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TNF-α promotes human retinal pigment epithelial (RPE) cell migration by inducing matrix metallopeptidase 9 (MMP-9) expression through activation of Akt/mTORC1 signaling

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ABSTRACT

Tumor necrosis factor-alpha (TNF-α) promotes *in vitro* retinal pigment epithelial (RPE) cell migration to initiate proliferative vitreoretinopathy (PVR). Here we report that TNF-α promotes human RPE cell migration by inducing matrix metallopeptidase 9 (MMP-9) expression. Inhibition of MMP-9 by its inhibitor or its neutralizing antibody inhibited TNF-α-induced *in vitro* RPE cell migration. Reversely, exogenously-added active MMP-9 promoted RPE cell migration. Suppression Akt/mTOR complex 1(mTORC1) activation by LY 294002 and rapamycin inhibited TNF-α-mediated MMP-9 expression. To introduce a constitutively active Akt (CA-Akt) in cultured RPE cells increased MMP-9 expression, and to block mTORC1 activation by rapamycin inhibited its effect. RNA interference (RNAi)-mediated silencing of SIN1, a key component of mTOR complex 2 (mTORC2), had no effect on MMP-9 expression or secretion. In conclusion, this study suggest that TNF-α promotes RPE cell migration by inducing MMP-9 expression through activation of Akt/ mTORC1, but not mTORC2 signaling.

1. Introduction

Although dramatic improvements have been made in vitreoretinal surgery in the past a few years, proliferative vitreoretinopathy (PVR) is still the common cause of severe visual loss and blindness [1–4]. Surgical treatment is the "gold standard" for PVR, however, it is often accompanied with a wide range of severe complications and high recurrence rate [5,6]. Blindness is caused by rhegmatogenous retinal detachment (RRD) due to the formation of contractile retinal fibrous membranes. As the initial step of fibrous membranes formation, tumor necrosis factor-alpha (TNF-α) and other cytokines leak into the sub-retinal space, induce resident retinal pigment epithelial (RPE) cells to migrate [7,8]. However, the cellular mechanisms involved in the migration process of TNF-α-activated RPE cells is not studied extensively, and how this is regulated would provide potential therapeutic targets against PVR.

The progression of PVR is characterized by the ability of resident RPE cells to produce matrix-degrading enzymes, giving them an advantage in disseminating from the monolayer, and to migrate through a provisional extracellular matrix (ECM) within the subretinal space. These migrated RPE cells eventually form pathologic membranes on both surfaces of the neural retina [9–12]. Matrix metallopeptidase 9 (MMP-9), a 92 kDa type IV collagenase, degrades type IV collagen, the major structural component of the basement membrane and extracellular matrix. Previous studies have suggested that MMP-9 is important to regulate cell migration by TNF-α [13–16], we here first tested TNF-α's effect on MMP-9 expression in RPE cells.

Many signaling transduction pathways are involved in the regulation of MMP-9 expression and production in human cells. Growth factors, nutrients, stresses and cytokines [17] all activate the phosphoinositide 3-kinase (PI3 K)/Akt/mTOR signaling pathway, which mediates cell growth, proliferation, survival, protein synthesis, transcription and migration [18–20]. There are two functionally distinct mTOR complexes, mTORC1 complex 1 (mTORC1) and mTORC1 complex 2 (mTORC2). It is now become clear that mTORC1 (rapamycin sensitive) is composed of mTOR, Raptor, mLST8, and PRAS40. Akt indirectly activates mTORC1 by phosphorylating and inactivating of tuberous sclerosis protein 2 (TSC2), an inhibitor of mTORC1. Activation of mTORC1 then activates S6K1 (p70 ribosomal protein S6 kinase) and inactivates 4E-BP1 (eIF4E binding protein 1) [18–20]. On the other hand, mTORC2, a complex that is composed

Abbreviations: TNF-α, tumor necrosis factor-alpha; MMP-9, matrix metallopeptidase 9; PVR, proliferative vitreoretinopathy; RRD, rhegmatogenous retinal detachment; RPE, retinal pigment epithelium; ECM, extracellular matrix; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; MAPK, mitogen-activated protein kinase; RNAi, RNA interference.

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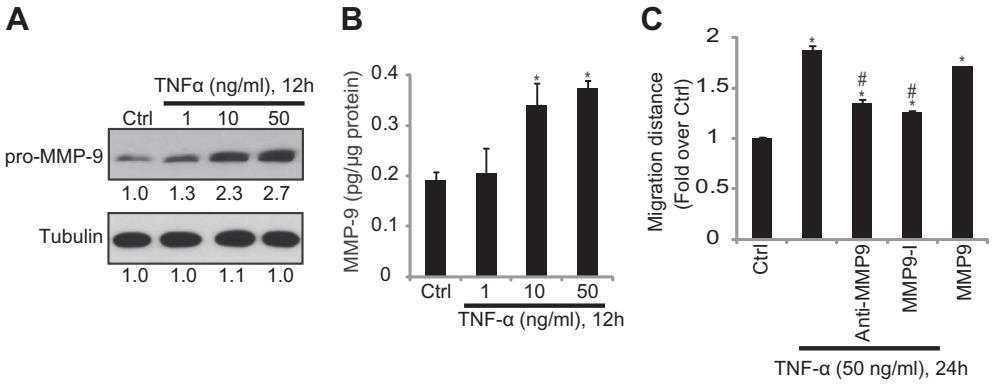

Fig. 1. TNF-α promotes MMP-9 expression and secretion in cultured human RPE cells. (A) Western-blots of RPE cell lysates after indicated TNF-α treatment confirmed pro-MMP-9 up-regulation. MMP-9 and tubulin (loading control) levels were quantified by Image J software, and were expressed as fold change vs. untreated control group. (B) The concentration of active MMP-9 in the cultured media of TNF-α-treated RPE cells was determined by ELISA and is expressed as picogram secreted protein per microgram cellular protein (pg/μg). (C) Average migration distance of RPE cells after 24 h of indicated treatments: untreated control (Ctrl), TNF-α (50 ng/ml), TNF-α (50 ng/ml) plus MMP-9 neutralizing antibody (2 μg/ml), TNF-α (50 ng/ml) plus MMP-9 inhibitor I (50 μM), active MMP-9 (10 ng/mL). Average migration distance of RPE cells was measured by "The Phagokinetic Track Motility Assay" as described, and was expressed as fold change vs. untreated control. The values in the figures are expressed as the means ± standard deviation (SD). $^{*}P < 0.05$ vs. Ctrl, $^{\#}P < 0.05$ vs. TNF-α only group (ANOVA). Experiments were repeated three times to insure consistency of results.

of mTOR, Rictor, SIN1, and mLST8, is required for Akt phosphorylation at Ser 473 [21,22]. Our previous study has confirmed that TNF-α activates Akt, mTORC1 and mTORC2 signaling in cultured RPE cells, however, Akt/mTORC1, but not mTORC2 signaling is required for TNF-α-mediated *in vitro* RPE cell migration [23]. In current study, we investigated the signaling mechanism of TNF-α-induced MMP-9 expression.

2. Material and methods

2.1. Cell culture

As reported early [23,24], human retinal pigment epithelial (RPE) cells (ARPE-19 line) were maintained in Dulbecco's Modified Eagle's Medium(DMEM)/Nutrient Mixture F-12 (DMEM/F12, Gibco Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Shanghai, China), penicillin/streptomycin (1:100, Sigma, St. Louis, MO), and 4 mM L-glutamine and 0.19% HEPES, in a humidified incubator at 37 °C and 5% CO_2.

2.2. Reagents and chemicals

Human recombinant MMP-9 (purified active form),TNF-α, rapamycin, SP 600125, SB 203580, PD 98059, and LY 294002 were purchased from Calbiochem (Darmstadt, Germany), MMP-9 inhibitor I (MMP-9-I) was purchased from EMD Millipore (La Jolla, CA), the monoclonal anti-human MMP-9 antibody is purchased from R&D Systems (Shanghai, China), all phosphorylation antibodies and their non-phosphorylated controls were obtained from Cell

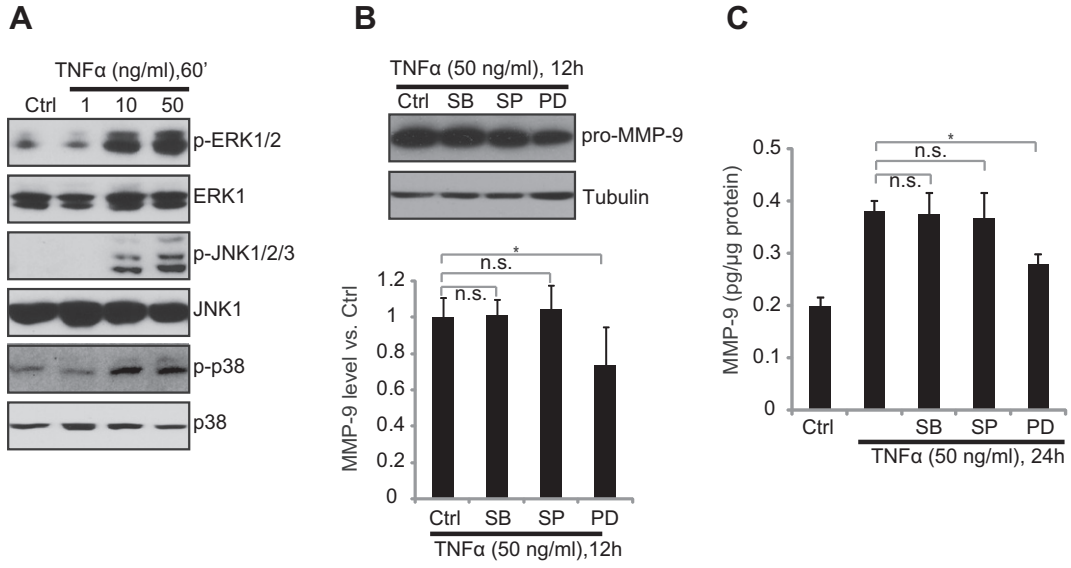

Fig. 2. MAPK activation is not critical for TNF-α-induced MMP-9 expression and secretion. (A) Western-blots of RPE cell lysates after indicated TNF-α treatment confirmed MAPK activation (Erk, JNK and p38 phosphorylation). (B, C) RPE cells were treated with TNF-α (50 ng/ml) plus p38 inhibitor SB 203580 (SB, 10 μM), JNK inhibitor SP 600125 (SP, 10 μM) or MEK/Erk inhibitor PD 98059 (PD, 10 μM) for 12–24 h, MMP-9 (pro-) expression level was examined by Western-blots (B), the amount of active MMP-9 in cultured medium was determined by ELISA (C). The values in the figures are expressed as the means ± standard deviation (SD). $^{*}P < 0.05$ (ANOVA), n.s.: no statistics difference. Experiments were repeated three times to insure consistency of results.

Signaling Tech. (Danvers, MA). Mouse mono-clone antibody against tubulin was purchased from Sigma (St. Louis, MO), the MMP-9 neutralizing antibody was obtained from CalBiochem (La Jolla, CA).

2.3. The phagokinetic track motility assay

The Phagokinetic Track Motility Assay was performed to determine RPE cell migration as previously described [23–25]. Cells were cultured for 36 h in the presence or absence of the appropriate reagents and then photographed with an Olympus 1×71 microscopes equipped with a Q Imaging Retiga 1300 system. At least 100 cells in 10 random views from each condition were collected, and each single cell's migration distance was quantified individually using Image J software (free download from NIH website) for statistic.

2.4. Mmp-9 elisa

RPE Cells were cultured at $5*10^3$ cells per well in 96-well plates (200 μL culture medium per well). At a confluence of approximately 80%, the cells were cultured in serum-free medium for 16 h. Subsequently, the culture medium was changed, and the cells were stimulated. Supernatants were collected after 12 h, and the levels of active MMP-9 (83 kDa) in the cultured media were determined by ELISA (R&D Systems, Shanghai, China).

2.5. Western-blot analysis

As previously described [23–25], aliquots of 30–40 μg of lysed proteins (lysed by 40 mM HEPES [pH 7.5], 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, EDTA-free protease inhibitors [Roche] and 1% Triton) from each treatment sample was separated by 10–12% SDS polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking with 10% instant non-fat dry milk for one hour, membranes were incubated with specific antibodies overnight at 4 °C followed by incubation with secondary antibodies for 45 min to one hour at room temperature. The Western-blots results were visualized by enhanced chemiluminescence (ECL).

2.6. RNA interference (RNAi) experiments

As reported early [23], custom SMART pool® RNAi duplexes targeting SIN 1 (79109: MAPKAP1) and mTOR (2475: FRAP1) were chemically synthesized by Dharmacon Research (Lafayette, CO). RNAi was transfected using FuGene6 protocol as previously described [23].

2.7. Constitutively active Akt (CA-Akt) transfection

As reported early [23], a constitutively active AKT1 plasmid (CA-Akt, plasmid 16244) and empty vector control were purchased

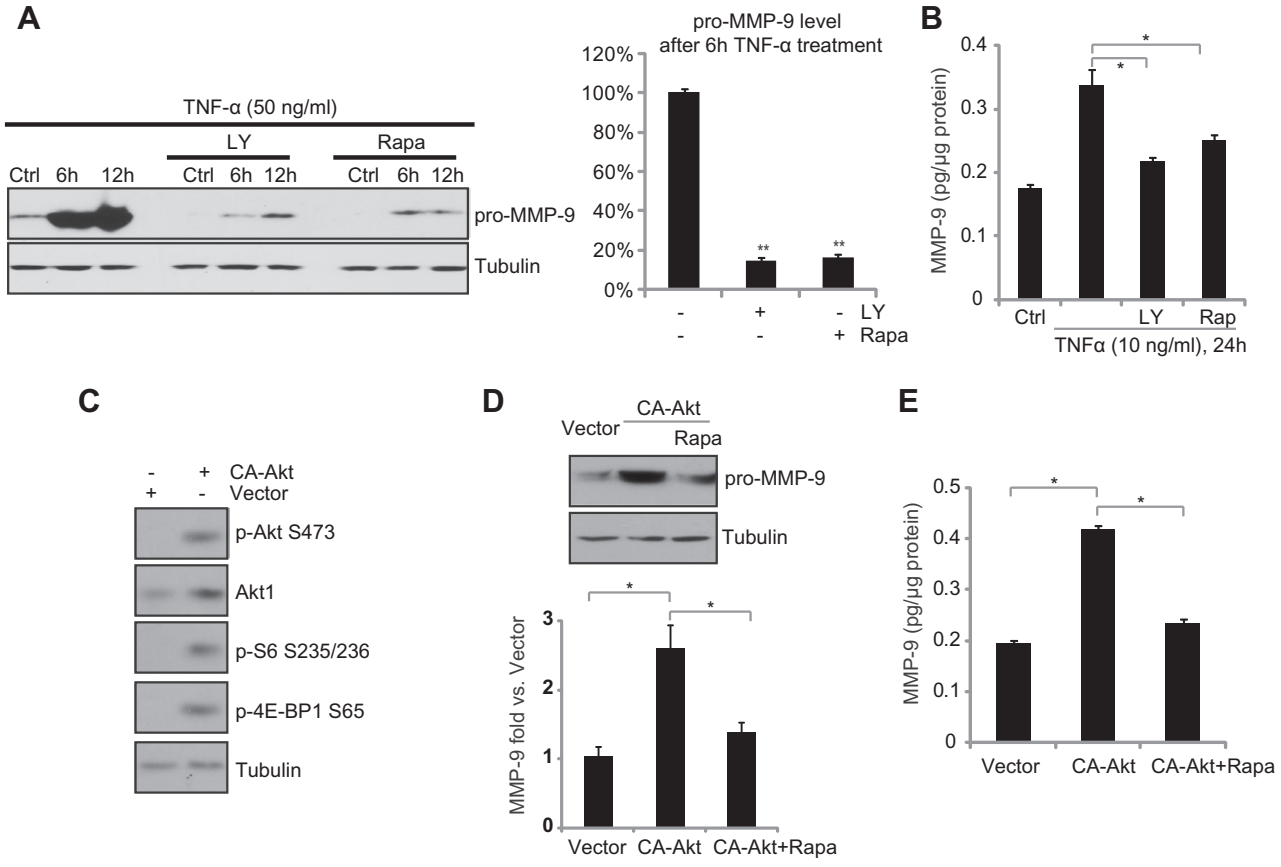

Fig. 3. Akt/mTORC1 signaling is important for TNF-α-induced MMP-9 expression. (A) Western-blot results show LY 294002 (LY, 10 μM) or rapamycin (rapa, 100 nM) largely inhibited TNF-α (50 ng/ml)-induced pro-MMP-9 expression in RPE cells. MMP-9 (pro-active) level after 6 h of TNF-α treatment was quantified by Image J. (B) The concentration of active MMP-9 in the cultured media after indicated TNF-α treatment with or without LY 294002 (LY, 10 μM) or rapamycin (RAP, 100 nM) was determined by ELISA. (C) Western-blots results confirmed Akt and downstream S6, 4E-BP1 phosphorylation after CA-Akt (2 μg) transfection in cultured RPE cells. (D) Western-blots of CA-Akt transfected RPE cell lysates confirmed pro-MMP-9 up-regulation, and rapamycin (100 nM) co-treatment almost reversed CA-Akt's effect on pro-MMP-9 expression, (E) Similarly, the amount of active MMP-9 in cultured medium was determined by ELISA. The values in the figures are expressed as the means ± standard deviation (SD). $^*P < 0.05$ (ANOVA). Experiments were repeated three times to insure consistency of results.

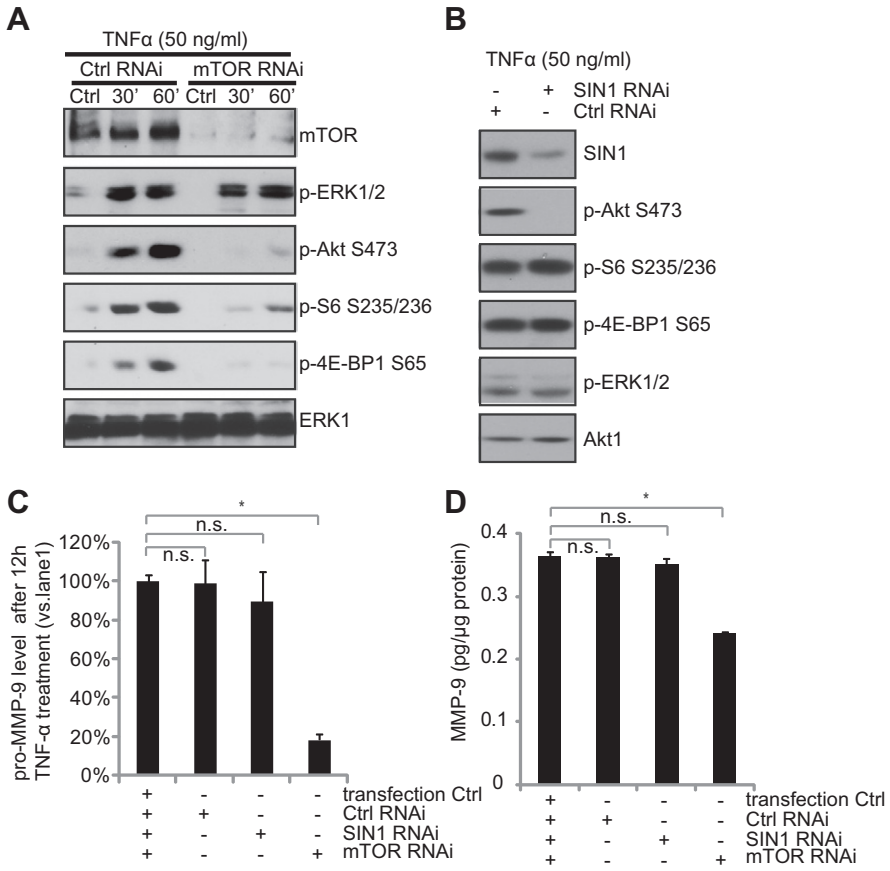

Fig. 4. Silencing mTOR, but not mTORC2 component Sin1, inhibits TNF-α-induced MMP-9 expression. (A, B) RPE cells transfected with scramble, mTOR or SIN1 RNAi (100 nM, 48 h) were treated with TNF-α (50 ng/ml) for indicated time points, activation of Akt/mTOR was analyzed by Western-blots detecting p-AKT (Ser 473), p-S6 (Ser 235/236) and p-4E-BP1 (Ser 65). mTOR, phospho- and total Erk1/2 levels were also measured. (C) TNF-α (50 ng/ml) induced pro-MMP-9 level in RPE cells transfected with scramble, mTOR or SIN1 RNAi (100 nM, 48 h) were analyzed by Western-blots, the results were quantified and normalized to lane 1. (D) The concentration of active MMP-9 in the cultured media after TNF-α (50 ng/ml) treatment in RPE cells transfected with scramble, mTOR or SIN1 RNAi (100 nM, 48 h). The values in the figures are expressed as the means ± standard deviation (SD). $^{*}P < 0.05$ (ANOVA), n.s.: no statistics difference. Experiments were repeated three times to insure consistency of results.

from addgene (Cambridge, MA). CA-Akt and the vector control (2 μg each) were transfected through Lipofectamine protocol described early [23].

2.8. Statistical analysis

Values of $p < 0.05$ were considered as statistically significant (ANOVA). All experiments were repeated at least three times and similar results were obtained.

3. Results

3.1. TNF-α promotes MMP-9 expression and secretion in cultured human RPE cells

Western-blot results in Fig. 1A showed that TNF-α (10, 50 ng/ml) significantly induced MMP-9 (pro-active form) expression in cultured human RPE cells. To determine whether this increase is associated with the secretion of MMP-9, the content of total active MMP-9 protein in the cultured media was measured by ELISA. Similar to the effect on protein expression, TNF-α induced a significant increase in the secretion of active MMP-9 (Fig. 1B). Inhibition of MMP-9 by its inhibitor (MMP-9 Inhibitor I) or its neutralizing antibody largely inhibited TNF-α-induced RPE cell migration, while exogenously-added active MMP-9 mimicked TNF-α's effect and enhanced RPE cell migration (Fig. 1C), these results suggest that TNF-α induces MMP-9 expression and secretion to mediate RPE cell migration.

3.2. MAPK activation is not critical for TNF-α-induced MMP-9 expression and secretion

TNF-α binds to its receptor and activates three major signaling pathways: mitogen-activated protein kinase (MAPK), NF-κB and PI3 K/Akt/mTOR [26], we then set to determine which pathway is important for MMP-9 expression. Western-blot results in Fig. 2A showed a significant MAPK activation (JNK/Erk/p38 phosphorylation) after TNF-α treatment in cultured RPE cells. Three different inhibitors were used to block MAPK activation, which included p38 inhibitor SB 203580, JNK inhibitor SP 600125 and MEK/Erk inhibitor PD 98059. Fig. 2B and C demonstrated that SB 203580 or SP 600125 had no effect on TNF-α-induced MMP-9 expression or secretion, and PD 98059, the MEK/Erk inhibition, only slightly diminished TNF-α's effect on MMP-9. These results suggest that although TNF-α induces a significant MAPK activation, it is not critical for MMP-9 induction.

3.3. Akt/mTORC1 signaling is important for TNF-α-induced MMP-9 expression

Our recent study has shown the critical role of Akt/mTORC1 signaling in TNF-α-induced RPE cell migration [23]. Here, we found that this signaling pathway is also important for TNF-α-induced MMP-9

expression. Both LY 294002 (LY, the broad PI3 K/Akt/mTOR inhibitor) and rapamycin (rapa, the mTORC1 inhibitor) significantly inhibited TNF-α-induced MMP-9 expression (Fig. 3A) and secretion (Fig. 3B). Conversely, forced-activation of Akt by introducing a constitutively active Akt (CA-Akt) (Fig. 3C) promoted MMP-9 expression and secretion, which was almost reversed by rapamycin (Fig. 3D and E). These data suggest that Akt and its downstream mTORC1 activation mediate TNF-α-induced MMP-9 expression.

3.4. RNAi silencing mTOR, but not SIN1, inhibits TNF-α-induced MMP-9 expression

To further dissect the role of mTOR signaling in TNF-α-induced MMP-9 expression, we used siRNA-mediated gene silencing to knockdown mTOR and SIN1, the latter is a key component of mTORC2. S6 and 4E-BP1 phosphorylation were tested as indicators of mTORC1 activation, while Akt phosphorylation at serine 473 was tested as the indicator of mTORC2 activation. As expected, silencing mTOR significantly inhibited TNF-α-induced mTORC1 and mTORC2 activation, as phosphorylation of Akt (Ser 473), S6 and 4E-BP1 were largely inhibited (Fig. 4A). Silencing of SIN1 only inhibited mTORC2 activation (Akt Ser 473), leaving mTORC1 activation unaffected (Fig. 4B). Western-blot and ELISA results in Fig. 4C and D showed that silencing mTOR, but not SIN1, inhibited TNF-α-induced MMP-9 expression and secretion. These data once again confirm that Akt and its downstream mTORC1, but not mTORC2 signaling, is important for TNF-α-induced MMP-9 expression in RPE cells.

4. Discussion

Recent studies have suggested that Akt/mTOR signaling is important to regulate the expression, activity and secretion of matrix metalloproteinases MMP-9 [27–29]. In multiple cancers, PTEN deficient activates Akt/mTOR signaling pathway, which is associated with MMP-9 up-regulation and cancer cell invasion [28]. Zhou et al., found that activation of S6K1 increase the expression of MMP-9 and cellular invasion, the authors suggest that MMP-9 up-regulation by S6 K is a key step for cell invasion and migration [29].

It appears that both mTORC1 and mTORC2 signaling are involved in processes of cytoskeleton rearrangement and migration, but the specific contributions of each complex remain to be established [30]. Activation of mTORC1 and S6K1 were shown to be essential for migration and invasion for both cancer and non-cancer cells [30–33], and cell migration and tumor progression are significantly suppressed by mTOR inhibition [31,34]. For example, rapamycin significantly suppressed tumor cell migration and tumor angiogenesis in gastric cancer cells *in vivo* [31,33]. In gastric cancer specimens, activated mTOR was detected predominantly at the invasive tumor front [31,33]. Moreover, mTOR positive tumor cells invading into lymphatic vessels were found [31,33]. Our previous study demonstrated that mTORC1 but not mTORC2 is required for TNF-α-induced RPE cell migration. Disrupting mTORC2 signaling complex by silencing its key components SIN1 or rictor had almost no effects on RPE cell migration. In the current study, we found that mTORC2 is also not important for MMP-9 expression, as SIN1 knockdown showed no significant effect on MMP-9 expression. Inhibition of Akt/mTORC1 signaling by its inhibitor or RNAi knockdown inhibited MMP-9 expression. It should be noted that the exact mechanisms by which Akt/mTORC1 signaling pathway regulates MMP-9 expression need to be further elucidated. Our study provides new insight of RPE cell migration and potential therapeutic targets (MMP-9) against PVR.

5. Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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