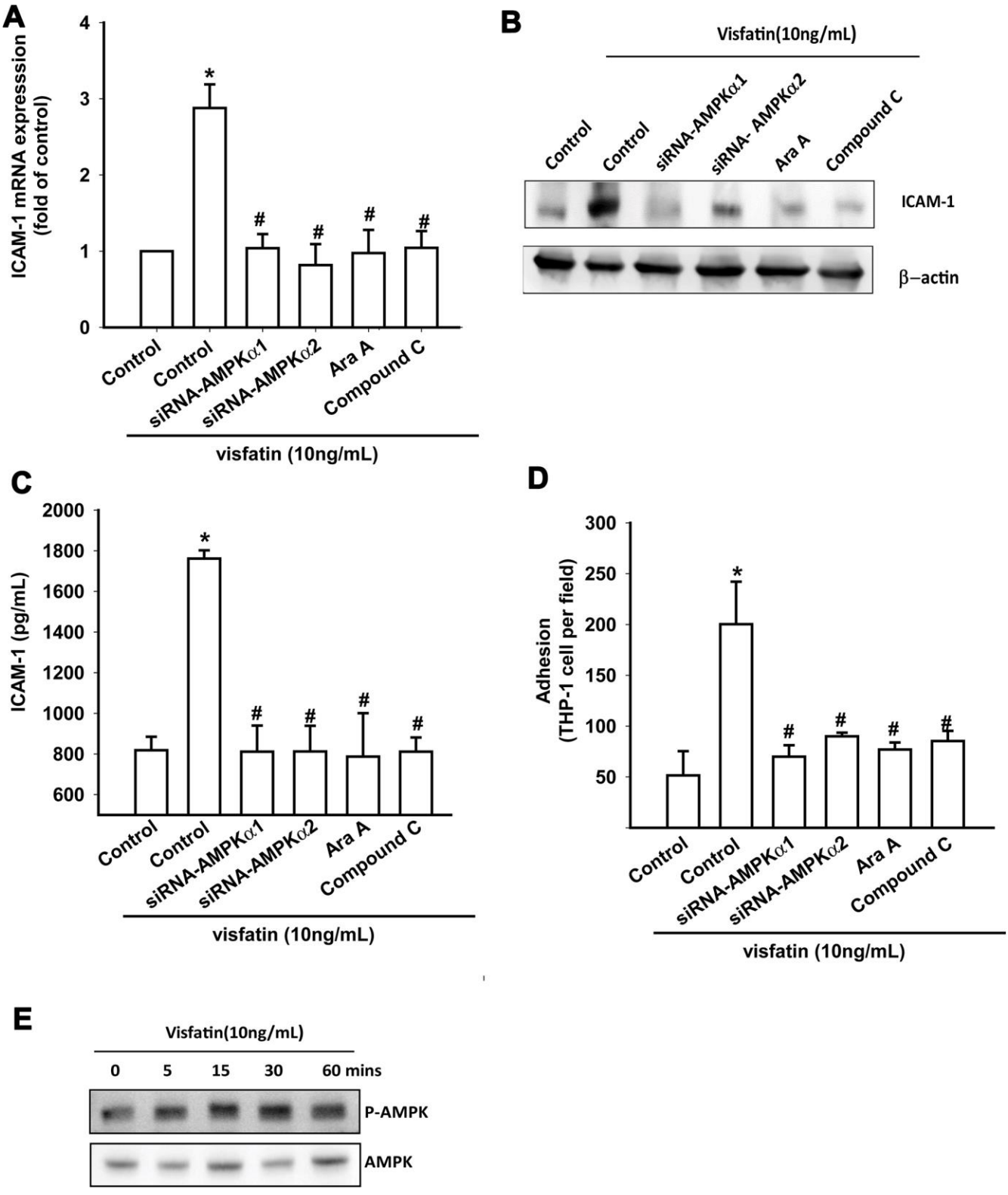

Figure 4. The AMPK pathway is involved in visfatin-induced ICAM-1 synthesis and monocyte adhesion. (**A**–**C**) OASFs were pretreated with AMPK inhibitors (Ara A, compound C) or transfected with AMPK siRNAs, then incubated with visfatin for 24 h. ICAM-1 levels were examined by RT-qPCR, Western blot and ELISA assays. (**D**) OASFs were treated with the same conditions as those described in (**A**). THP-1 cells loaded with BCECF-AM were added to OASFs for 6 h, then THP-1 cell adherence was measured by fluorescence microscopy. (**E**) OASFs were incubated with visfatin for the indicated time intervals, and AMPK phosphorylation was examined by Western blot. * $p<0.05$ compared with the control group; # $p<0.05$ compared with the visfatin-treated group.

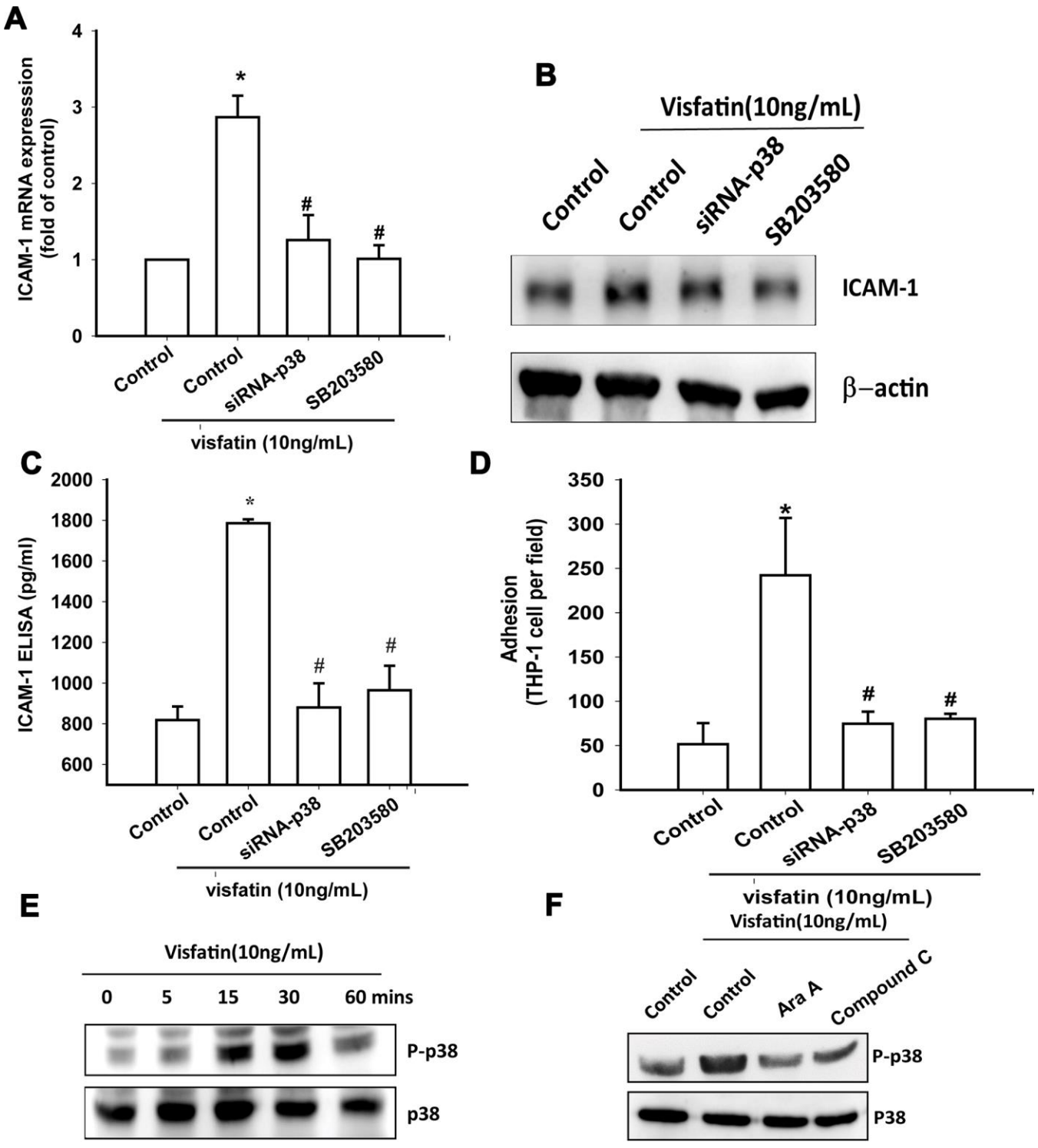

Figure 5. The p38 pathway is involved in visfatin-induced ICAM-1 synthesis and monocyte adhesion. (**A**–**C**) OASFs were pretreated with a p38 inhibitor (SB203580) or transfected with p38 siRNA, then incubated with visfatin for 24 h. ICAM-1 levels were examined by RT-qPCR, Western blot and ELISA assays. (**D**) OASFs were treated with the same conditions as those described in (**A**). THP-1 cells loaded with BCECF-AM were added to OASFs for 6 h, then THP-1 cell adherence was measured by fluorescence microscopy. (**E, F**) OASFs were incubated with visfatin for the indicated time intervals or pretreated with AMPK inhibitors then stimulated with visfatin, and p38 phosphorylation was examined by Western blot. * $p<0.05$ compared with the control group; # $p<0.05$ compared with the visfatin-treated group.

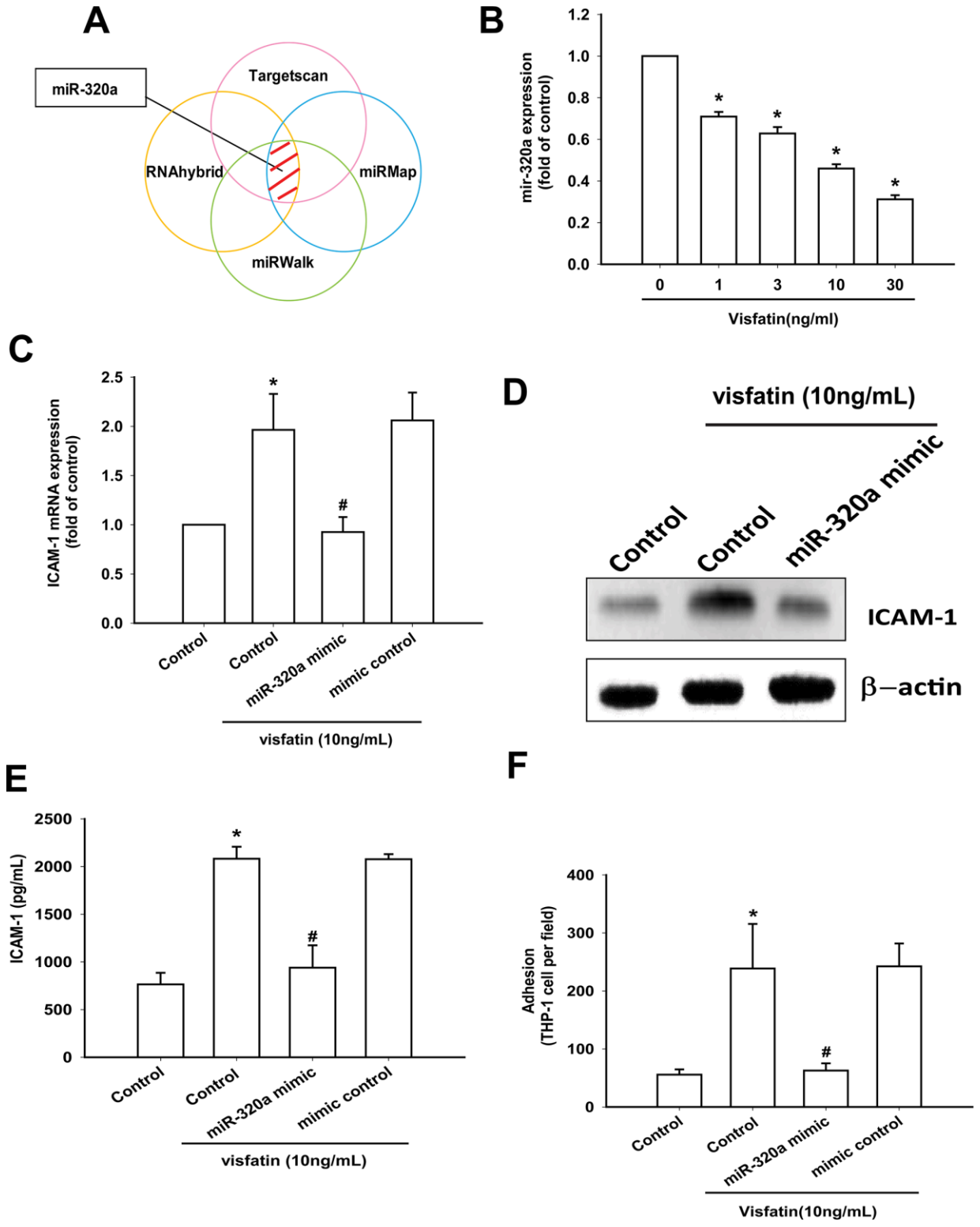

Figure 6. Visfatin promotes ICAM-1 production and monocyte adhesion by suppressing miR-320a. (**A**) Open-source software (TargetScan, miRMap, RNAhybrid, and miRWalk) was used to identify which miRNAs could possibly interfere with ICAM-1 transcription. (**B**) OASFs were incubated with visfatin (1–30 ng/mL). miR-320a expression was examined by RT-qPCR. (**C**–**E**) OASFs were transfected with miR-144-3p mimic and mimic control (serving as the vehicle control) and then stimulated with visfatin. ICAM-1 levels were examined by RT-qPCR, Western blot and ELISA assays. (**F**) OASFs were treated with the same conditions as those described in (**C**). THP-1 cells loaded with BCECF-AM were added to OASFs for 6 h, then THP-1 cell adherence was measured by fluorescence microscopy. * $p<0.05$ compared with the control group; # $p<0.05$ compared with the visfatin-treated group.

pivotal part in its pathogenesis [19], which highlights the importance of synovium-targeted therapy in this disease [6, 20]. Adhesion molecules in the synovial lining assist with monocyte infiltration into inflamed OA synovium [14]. In this study, we found that ICAM-1 acts as a target protein for visfatin and facilitates monocyte adhesion to OASFs. We also found that visfatin enhances ICAM-1 production by inhibiting miR-320a expression via the AMPK and p38 signaling pathways, and facilitates monocyte adhesion to human OASFs.

Previous findings of higher visfatin concentrations in synovial fluid from OA patients compared with healthy synovial fluid [21, 22] were confirmed in this study. Our analysis of records from the GEO database also found higher visfatin levels in inflammatory synovial tissue than in normal synovial tissue. Those records and our study findings revealed positive correlations between visfatin and ICAM-1 concentrations, highlighting the importance of visfatin as a molecular target in OA therapy. Adiponectin and leptin are also key adipokines in OA disease [23]. Adiponectin has been documented to promote inflammatory cytokine release and matrix metalloproteinase production in OASFs [24, 25], while leptin increases inflammatory cytokine production and ADAM expression during OA pathogenesis [26–28]. Clearly, visfatin, adiponectin and leptin play critical roles in OA disease. We have also confirmed novel functioning of visfatin that is similar to activities of the previously identified adipokines in OA pathogenesis.

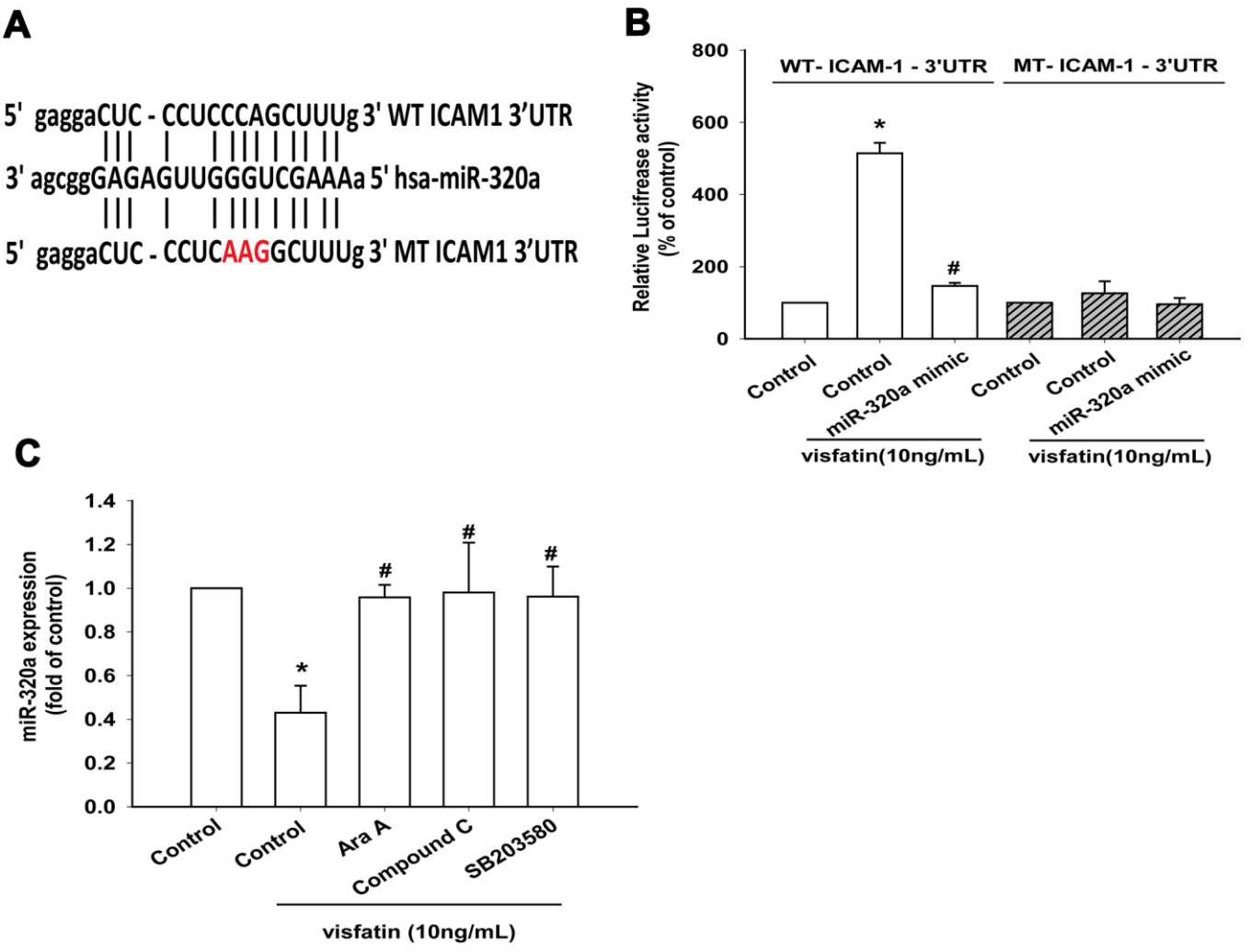

Figure 7. Visfatin suppresses miR-320a synthesis via the AMPK and p38 pathways. (**A**) Schematic 3′-UTR representation of human ICAM-1 containing the miR-320a binding site. (**B**) OASFs were transfected with the indicated luciferase plasmid with or without miR-320a mimic, then stimulated with visfatin. Relative luciferase activity was examined. (**C**) OASFs were pretreated with Ara A, compound C and SB203580 for 30 min, then incubated with visfatin for 24 h. The expression of miR-320a was examined by qPCR. * p<0.05 compared with the control group; # p<0.05 compared with the visfatin-treated group.

Activation of AMPK signaling regulates multiple cellular functions [29], including the expression of adhesion molecules [30, 31]. We have found that visfatin facilitates AMPK phosphorylation, while AMPK inhibitors and siRNAs attenuate visfatin-enhanced ICAM-1 production and monocyte adhesion to OASFs. AMPK-dependent p38 activation is critical for controlling cell adhesion and motility [30, 32]. Some papers have mentioned that p38 activates AMPK and is upstream of AMPK [31]. Thus, our evidence indicates that visfatin facilitates p38 phosphorylation, and that this is reversed by AMPK inhibitors. Our data therefore confirms that AMPK is upstream of p38 and that p38 is required for visfatin-promoted ICAM-1 production and monocyte adhesion.

Regulating miRNA expression should help to lessen OA inflammation [33, 34]. Open-source miRNA software identified that miR-320a potentially interferes with ICAM-1 transcription, which was supported by our findings showing that visfatin reduced miR-320a synthesis, while overexpression of miR-320a mimic mitigated the stimulatory effects of visfatin on ICAM-1 expression and monocyte adhesion. It appears that visfatin facilitates ICAM-1 production and monocyte adhesion by reducing miR-320a expression through AMPK and p38 signaling.

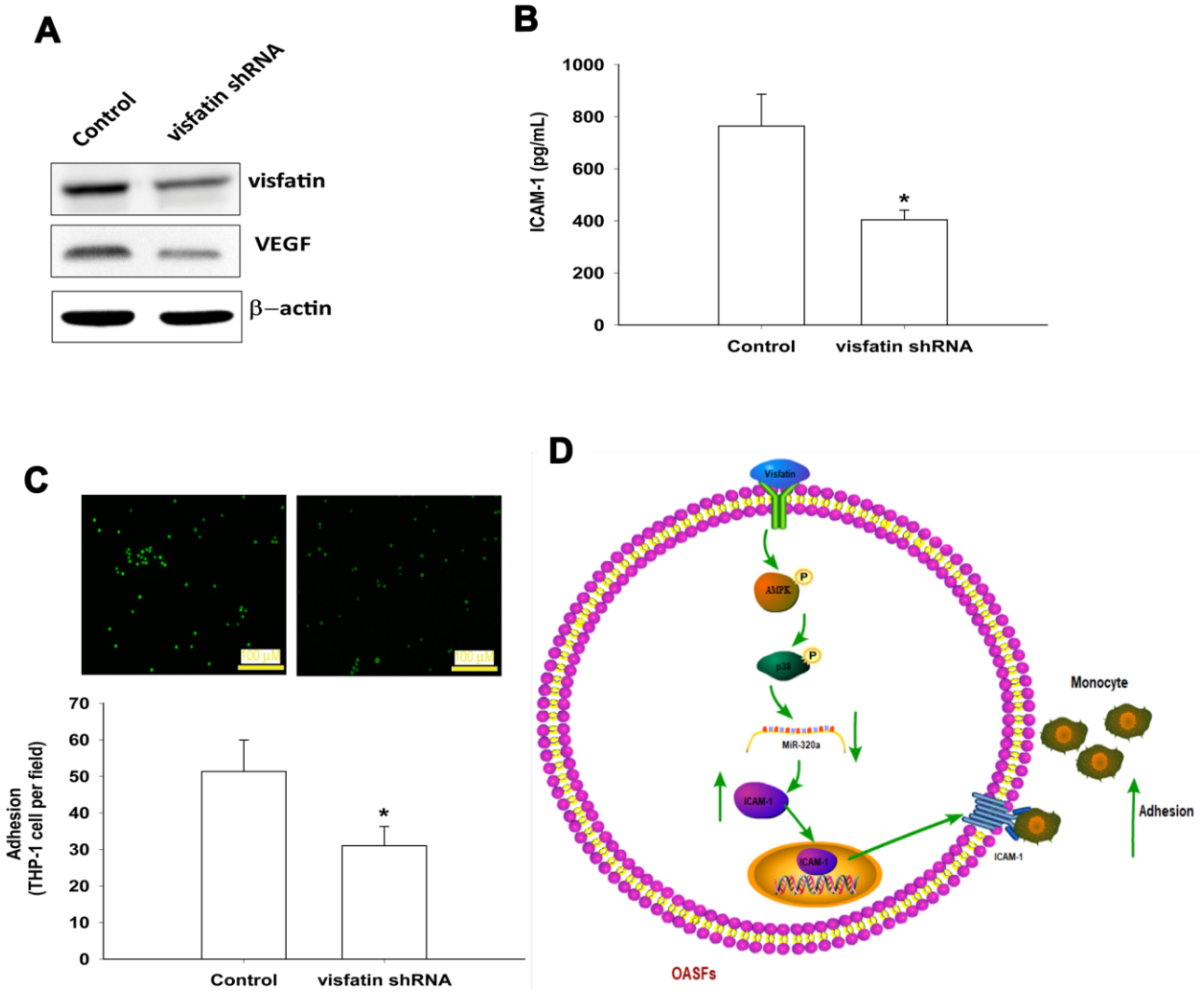

Figure 8. Knockdown visfatin reduces ICAM-1 expression and monocyte adhesion in OASFs. (**A**, **B**) OASFs were transfected with visfatin shRNA. Visfatin and ICAM-1 expression was examined by Western blot and ELISA. (**C**) OASFs were treated with the same conditions as those described in (**A**). THP-1 cells loaded with BCECF-AM were added to OASFs for 6 h, then THP-1 cell adherence was measured by fluorescence microscopy. (**D**) The schematic diagram summarizes the mechanism whereby visfatin promotes ICAM-1 expression and monocyte adhesion in OASFs. Visfatin promotes ICAM-1 expression and enhances monocyte adhesion to OASFs by downregulating miR-320a through the AMPK and p38 signaling pathways. * $p<0.05$ compared with the control group.

A limitation of our research is that demographic details and any other general information of our study participants were not recorded, to maintain patient confidentiality. Thus, we could not compare demographic details with levels of visfatin and ICAM-1 expression.

Previous research has described how 3 months of garlic supplementation in postmenopausal overweight or obese women with knee OA was associated with significant reductions from baseline in resistin levels and also pain scores, which suggests that effectively lowering concentrations of a proinflammatory adipocytokine such as resistin may reduce pain severity [35]. Our study focused on whether changes in visfatin concentrations induce changes in monocyte adhesion to the synovium and consequently the severity of OA disease. We observed that knockdown of visfatin resulted in lower levels of monocyte adhesion, which may attenuate OA disease. For the purposes of this study, we did not seek to determine levels of any other proinflammatory cytokines, such as TNF-α. More research is needed in this area.

In summary, visfatin increased ICAM-1 expression and promoted monocyte adhesion to OASFs by inhibiting miR-320a synthesis through the AMPK and p38 signaling pathways (Figure 8D). Targeting visfatin may improve the pathogenesis of OA.

MATERIALS AND METHODS

Antibodies against ICAM-1 (SC-107), p-AMPK (SC-33524), AMPK (SC-25792), p-p38 (SC-166182), p38 (SC-271120) and β-actin (SC-47778) were all bought from Santa Cruz (Santa Cruz, CA, USA). All ON-TARGET*plus* siRNAs were purchased from Dharmacon (Lafayette, CO, USA). Cell culture supplements were purchased from Invitrogen (Carlsbad, CA, USA). A Dual-Luciferase® Reporter Assay System was bought from Promega (Madison, WI, USA). qPCR primers and probes, as well as the Taqman® one-step PCR Master Mix, were supplied by Applied Biosystems (Foster City, CA, USA). All other chemicals not mentioned above were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Synovial tissue from the suprapatellar pouch of the knee was obtained from patients whose radiographically-detected OA of the knee was classified under the Ahlbäck criteria as stage IV OA [36]. Synovial fibroblasts were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin and 50 μg/mL streptomycin, as previously described [37, 38].

THP-1, a human leukemia cell line of monocyte/macrophage lineage, was obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in RPMI-1640 medium containing 10% FBS.

Clinical samples

Clinical samples were collected from patients meeting the following inclusion criteria: (1) aged over 20 years presenting with an accidental or sports injury requiring joint replacement and repair; or (2) degenerative arthritis. Exclusion criteria specified patients who did not satisfy either of these two categories. Serum (2 mL) and synovial tissue samples (which could be of unlimited sizes) were obtained from patients with OA undergoing knee replacement surgery and also from those undergoing arthroscopy after trauma/joint derangement (who served as healthy controls) at China Medical University Hospital, Taichung, Taiwan. The study protocol was approved by the Institutional Review Board (IRB) of China Medical University Hospital and all methods were performed in accordance with the IRB's guidelines and regulations. Informed written consent was obtained from all patients.

Real-time quantitative PCR analysis of mRNA and miRNA

Total RNA was extracted from OASFs by TRIzol; reverse transcription used 1 μg of total RNA transcribed into cDNA by oligo (dT) primers. RT-qPCR used the Taqman® One-Step RT-PCR Master Mix. All RT-qPCR assays were performed using the StepOnePlus sequence detection system (Applied Biosystems) [39, 40].

Western blot analysis

Cell lysate was separated by SDS-PAGE electrophoresis then transferred to polyvinylidene difluoride (PVDF) membranes, following the method described in our previous work [41, 42]. After blocking the blots with 4% bovine serum albumin, the blots were treated with primary antibody and then secondary antibody. Enhanced chemiluminescent imaging of the blots was visualized with the UVP Biospectrum system (UVP, Upland, CA, USA) [43–45].

ELISA assay

OASFs were cultured in 24-well plates until they reached 90% confluence, when they were then changed to serum-free medium, in which they were treated with visfatin for 24 h with or without the transfection of

siRNAs or inhibitors. The CM was collected and ICAM-1 levels were quantified with the ICAM-1 ELISA kit.

Serum was collected from patients with OA or normal healthy controls. Visfatin and ICAM-1 levels were quantified by the Visfatin ELISA kit (EIA-VIS-1; RayBiotech, Peachtree corners, GA, USA; detection ranges 100–1,000,000 pg/mL) and the ICAM-1 ELISA kit (DY720; R&D Systems, Minneapolis, MN, USA).

Analysis of the GEO database

Data on visfatin and ICAM-1 mRNA expression for normal healthy controls and OA patients were retrieved from the GEO dataset records [46].

Luciferase assays

Wild-type and mutant ICAM-1 3'-UTR plasmids were purchased from Invitrogen (Carlsbad, CA, USA). Luciferase activity was assayed using the method described in our previous publications [2, 37, 47].

Cell adhesion assay

THP-1 cells were loaded with BCECF-AM (10 μM) for 1 h at 37°C in RPMI-1640 medium and subsequently washed by centrifugation. OASFs grown on glass coverslips were incubated with visfatin then incubated with THP-1 cells at 37°C for 1 h. Nonadherent THP-1 cells were removed and gently washed with PBS. The number of adherent THP-1 cells was counted using a fluorescent microscope.

Statistics

All values are given as the mean ± standard error of the mean ± S.D. All ELISA procedures were repeated three times. The Student's *t*-test assessed between-group differences. A p value of <0.05 was considered to be statistically significant.

AUTHOR CONTRIBUTIONS

C.K. Wang and C.H. Tang initiated the research project. Y.Y. Law, S.C. Liu and W.H. Chung performed research. Y.M. Lin, M.H. Wu, C.H. Tsai and Y.C. Fong provided the material. C.K. Wang and C.H. Tang wrote the paper.

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CONFLICTS OF INTEREST

None of the authors has any financial or personal relationships with other people or organizations that could inappropriately influence this work.

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