

Original Paper

Resistin Enhances Monocyte Chemoattractant Protein-1 Production in Human Synovial Fibroblasts and Facilitates Monocyte Migration

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Key Words

Osteoarthritis • Resistin • MCP-1 • Monocyte migration

Abstract

Background/Aims: The adipocyte-secreting adipokine, resistin, may play a critical role in the modulation of inflammatory diseases. Migration and infiltration of mononuclear cells into inflammatory sites are critical events during the development of osteoarthritis (OA). Monocyte chemoattractant protein-1 (MCP-1), also known as chemokine ligand 2 (CCL2),

plays a critical role in the regulation of monocyte migration and infiltration. In this study, we show how resistin promotes MCP-1 expression in OA synovial fibroblasts and monocyte migration. **Methods:** We used qPCR to detect MCP-1 and miRNA expression. THP-1 migration was investigated by Transwell assay. The Western blotting was used to examine the resistin-mediated signaling pathways. **Results:** Resistin activated the phosphatidylinositol-3-kinase (PI3K), Akt and mammalian target of rapamycin (mTOR) signaling pathways, while PI3K, Akt and mTOR inhibitors or small interfering RNAs diminished resistin-induced MCP-1 expression and monocyte migration. We also demonstrate that resistin stimulates MCP-1-mediated monocyte migration by suppressing microRNA (miR)-33a and miR-33b via the PI3K, Akt and mTOR signaling pathways. **Conclusion:** These results provide new insights into the mechanisms of resistin action that may have therapeutic implications for patients with OA.

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Introduction

Osteoarthritis (OA) is the most common form of arthritis and the single most important determinant of disability in older adults [1]. No specific cause has been identified for OA, although aging, gender, obesity, genetic factors and injury all increase the risk of OA [2, 3]. Inflammation, even in the early stage of the disease, is believed to be involved in OA progression and development [4]. Synovial membrane inflammation is also implicated in the disease pathophysiology. Biochemical mediators such as interleukins, cytokines, chemokines and growth factors in OA synovial fibroblasts (OASFs) affect knee joint cellular functioning [5]. In particular, cytokines and chemokines enhance inflammation, neovascularization and cartilage degradation, which facilitates OA progression [6].

Increasing evidence indicates migration and infiltration of mononuclear cells to inflammatory sites are regulated by adhesion molecules, such as monocyte chemoattractant protein-1 (MCP-1), also known as the chemokine (C-C motif) ligand 2 (CCL2) [7, 8]. MCP-1 initiates chemotaxis and transendothelial migration of monocytes to inflammatory lesions [9]. In various inflammatory conditions such as arthritis [10], multiple sclerosis, atherosclerosis [11] and cancer [12], MCP-1 attracts monocytes and lymphocytes to the site of inflammation. OA and rheumatoid arthritis (RA) are characterized by high levels of MCP-1 expression in the blood, synovial fluid and synovial tissue [13-15]. In preclinical investigations, increased production of MCP-1 promoted the development of synovitis [16], and macrophage infiltration was markedly increased after MCP-1 injections into rabbit joints [14]. MCP-1 is thought to act as a proinflammatory agent and increase monocyte expression in synovial fluid and tissue during the OA disease process [17-19].

Resistin is a 12.5-kDa cysteine-rich adipokine that is constitutively secreted by adipose tissue [20]; resistin levels in plasma correlate with inflammatory markers and coronary artery calcification, a measure of coronary atherosclerosis [21]. Moreover, resistin promotes VEGF expression and angiogenesis in EPCs in the pathogenesis of RA [22] and resistin single nucleotide polymorphisms have been linked to RA susceptibility and clinicopathological characteristics [23]. Elevated resistin may be a predictor of OA: patients with OA have high levels of resistin levels in serum and synovial fluid [24] and resistin levels might correlate with markers of inflammation.

Resistin promotes an inflammatory response in OA and RA [7, 25]. However, although it is recognized that resistin is involved in OA pathogenesis, the role of resistin in MCP-1 expression and monocyte accumulation is unclear. Our study shows that resistin upregulates MCP-1 expression in OASFs and promotes monocyte migration. We also show that resistin-induced downregulation of miR-33a/miR-33b involves the PI3K, Akt and mammalian target of rapamycin (mTOR) signaling pathways. These results provide new therapeutic insights into the mechanisms of resistin in OA.

Materials and Methods

Materials

Recombinant human resistin was purchased from R&D Systems (Minneapolis, MN, USA). We purchased p85, Akt and mTOR primary antibodies (Santa Cruz Biotechnology, CA, USA), as well as rabbit polyclonal antibodies specific for p-p85, p-Akt and p-mTOR (Cell Signaling Technology, Danvers, MA, USA). miR-33a and miR-33b mimics, miRNA control, Lipofectamine 2000, and Trizol were purchased from Life Technologies (Carlsbad, CA, USA). Dharmacon Research (Lafayette, CO, USA) supplied ON-TARGETplus™ siRNAs. Gibco-BRL life technologies (Grand Island, NY, USA) supplied fetal bovine serum (FBS), RPMI-1640, α -MEM, and all other cell culture reagents. All other chemicals or inhibitors were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Human synovial fibroblasts were isolated by collagenase treatment of synovial tissue samples obtained from 6 patients with OA undergoing knee replacement surgery in China Medical University Hospital. All study participants gave written consent before enrollment. The study protocol was approved by China Medical University Hospital's Institutional Review Board. OASFs were isolated, cultured, and characterized as previously described [26, 27]. Experiments were performed using cells from passages 3 to 6.

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 with 10% FBS.

Quantitative real-time PCR (qPCR) of mRNA and miRNA

Total RNA was extracted from OASFs cells using TRIzol reagent. qPCR analysis was carried out according to an established protocol [28, 29].

Western blot analysis

Cell lysates underwent electrophoresis with SDS-PAGE and were transferred to PVDF membranes according to the method described in our previous studies [30, 31]. After blocking the blots with 4% bovine serum albumin, we treated them with primary antibody and then peroxidase-conjugated secondary antibody. The blots were visualized using the chemiluminescence UVP BioSpectrum system (UVP, Upland, CA, USA).

In vitro chemotaxis assay

Conditioned medium obtained from resistin-treated OASFs were placed in the lower compartment of the Transwell assay (Costa Corning, Cambridge, MA, USA). THP-1 cells (5×10^4 cells) were added to the upper chamber and cell migratory ability was assayed using the method described in our previous work [8, 32].

Statistical Analysis

Data are presented as mean \pm standard error of the mean (SEM). The Student's *t*-test determined statistical differences between samples and the Bonferroni *post hoc* procedure was performed for a one-way analysis of variance (ANOVA) of statistical comparisons between more than two samples; *p*-values of less than 0.05 were considered to be statistically significant.

Results

Resistin promotes MCP-1 production in human synovial fibroblasts and facilitates monocyte migration

MCP-1 is indicated to be a critical chemokine that regulates monocyte migration and infiltration [9]. In this study, directly applying resistin to OASFs promoted mRNA expression of MCP-1 in a concentration-dependent manner (Fig. 1A). We next investigated whether resistin-induced MCP-1 expression facilitates monocyte migration. An *in vitro* chemotaxis assay revealed that conditioned medium from resistin-treated OASFs increased monocyte

migration, while incubation with MCP-1 neutralizing antibody abolished resistin-promoted monocyte migration (Fig. 1B). These results suggest that resistin induces MCP-1 expression in synovial fibroblasts and thereby promotes monocyte migration.

The PI3K/Akt signaling pathway plays a role in resistin-induced MCP-1 expression and monocyte migration

The PI3K/Akt signaling pathway is frequently implicated in inflammatory responses [33]. We found that pretreating OASFs with PI3K inhibitors (Ly294002, wortmannin) or an Akt inhibitor abolished resistin-enhanced MCP-1 expression (Fig. 2A&3A). These inhibitors also diminished resistin-induced migration of monocytes (Fig. 2C&3C). PI3K and Akt siRNA showed similar effects (Fig. 2B&D and 3B&D). The PI3K-dependent signaling pathway activates Akt residue phosphorylation [34]. We identified a significant, time-dependent induction of p85 and Akt phosphorylation in response to resistin treatment (Fig. 2E&3E). Pretreating the cells with a PI3K inhibitor blocked resistin-induced Akt phosphorylation (Fig. 3F). It appears that resistin acts via the PI3K/Akt-dependent signaling pathway to increase MCP-1 expression and promote monocyte migration.

Resistin increases MCP-1 production and facilitates monocyte migration via the mTOR pathway

mTOR activation is a common downstream event of the PI3/Akt signaling pathway [35]. In our investigation, resistin-induced MCP-1 mRNA expression and monocyte migration was reduced by an mTOR inhibitor and an siRNA (Fig. 4A-D). In addition, resistin increased mTOR phosphorylation in OASFs (Fig. 4E), which was antagonized when cells were incubated with PI3K or Akt inhibitors (Fig. 4F). Thus, the PI3K/Akt/mTOR signaling pathway mediates the effects of resistin upon MCP-1 expression and monocyte migration.

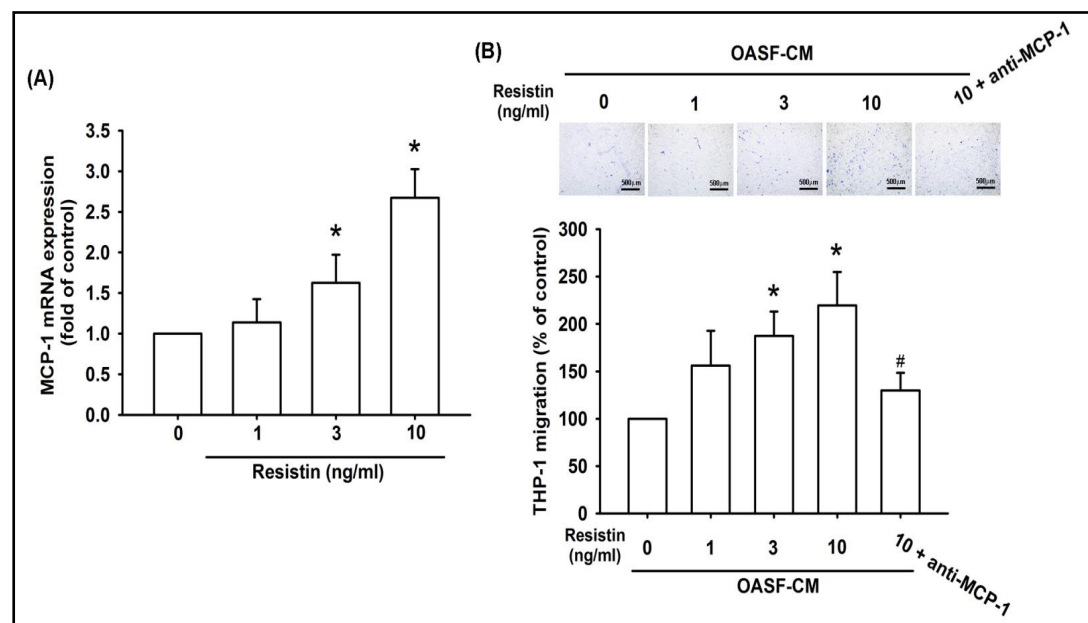


Fig. 1. Resistin promotes MCP-1 expression in OASFs and facilitates monocyte migration. (A) OASFs were incubated with resistin (1–10 ng/ml) for 24 h; MCP-1 mRNA expression was examined by qPCR. (B) OASFs were incubated with resistin for 24 h then stimulated with MCP-1 antibody (1 µg/ml) for 30 min. The conditioned medium (CM) was then collected and applied to THP-1 cells. THP-1 migration was measured. Results are expressed as the mean ± SEM. * $p < 0.05$ as compared with the control group; # $p < 0.05$ as compared with the resistin-treated group.

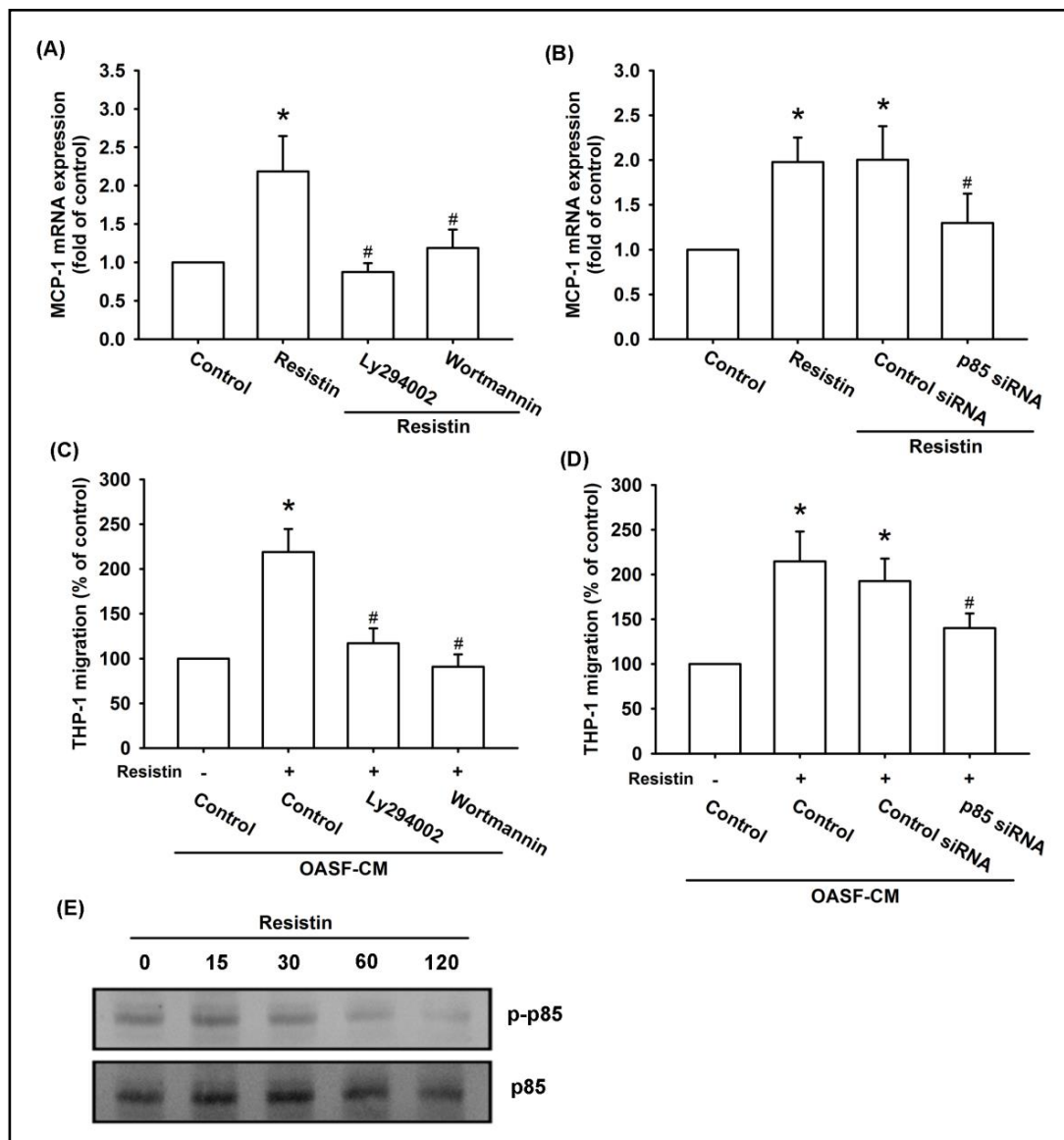


Fig. 2. The PI3K pathway is involved in resistin-induced MCP-1 expression and monocyte migration. (A&B) OASFs were pretreated for 30 min with Ly294002 (10 μ M) and wortmannin (5 μ M) or transfected with p85 siRNA, then stimulated with resistin. MCP-1 expression was examined by qPCR. (C&D) CM was applied to THP-1 cells and analyzed for migration activity. (E) OASFs were incubated with resistin and p-p85 expression was examined by Western blot. Results are expressed as the mean \pm SEM. * p <0.05 as compared with the control group; # p <0.05 as compared with the resistin-treated group.

Resistin facilitates MCP-1-related monocyte migration by suppressing miR-33a/miR-33b

Accumulating evidence suggests that miRNAs are crucial regulators of cell motility [33, 36]. Using open-source software, we found that the 3'UTR region of MCP-1 mRNA harbors potential binding sites for 5 candidate miRNAs, and that miR-33a (GTGCATTGTAGTTGCATTGCA) and miR-33b (GTGCATTGCTGTTGCATTGC) are markedly downregulated after resistin treatment (Fig. 5A). Exogenous resistin concentration-dependently inhibited miR-33a and miR-33b expression (Fig. 5B). Transfection of cells with miR-33a and miR-33b mimics diminished resistin-enhanced MCP-1 expression (Fig. 5C) and also inhibited resistin-boosted monocyte migration (Fig. 5D).

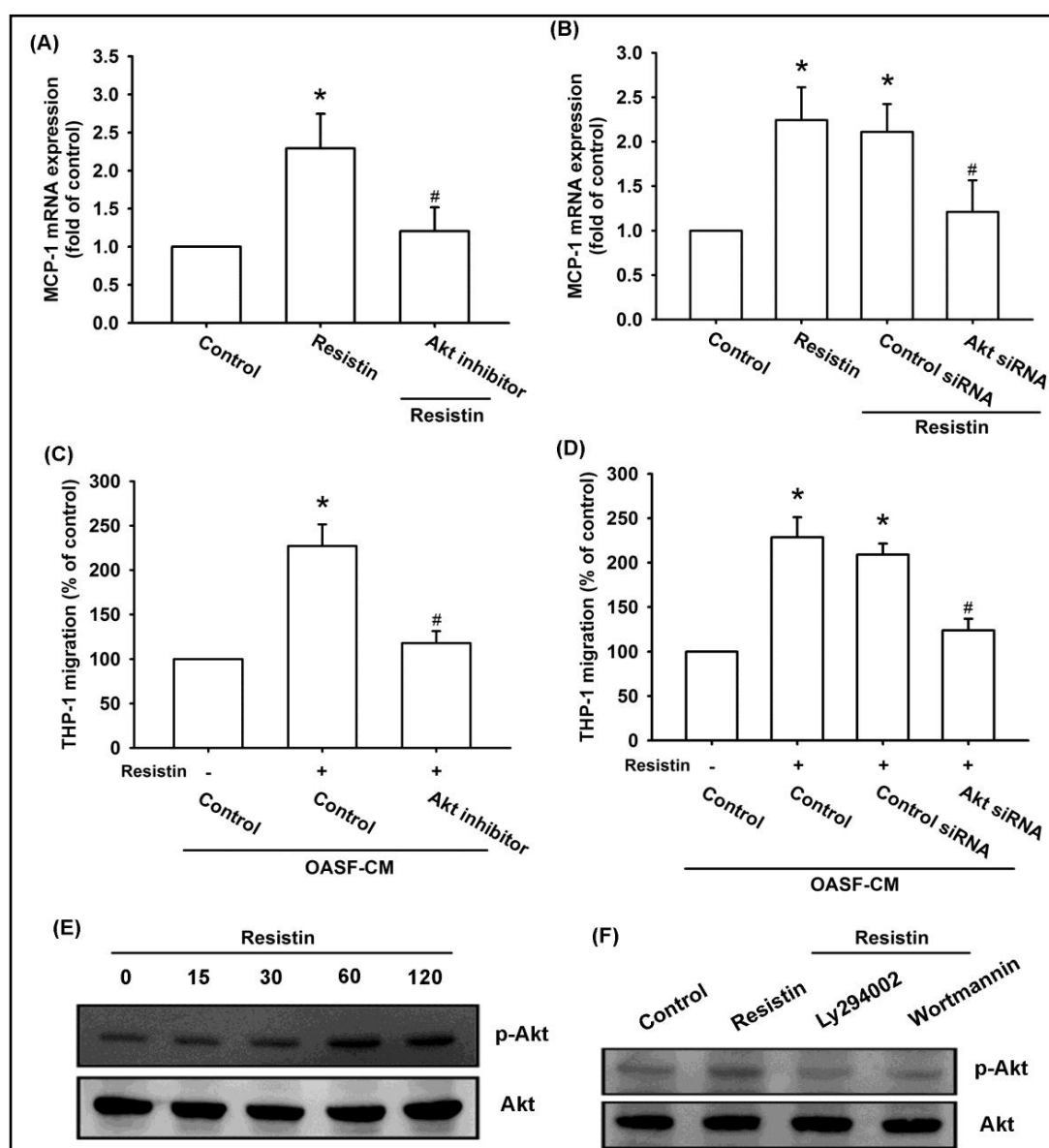


Fig. 3. Akt is involved in resistin-induced MCP-1 expression and monocyte migration. (A&B) OASFs were pretreated for 30 min with an Akt inhibitor (10 μ M) or transfected with the Akt siRNA then stimulated with resistin. MCP-1 expression was examined by qPCR. (C&D) CM was applied to THP-1 cells and analyzed for migration activity. (E&F) OASFs were incubated with resistin or pretreated with PI3K inhibitors then stimulated with resistin and p-Akt expression was examined by Western blot. Results are expressed as the mean \pm SEM. * p <0.05 as compared with the control group; # p <0.05 as compared with the resistin-treated group.

Next, we examined the relationship between the PI3K/Akt/mTOR pathway and miR-33a/miR-33b. Resistin-induced reductions in miR-33a and miR-33b expression were reversed when the cells were treated with PI3K, Akt and mTOR inhibitors (Fig. 6A&C&E) and also by PI3K, Akt and mTOR siRNAs (Fig. 6B&D&F), indicating that resistin induces MCP-1 expression and monocyte migration by inhibiting miR-33a/miR-33b via the PI3K, Akt and mTOR pathways.

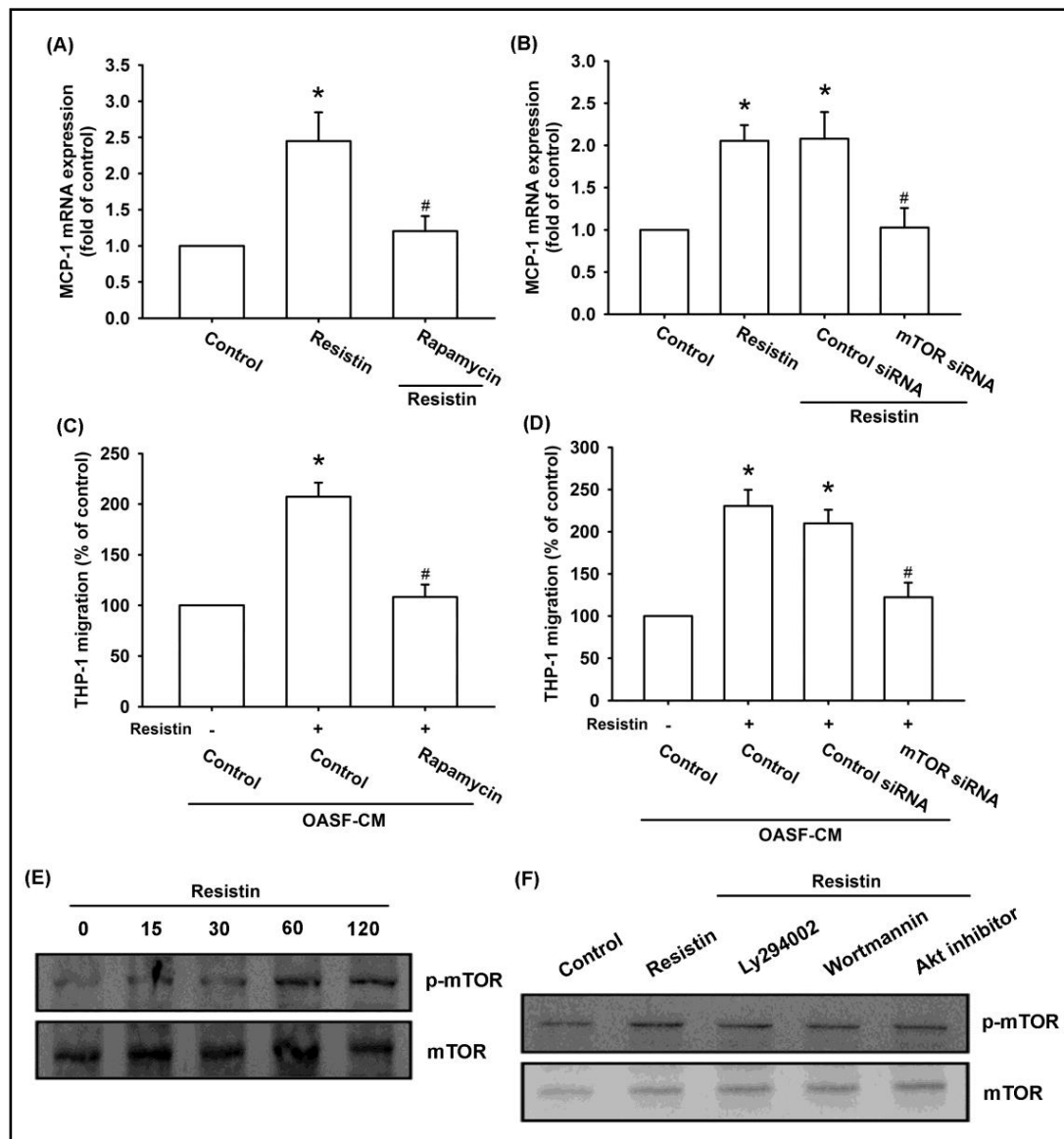


Fig. 4. mTOR is involved in resistin-induced MCP-1 expression and monocyte migration. (A&B) OASFs were pretreated for 30 min with rapamycin (10 μ M) or transfected with the mTOR siRNA then stimulated with resistin. MCP-1 expression was examined by qPCR. (C&D) CM was applied to THP-1 cells and analyzed for migration activity. (E&F) OASFs were incubated with resistin or pretreated with PI3K and Akt inhibitors then stimulated with resistin and p-mTOR expression was examined by Western blot. Results are expressed as the mean \pm SEM. * p <0.05 as compared with the control group; # p <0.05 as compared with the resistin-treated group.

Discussion

OA pathogenesis is little understood. It is well accepted, however, that effective immune surveillance involves the monitoring of leukocyte infiltration to sites of inflammatory stimuli. MCP-1 is the main regulator of monocyte infiltration into inflammatory sites during OA pathogenesis. Resistin is an adipokine that is associated with obesity, inflammation, and various cancers [22, 25, 37]. High levels of resistin have been found in serum and joint fluids of OA and RA patients [24, 25]. We hypothesized that resistin would influence monocyte

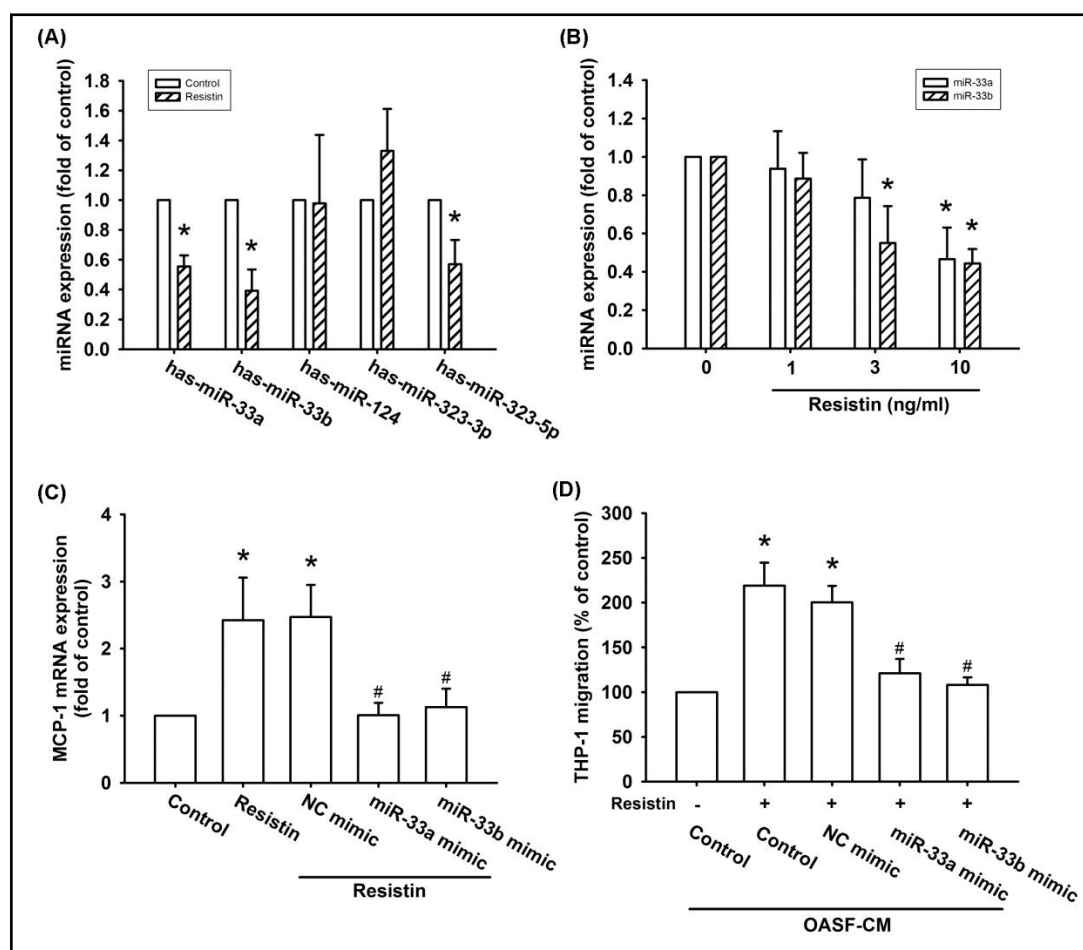


Fig. 5. Resistin promotes MCP-1-mediated monocyte migration by inhibiting miR-33a and miR-33b. (A&B) OASFs were incubated with resistin for 24 h and miRNA expression was examined by qPCR. (C) OASFs were transfected with the miR-33a and miR-33b mimics then incubated with resistin for 24 h; MCP-1 expression was measured by qPCR. (D) CM was applied to THP-1 cells and analyzed for migration activity. Results are expressed as the mean \pm SEM. * $p < 0.05$ as compared with the control group; # $p < 0.05$ as compared with the resistin-treated group.

infiltration during OA pathogenesis. In this study, we provide evidence showing that resistin induces MCP-1 production in human OASFs and contributes to monocyte migration by suppressing miR-33a/miR-33b expression through the PI3K/Akt/mTOR signaling pathway (Fig. 7). This is the first indication that adipokine resistin boosts MCP-1-associated monocytes migration via the downregulation of miR-33a/miR-33b. Similar effects have been reported with other adipokines, such as adiponectin, which increases IL-6 and ICAM-1 expression during the development of arthritis [38, 39], while leptin enhances IL-8 and oncostatin M production in arthritis [40, 41]. In addition, the adipocyte hormone visfatin upregulates levels of IL-6 and TNF- α expression in arthritis disease [27]. These findings suggest that adipokines play a pro-inflammatory role in arthritis.

Activation of the PI3K/Akt signaling pathway is a critical event in inflammatory responses and represents a potential therapeutic target against inflammatory disorders. This pathway mediates multiple cellular functions, including cell survival, proliferation, migration, and autophagy [42]. In this study, we demonstrated that PI3K and Akt inhibitors are capable of inhibiting resistin-induced MCP-1 expression and monocyte migration. Furthermore, we observed that p85 and Akt siRNAs reduced MCP-1 expression in OASFs as well as monocyte migration. Incubation of cells with resistin increased PI3K and Akt phosphorylation. The

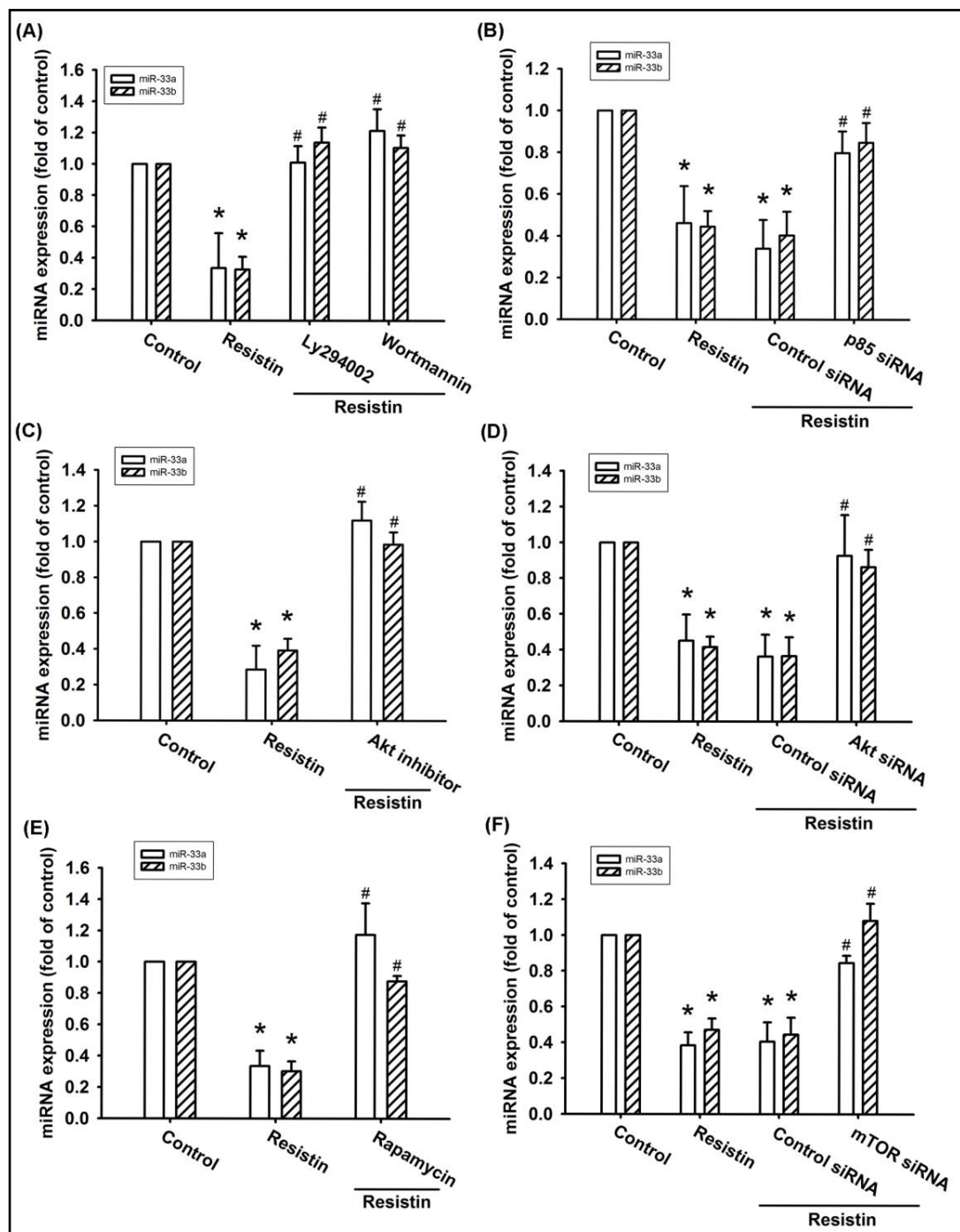


Fig. 6. Resistin inhibits miR-33a and miR-33b expression through the PI3K, Akt and mTOR pathways. OASFs were pretreated for 30 min with PI3K, Akt and mTOR inhibitors or siRNAs, then stimulated with resistin. miRNA expression was examined by qPCR. Results are expressed as the mean \pm SEM. * $p < 0.05$ as compared with the control group; # $p < 0.05$ as compared with the resistin-treated group.

resistin-induced increase in Akt phosphorylation was suppressed when cells were pretreated with a PI3K inhibitor. This indicates that the PI3K/Akt signaling pathway is involved in resistin-mediated MCP-1 expression in OASFs and monocyte migration.

Increasing reports have shown that mTOR is a major downstream signaling molecule of the PI3K/Akt pathway [43, 44]. We therefore examined the role of mTOR in resistin-

mediated MCP-1 production and monocyte migration. We found that an mTOR inhibitor (rapamycin) reduced resistin-promoted MCP-1 expression in OASFs. Furthermore, resistin-enhanced MCP-1 production and monocyte migration were diminished by treatment with an mTOR inhibitor and an siRNA. We also observed that mTOR phosphorylation was increased after treating OASFs with resistin. Stimulation of cells with PI3K or Akt inhibitors reduced resistin-promoted phosphorylation of mTOR, suggesting that resistin promotes MCP-1 production in OASFs and subsequently promotes monocyte migration through the PI3K/Akt/mTOR signaling cascades.

miRNAs inhibit and regulate gene expression [45]. In particular, they help to regulate various inflammatory diseases, including OA and RA [46]. We therefore sought to examine whether miRNAs are implicated in MCP-1 expression following resistin treatment. Our results demonstrate that resistin significantly reduces miR-33a and miR-33b expression in OASFs, while transfecting OASFs with miR-33a and miR-33b mimics antagonizes resistin-enhanced MCP-1 expression and monocyte migration. Strikingly, treatment with PI3K, Akt and mTOR inhibitors or an siRNA reversed resistin-inhibited miR-33a and miR-33b expression, indicating that resistin promotes MCP-1-mediated monocyte migration by suppressing miR-33a and miR-33b expression through the PI3K, Akt and mTOR signaling cascades.

Conclusion

This study has identified that resistin increases MCP-1 production in OASFs by suppressing miR-33a/miR-33b expression through the PI3K/Akt/mTOR signaling cascades, facilitating monocyte migration. Resistin may be a novel therapeutic target in OA.

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Disclosure Statement

The authors have no financial or personal relationships that could inappropriately influence this research.

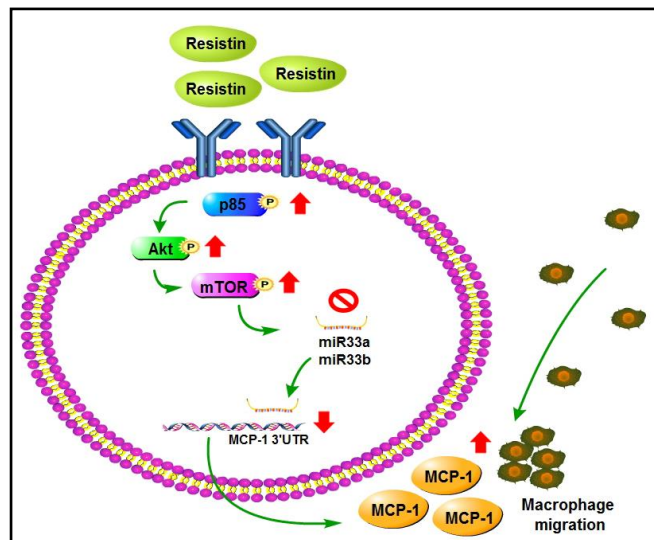


Fig. 7. Schematic diagram summarizes the mechanism of resistin-promoted MCP-1 expression and boosted monocyte migration. Resistin induces MCP-1 production in OASFs by downregulating miR-33a/miR-33b through the PI3K/Akt/mTOR pathway and subsequently facilitates monocyte migration.

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