• Basic Research •

Hepatocyte growth factor promotes retinal pigment epithelium cell activity through MET/AKT signaling pathway

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Abstract

● **AIM:** To explore the effects of hepatocyte growth factor (HGF) on retinal pigment epithelium (RPE) cell behaviors.

● **METHODS:** The human adult retinal pigment epithelial cell line-19 (ARPE-19) were treated by HGF or mesenchymal-epithelial transition factor (MET) inhibitor SU11274 *in vitro*. Cell viability was detected by a Cell Counting Kit-8 assay. Cell proliferation and motility was detected by a bromodeoxyuridine incorporation assay and a wound healing assay, respectively. The expression levels of MET, phosphorylated MET, protein kinase B (AKT), and phosphorylated AKT proteins were determined by Western blot assay. The MET and phosphorylated MET proteins were also determined by immunofluorescence assay.

● **RESULTS:** HGF increased ARPE-19 cells' viability, proliferation and migration, and induced an increase of phosphorylated MET and phosphorylated AKT proteins. SU11274 significantly reduced cell viability, proliferation, and migration and decreased the expression of MET and AKT proteins. SU11274 suppressed HGF-induced increase of viability, proliferation, and migration in ARPE-19 cells. Additionally, SU11274 also blocked HGF-induced phosphorylation of MET and AKT proteins.

● **CONCLUSION:** HGF enhances cellular viability, proliferation, and migration in RPE cells through the MET/AKT signaling pathway, whereas this enhancement is suppressed by the MET inhibitor SU11274. HGF-induced MET/AKT signaling might be a vital contributor of RPE cells survival.

● **KEYWORDS:** hepatocyte growth factor; mesenchymal epithelial transition factor; SU11274; retinal pigment epithelial cells

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INTRODUCTION

Macular hole is a retinal disease that seriously threatens vision, and atrophy of the retinal epithelium is common following macular hole surgery[1-2]. In recent years, the use of human amniotic membrane (AM) tissue to cover the retinal layer has emerged as a novel approach to treat refractory macular holes[3]. AM also has better clinical efficacy in the treatment of retinal detachment after macular hole surgery[4]. As the innermost layer of the placenta, human AM is smooth and devoid of blood or lymph vessels[5]. It was reported that human AM transplanted into the subretinal region reduce vitreous-retinal pigment epithelium (RPE) contact and encourage RPE cells proliferation *in vitro*[6]. However, up to date, the functional mechanism of AM on RPE cells is still not fully elucidated.

Hepatocyte growth factor (HGF), a multifunctional cytokine, plays a crucial role in cell growth, survival, movement, and morphogenesis[7]. HGF is primarily released by cells with mesenchymal ancestry and is the highest expressed in the AM tissues[8]. HGF can bind to its receptor, mesenchymal-epithelial transition factor (MET or c-MET), triggers the phosphorylation

of MET (p-MET) and initiates a signaling cascade[9]. This HGF/ MET signaling pathway is vital for cell growth, motility as well as tissues regeneration[10]. Some studies showed the protein kinase B (AKT) activated by the HGF/MET axis involved in regulation of cells' proliferation, differentiation, migration[11]. Some researcher reported that HGF and its receptor expressed in RPE cells and HGF was mitogenic for RPE cells[12]. Some studies also showed that HGF promoted RPE cell migration and proliferation and prevented oxidative damage induced by glutathione deficiency in RPE cells[13-14]. SU11274, a selective MET inhibitor, not only significantly suppressed HGF-induced phosphorylation of MET, but also caused autophagy, apoptosis, and cell cycle arrest[15-16]. It is proven that the increase of phosphorylated form of Mer tyrosine kinase in the progranulin-exposed RPE cells was attenuated by SU11274[17]. Whereas the effects of HGF and SU11274 on RPE cells is not fully clarified.

In the present study, in order to elucidate the effects of the HGF and MET inhibitor SU11274 on RPE cells, we use different concentrations of HGF and SU11274 on RPE cells *in vitro* and measured cell viability, migration, proliferation and MET/AKT signaling pathway.

MATERIALS AND METHODS

Materials The human RPE cell line, adult retinal pigment epithelial cell line-19 (ARPE-19) cells were from American Type Culture Collection (Manassas, VA, USA). HGF and SU11274 were purchased from PeproTech Inc. (Rocky Hill, NT, USA) and Selleck Chemicals LLC (Houston, Texas, USA), respectively. The cell counting kit (CCK)-8 viability assay and radio immunoprecipitation assay (RIPA) buffer lysis were obtained from APExBIO (Houston, USA). FluoroShield™ with DAPI was purchased from Sigma-Aldrich (St. Louis, MO, USA). The 5-bromodeoxyuridine (BrdU), MET, AKT, phosphorylated MET, phosphorylated AKT, and β-actin primary antibodies were from Cell Signaling Technology (Danvers, MA, USA).

Cell Viability Assay ARPE-19 cells (0.5×10^4/well) in 96-wells plate were treated with HGF or SU11274. Next, each well was added 10 μL of the CCK-8 reagent and cells were incubated at 37°C in the dark for 60min. The absorbance at 450 nm was detected by a microplate reader (ELx808IU, Gene company limited, HK, China).

Cell Proliferation Assay ARPE-19 cells were treated with HGF or SU11274 for 24h and cultured in medium with BrdU (30 μmol/L) for 4h. Then cells were fixed in 4% paraformaldehyde (PFA) solution for 30min, rinsed thrice with phosphate buffer solution (PBS), and permeabilized with 2 mmol/L hydrochloric acid solution for 3min. Next, the cells were rinsed thrice in PBS, blocked for 2h with 5% bovine serum albumin, and incubated in BrdU primary antibody dilution buffer (1:100) for 2h. After rinsing thrice in PBS, cells were incubated in the dark for one hour with fluorescently labeled secondary antibody (1:1000). Following three PBS washes and sealing, the cells were observed by using a fluorescence microscope (IX73, Olympus, Japan).

Wound Healing Assay After ARPE-19 cells were cultivated to confluence, a 200 μL pipet tip was used to scrape the monolayer. Then cells were treated with HGF or SU11274 in DMEM/F-12 culture medium containing 3% fetal bovine serum (FBS) for 24h. The scratches of "wound injury" were observed and analyzed by using by microscope and Image J Software, respectively.

Western Blot Analysis After treatment with HGF or SU11274, cells were rinsed twice in PBS and lysed in RIPA lysis buffer. The sample proteins were heated to denature at 100°C for 10min. Next, the sample proteins were separated and transferred to polyvinylidene fluoride (PVDF) membranes. Then 5% defatted milk was used to block the membranes for one hour. Next step, the membranes were washed thrice in PBS, incubated in primary antibody dilution solution (1:1000) at 4°C overnight, rinsed thrice, incubated with secondary antibodies for 1h, rinsed thrice, and incubated in chemiluminescence solution for 10min. The blots of sample proteins were detected by using the G: Box Chemi-XR (Gene company limited, HK, China) and analyzed by Image J Software.

Immunofluorescence ARPE-19 cells were cultured on glass slides covered with 1% gelatin solution and treated with HGF or SU11274 for 24h. Then cells were immobilized with 4% PFA for 30min and permeabilized in 1% Triton X-100 solutions for 5min. Next, cells were blocked in 5% bovine serum albumin for 120min and incubated in primary antibody diluents (1:100) for 2h, rinsed thrice in PBS, and incubated in secondary antibody diluent (1:1000) for 60min. Finally, the cells were rinsed thrice in PBS, closed with an anti-fluorescent sealer, and observed with a fluorescence microscope (IX73, Olympus, Japan).

Statistical Analysis All data were presented as the mean±standard error of the mean (SEM) and analysed by GraphPad Prism 8 software (GraphPad Software, LaJolla, CA, USA). The differences among groups were conducted by one-way ANOVA (Bonferroni).

RESULTS

HGF Enhances Cellular Viability, Proliferation, and Migration in ARPE-19 Cells The images from phase contrast microscope showed the density and the quantity of the ARPE-19 cells raised as the increase of the concentration of the HGF after treatment with HGF (0, 1.0, 5.0, 10, 50, and 100 ng/mL) for 24h (Figure 1A). The CCK-8 viability assay showed that, compared with control group, treatment with 5.0, 10, 50, and 100 ng/mL of HGF increased cell viability by 10.83%, 15.98%,

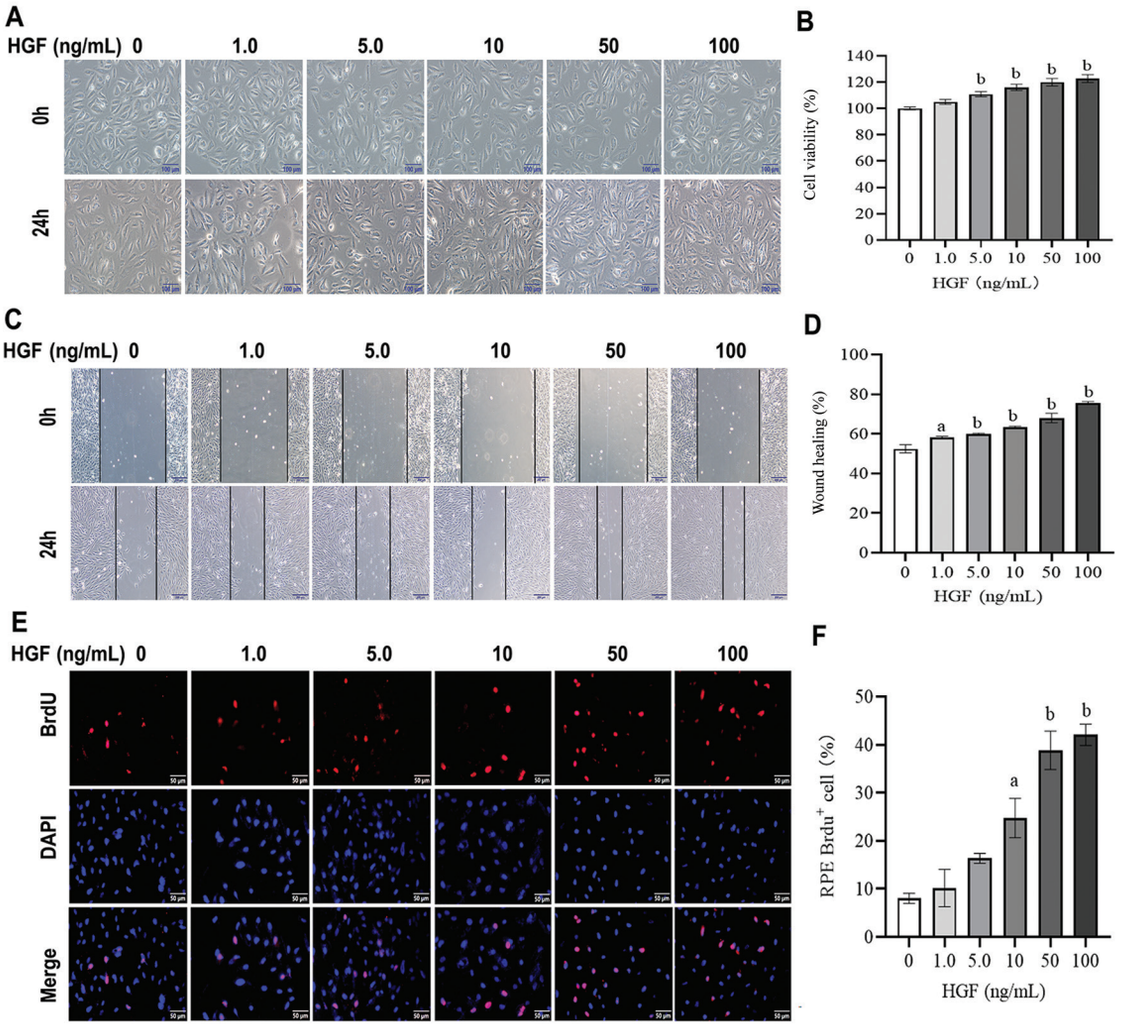

Figure 1 HGF increases cellular activity in ARPE-19 cells The ARPE-19 cells were dealt with 0, 1.0, 5.0, 10, 50, and 100 ng/mL of HGF for 24h. A: The cytomorphology was observed by microscopy (scale bar: 100 μm); B: Cellular viability was determined by a CCK-8 viability assay; C, D: Cell migration was detected and analyzed by a wound healing assay (scale bar: 200 μm); E, F: Cellular proliferation was determined and analyzed by BrdU incorporation assay (scale bar: 50 μm). $^{a}P<0.05$, $^{b}P<0.01$. HGF: Hepatocyte growth factor; CCK: Cell counting kit; ARPE-19: Adult retinal pigment epithelial cell line-19.

19.94%, and 22.71%, respectively (Figure 1B). The wound healing assay showed that when treatment with 0, 1.0, 5.0, 10, 50, and 100 ng/mL of HGF for 24h, the migration distances of ARPE-19 cells were 55.10%, 58.11%, 60.04%, 63.32%, 68.01%, and 75.49% of the scratches, respectively. Compared with control group, HGF induced a significant increase of ARPE-19 cells' migration (Figure 1C, 1D). The results of BrdU incorporation assay showed the BrdU staining positive cells were 8.05% in the control group and were 10.16%, 16.35%, 24.72%, 36.05%, 42.08% in the treatment groups with 1.0, 5.0, 10, 50, and 100 of HGF, respectively (Figure 1E). Quantitative analysis of the BrdU staining positive cells indicated that, compared to the control group, treatment with HGF (10, 50, and 100 ng/mL) induced a significant increase of cell proliferation (Figure 1F).

HGF Activates the MET/AKT Signaling in ARPE-19 Cells

The data of Western blots showed that treatment with 1.0, 5.0, 10, 50 ng/mL of EGF did not cause phosphorylation of MET and AKT proteins, while treatment with HGF (100 ng/mL) for 24h induced a significant increase of phosphorylated MET and phosphorylated AKT proteins expression. There were no appreciable variations in expression of MET and AKT proteins compared to the control group (Figure 2A, 2B). We also detected the effects of treatment with of HGF (100 ng/mL) for 0, 0.25, 0.5, 1.0, 3.0, 6.0, and 12h on MET/AKT signaling in RPE cells. Western blot assay showed the expression of phosphorylated MET and phosphorylated AKT proteins were highest at 0.5h compared to 0h (Figure 2C, 2D).

Effects of MET Inhibitor SU11274 on ARPE-19 Cells

The results of observation from inverted phase contrast microscopy showed that treatment with 5.0 and 10 μmol/L of SU11274 induced the cells' shape changed from irregular to round (Figure 3A). The data of the CCK-8 viability assay indicated that ARPE-19 cells' viability decreased by 7.67%,

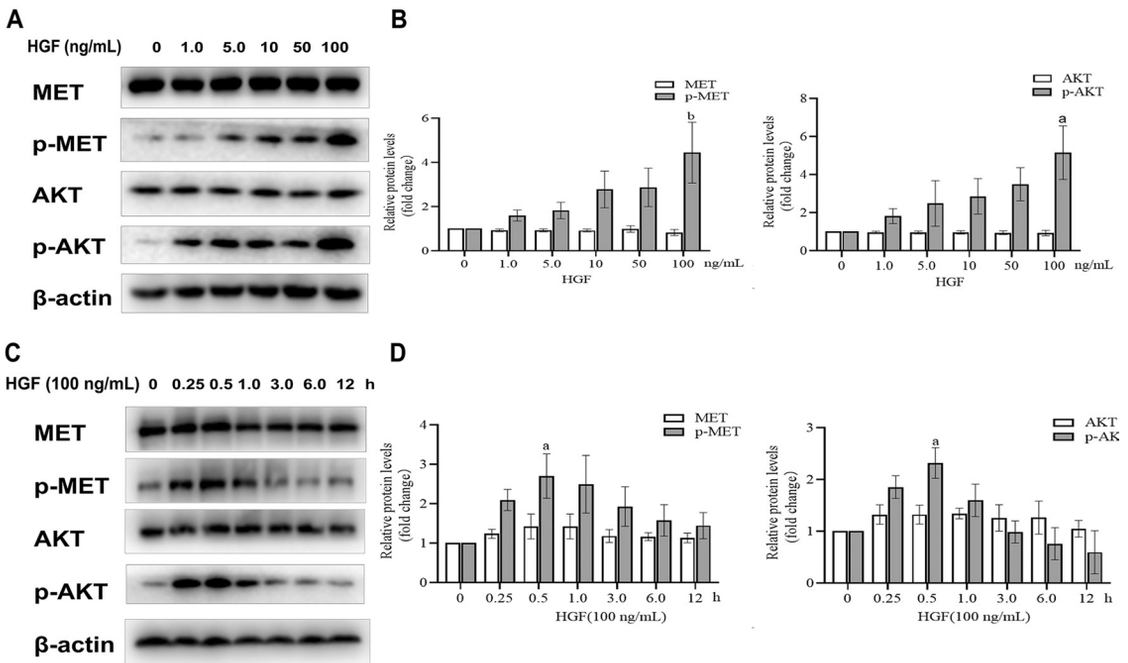

Figure 2 HGF activates the MET/AKT signaling pathway in ARPE-19 cells A, B: The MET, phosphorylated MET, AKT, and phosphorylated AKT proteins in ARPE-19 were detected and analyzed by Western blot assay after treatment with 0, 1.0, 5.0, 10, 50, or 100 ng/mL of HGF for 24h; C, D: The MET, phosphorylated MET, AKT, and phosphorylated AKT proteins in ARPE-19 were detected and analyzed by Western blot assay after treatment with 100 ng/mL of HGF for 0, 0.25, 0.5, 1.0, 3.0, 6.0, 12h. $^{a}P<0.05$, $^{b}P<0.01$. HGF: Hepatocyte growth factor; MET: Mesenchymal-epithelial transition factor; p-MET: Phosphorylation of MET; AKT: Protein kinase B; p-AKT: Phosphorylated AKT; ARPE-19: Adult retinal pigment epithelial cell line-19.

11.27%,13.15%, and 13.29 % in treatment groups with 1.0, 2.5, 5.0, and 10 μmol/L of SU11274, respectively, compared to control group (Figure 3B). The data from wound healing assay showed when treatment with 0, 1.0, 2.5, 5.0, and 10 μmol/L of SU11274, the distances of cell migration were 68.10%, 55.00%, 47.24%, 40.38%, and 31.96% of the scratches, respectively (Figure 3C, 3D). 5.0 and 10 μmol/L of SU11274 obviously caused a decrease of distances of cell migration compared with control group. The data from BrdU incorporation assay showed when treatment with 0, 1.0, 2.5, 5.0 and 10 μmol/L of SU11274, the BrdU staining positive cells were 11.74%, 9.88%, 7.04%, 5.09%, and 3.03%, respectively (Figure 3E). Quantitative analysis of the BrdU staining positive cells showed that, compared to the control group, treatment with 5.0 and 10 μmol/L of SU11274 significantly reduced cell proliferation rates (Figure 3F).

SU11274 Down Regulates MET and AKT Proteins in ARPE-19 Cells The results of Western blots showed treatment with 1.0 and 2.5 μmol/L of SU11274 did not affected expression of MET protein, but 5.0 and 10 μmol/L of SU11274 caused a significant decrease of MET protein. Meanwhile, treatment with 1.0 and 2.5 μmol/L of SU11274 did not affected expression of AKT protein, whereas 5.0 and 10 μmol/L of SU11274 significantly decreased expression of AKT protein (Figure 4A, 4B). Immunofluorescence staining results showed that SU11274 (10 μmol/L) caused contraction of cell morphology and decreased MET protein expression compared to the control group (Figures 4C).

SU11274 Inhibits HGF-induced Activation in ARPE-19 Cells Through the MET/AKT Signaling Pathway The data of CCK-8 viability assay indicated that, compared to the control group, HGF treatment increased cell viability by 5.27%, while SU11274 treatment decreased cell viability by 9.72%. Compared to the HGF treatment group, cell viability in the group with both SU11274 and HGF was decreased by only 4.4% (Figure 5A). The data from wound healing assay indicated that the distance of cellular migration in the scratch was 55.25% in the control group, 76.58% in the group with HGF, 37.53% in the group with SU11274 group, and 54.60% in the group with both HGF and SU11274 (Figure 5B). Compared to the control group, HGF treatment increased cellular migration by 21.33%, while SU11274 treatment decreased cellular migration by 17.72%. In contrast to the HGF group, cellular migration in the group treatment with both SU11274 and HGF was decreased by 21.98% (Figure 5C). Data from BrdU incorporation assays showed that the BrdU staining positive cells were 6.3% in the control group,12.35% in the group with HGF treatment, 0 in the group with SU11274, 1.67% in the group with both HGF and SU11274 (Figure 5D). Compared to the control group, HGF treatment increased the BrdU staining

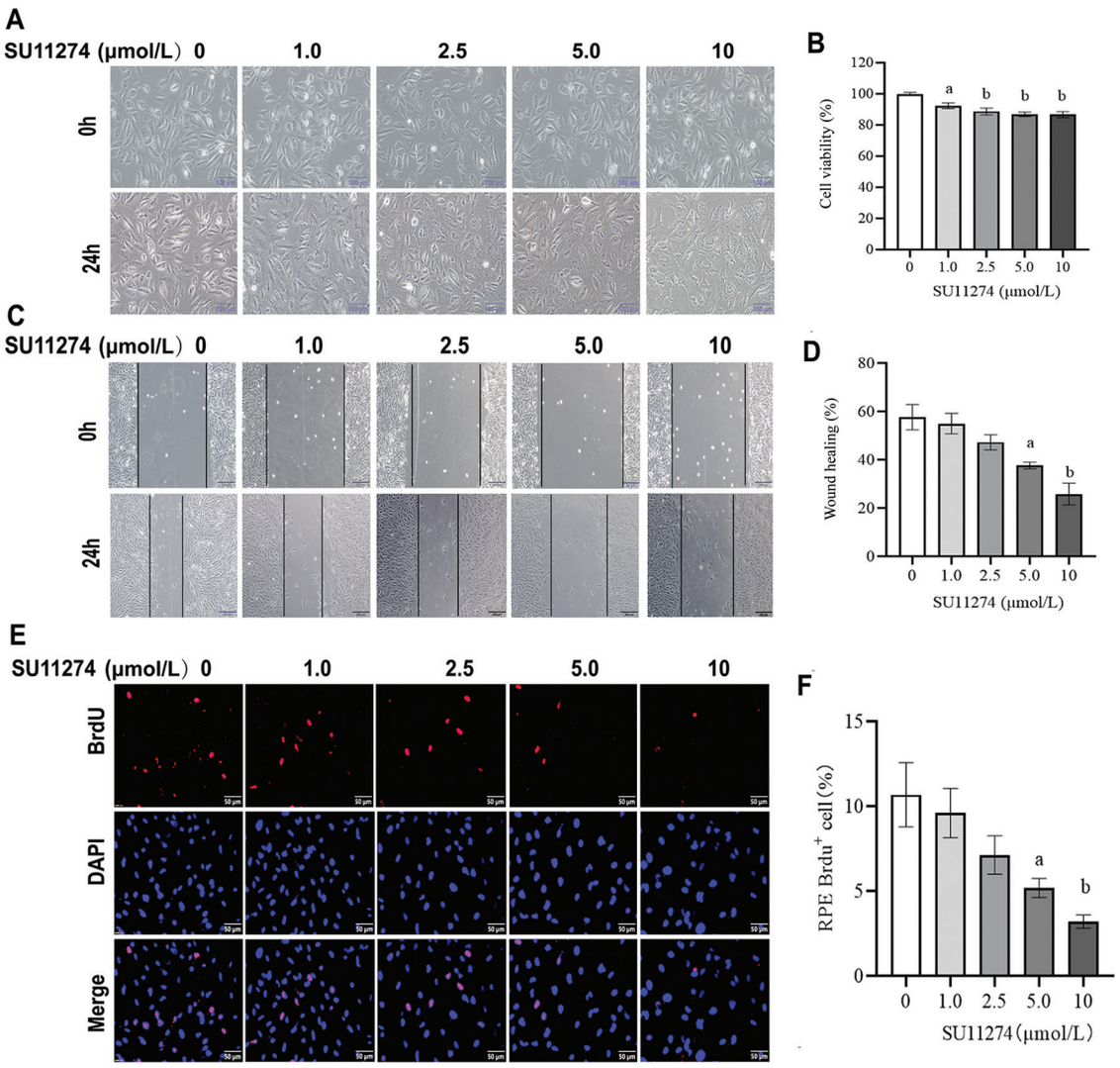

Figure 3 The results of SU11274 on cellular morphology, viability, migration, and proliferation in ARPE-19 cells The MET inhibitor SU11274 (0, 1.0, 2.5, 5.0, and 10 μmol/L) was used to deal with ARPE-19 cells for 24h. A: The cellular cytomorphology was observed by microscopy (scale bar: 100 μm); B: The cellular viability was analyzed by a CCK-8 viability assay; C, D: The cellular migration was detected and analyzed by a wound healing assay (scale bar: 200 μm); E, F: The cellular proliferation was determined and analyzed by BrdU incorporation assay (scale bar: 50 μm). $^{a}P<0.05$, $^{b}P<0.01$. CCK: Cell counting kit; ARPE-19: Adult retinal pigment epithelial cell line-19.

positive cells by 6.05%, while SU11274 treatment decreased the BrdU staining positive cells by 6%. Compared to the HGF treatment group, treatment with both SU11274 and HGF was decreased BrdU staining positive cells by 10.68% (Figure 5E). The data from Western blot indicated that treatment with HGF caused a significant increase of phosphorylated MET and phosphorylated AKT proteins compared to the control group. This HGF-caused phosphorylation of MET and AKT proteins was significantly suppressed by SU11274 (Figure 5F, 5G). The immunofluorescence staining assay also showed that treatment with HGF for 30min induced significant expression of phosphorylated MET, whereas this HGF-included an increase of phosphorylated MET protein express was significantly suppressed by SU11274.

DISCUSSION

HGF consists of an α-chain and a β-chain and is known as a pleiotropic cytokine, which can bind its specific receptor tyrosine kinase, MET, to be involved in cell survival, tissues regeneration, and neurotrophic action[18-21]. A previous study has demonstrated that HGF can enhance cell proliferation in RPE cells, and HGF has been proposed as a therapeutic candidate for RPE cell recovery[22]. Some researchers used HGF to treat bovine RPE cells and found that HGF could accelerate the migration of RPE cells[23]. Furthermore, some data showed that injection of HGF can protect the RPE from degeneration by $NaIO_3$[24]. Some studies showed that HGF (10 ng/mL) could promote the proliferation of RPE cells that were derived from human embryonic stem cell[13]. In this study, we used the concentration of HGF (0, 1.0, 5.0, 10, 50, and 100 ng/mL) to treat RPE cells and found similar results that HGF caused a significant increase of REP cells' viability, proliferation, and migration in a concentration-dependent

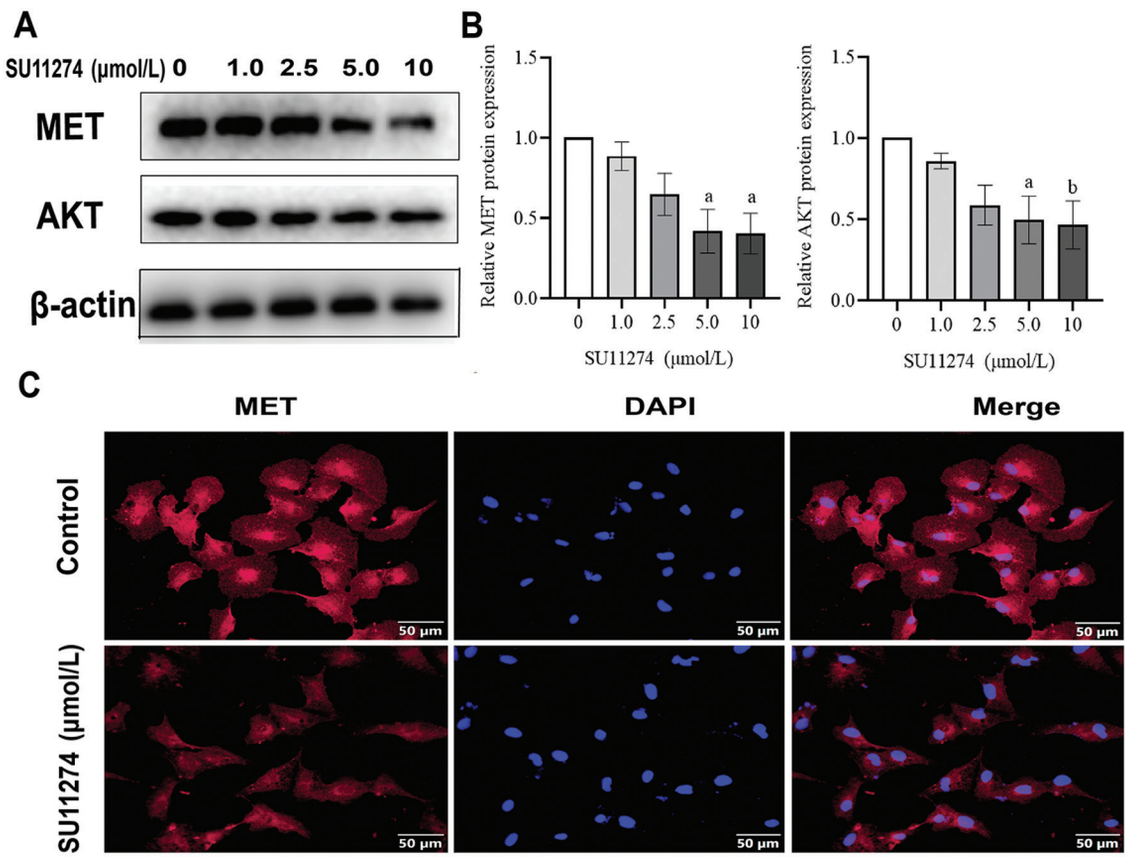

Figure 4 The effects of SU11274 on MET and AKT proteins in ARPE-19 cells A, B: ARPE-19 cells were dealt with MET inhibitor SU11274 (0, 1.0, 2.5, 5.0, and 10 μmol/L) for 24h, the expression levels of c-MET and AKT proteins were determined and analyzed by Western blot assay, $^{a}P<0.05$, $^{b}P<0.01$; C: ARPE-19 cells were dealt with SU11274 (10 μmol/L) and MET protein was detected by immunofluorescence staining (scale bar: 50 μm). MET: Mesenchymal-epithelial transition factor; AKT: Protein kinase B; ARPE-19: Adult retinal pigment epithelial cell line-19.

manner. To sum up, these studies indicated that HGF might be a valuable drug candidate for maintaining RPE cell survival and treating RPE atrophy.

HGF/MET signaling has multiple functions in different kinds of cells[25-27]. The bind of HGF to MET initiates the receptor's innate kinase activity including activation of PI3K/AKT, extracellular regulated protein kinases (ERK), and transcription 3 signaling pathways, and promotes cell growth, proliferation, and motility, as well as invasion, blood vessel development, epithelial-mesenchymal transition, and branched morphology[28-30]. Some studies showed HGF induced phosphorylation of MET-1234Y and the MET signaling intermediaries Gab1 and ERK1/2 in human induced pluripotent stem cells[31]. A recent study showed that HGF induced phosphorylated AKT expression in osteoclasts, and phosphorylated AKT expression was the highest when treatment with HGF (50 ng/mL) for 0.5h[32]. In this study, our data are consistent with above studies and showed 100 ng/mL of HGF can induced a significant phosphorylated MET and phosphorylated AKT proteins. Taken together, these results suggested that the function of HGF-promoted activity of RPE cells might carried out through MET/AKT signaling pathway.

SU11274, a selective small molecule inhibitor of MET, can inhibit EGF-induced cell viability and proliferation, migration and epithelial-mesenchymal transition[33-34]. Some data showed that 5.0 μmol/L of SU11274 inhibited tongue cancer cell proliferation[35], 1.0 and 5.0 μmol/L of SU11274 inhibited the proliferation of cancer cells *in vitro* and 0.5 mg/kg of SU11274 decreased primary tumor masses and liver tumor colonies *in vivo*[36], and 2 μmol/L of SU11274 suppressed MET receptors activated by tissue-type plasminogen activator[37]. In present study, we analyzed the effects of MET inhibitor SU11274 (0, 1.0, 2.5, 5.0, and 10 μmol/L) on HGF/MET signaling pathway in RPE cells.

Our data showed that SU11274 altered the morphology of RPE cells and inhibited their viability and motility. It is reported that 0.5 μmol/L of SU11274 could completely inhibit the expression of phosphorylated MET protein[38]. Some researchers have found that HGF increased the phosphorylated MET protein at the active phosphoric acid site, while SU11274 significantly reduced this HGF-induced phosphorylation of MET protein[39]. Additionally, it has also been found that SU11274 can inhibit phosphorylated MET in ovarian carcinomas cells and inhibit tumor growth[40]. Similarly, our

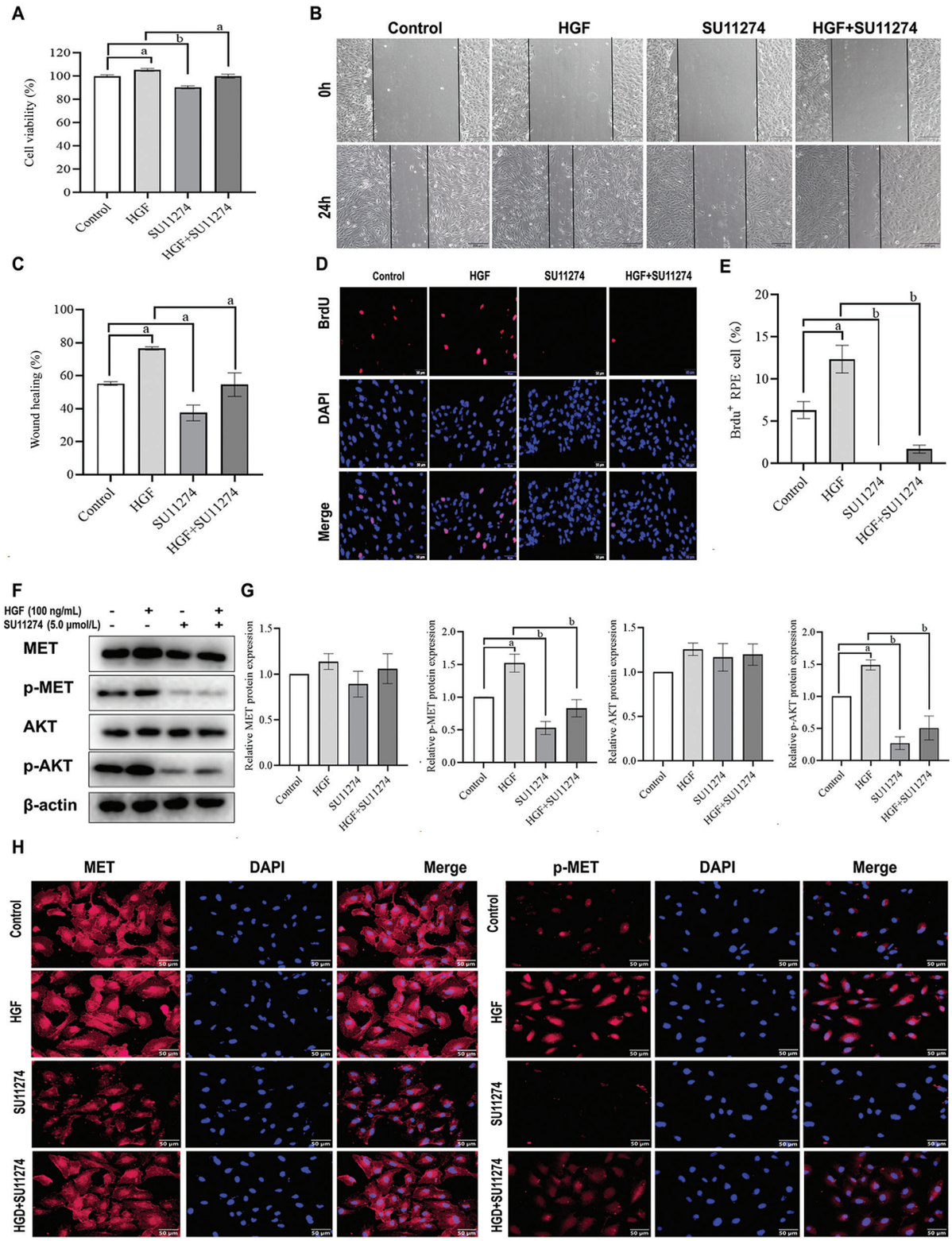

Figure 5 SU11274 inhibits HGF-caused activation in ARPE-19 cells *via* the MET/AKT signaling pathway ARPE-19 cells were dealt with HGF (100 ng/mL), SU11274 (5.0 μmol/L), HGF (100 ng/mL) combined SU11274 (5.0 μmol/L) for 24h, respectively. A: Cellular viability was detected by a CCK-8 viability assay; B, C: A wound healing assay was used to detect and analyze cellular migration (scale bar: 200 μm); D, E: A BrdU incorporation assay was used to measure and analyze cellular proliferation (scale bar: 50 μm). After pretreatment with SU11274 (5 μmol/L) for 6h, ARPE-19 cells were treated with HGF (100 ng/mL) for 30min. F, G: The MET, phosphorylated MET, AKT, and phosphorylated AKT proteins were evaluated and analyzed by Western blot assay, $^{a}P<0.05$, $^{b}P<0.01$. H: Immunofluorescence staining assay was used to detected MET and phosphorylated MET proteins (scale bar: 50 μm). HGF: Hepatocyte growth factor; MET: Mesenchymal-epithelial transition factor; p-MET: Phosphorylation of MET; AKT: Protein kinase B; p-AKT: Phosphorylated AKT; ARPE-19: Adult retinal pigment epithelial cell line-19; DAPI: Diamidino-2-phenylindole; RPE: Retinal pigment epithelium.

results indicated that SU11274 not only inhibited RPE cells' viability, proliferation, and migration through down regulating the expression of MET and AKT proteins, but also suppressed the HGF-activated RPE cells' viability, proliferation, and migration through blocking expression of phosphorylated MET and phosphorylated AKT proteins. These results suggested that HGF/MET signaling pathway was crucial to RPE cells survival and if this pathway was blocked, RPE cells might become dysfunctional. Certainly, our data in the present study only indicate the concentration values of HGF and SU11274 treatment are effective to RPE cells *in vitro* and the promoting effects of HGF on RPE cell survival need to be verified by further experiments *in vivo* and clinical studies.

In conclusion, our research showed that HGF improved RPE cells' viability, migration, and proliferation, whereas HGF-induced activation of RPE cells was inhibited by SU11274. Our results suggested that HGF-induced MET/AKT signaling might be a vital mechanism of activation of RPE cells.

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Conflicts of Interest: Zhou SR, None; **Zhu YS,** None; **Yuan WT,** None; **Pan XY,** None; **Wang T,** None; **Chen XD,** None.

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