Hepatocyte Growth Factor Induces Proliferation of Lens Epithelial Cells through Activation of ERK1/2 and JNK/SAPK

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**Purpose.** Posterior capsule opacification (PCO) is caused by proliferation and migration of lens epithelial cells (LECs) remaining after cataract surgery. In this study, the effect of HGF in LECs and the signaling pathways that contribute to HGF-induced proliferation were investigated.

**Methods.** Capsular bags prepared from porcine eyes were maintained in serum-free DMEM. The human lens epithelial B3 cells (HLE B3) and rat lens epithelial explants were cultured in MEM supplemented with 20% FCS and medium 199 with 0.1% BSA, respectively. Cell proliferation was determined by MTT assay, proliferating cell nuclear antigen (PCNA) expression, or flow cytometry. An antisense oligonucleotide was used to inhibit cyclin D1 expression. Activation of the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways was detected by immunoblot analysis.

**Results.** The proliferation of LECs in a capsular bag culture was significantly inhibited by treatment with the neutralizing antibody for HGF receptor. Stimulation of HLE B3 with hepatocyte growth factor (HGF) activated the MAPKs, ERK, and JNK/SAPK, but not p38. Activation of both ERK and JNK/SAPK was necessary for the HGF-stimulated induction of cyclin D1, which in turn was necessary for the HGF-induced proliferation of LECs. PI3K also participated in the regulation of cyclin D1 expression upstream of ERK and JNK/SAPK.

**Conclusions.** The data indicate that HGF is a potent growth factor for LECs and may contribute to the development of PCO and that the signaling pathways involved in HGF-stimulated proliferation may constitute potential therapeutic targets in the treatment of PCO. (Invest Ophthalmol Vis Sci. 2004;45:2696–2704) DOI:10.1167/iovs.03-1371

**Posterior capsule opacification (PCO) is the most frequent postoperative complication of cataract surgery. Approximately 35% of patients who undergo cataract surgery experience a secondary loss of vision that requires further corrective surgery within 2 years. PCO is mainly caused by the proliferation and migration of postoperative remnants of lens epithelial cells (LECs) in the posterior lens capsule.** Although a variety of studies have increased our understanding of the pathogenesis of PCO, the cellular mechanisms underlying the development of PCO are still unclear.

Recent studies have concentrated on the cytokines involved in the development of PCO. The levels of cytokines, such as TGF-β, bFGF, IL-6, and EGF, in the aqueous humor are elevated after cataract surgery, and these cytokines influence LECs in the early phase. However, these effects are short-lived. Considerably, in most cases, PCO does not become clinically relevant until months or years after surgery and that lens cells can survive, grow, and maintain metabolic activity in protein-free medium for more than 100 days (Wormstone, IM et al. IOVS 2000;41:ARVO Abstract 510), the cytokines that are produced by the lens cells themselves are believed to play an important role in the development of PCO. Early studies have analyzed growth factors produced by LECs on human capsular bags in a protein-free medium, which has simulated autocrine conditions. Those results demonstrated that HGF is highly expressed in human capsular bag cultures compared with other cytokines, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), and suggest that HGF may play an important role in the development of PCO (Wormstone, IM et al. IOVS 1998;39:ARVO Abstract 983). In fact, it has been reported that HGF induced the proliferation, protein synthesis, and migration of the human lens FIH124 cell line in vitro. However, there is no information on the signaling pathway used by HGF to stimulate mitosis in LECs.

Hepatocyte growth factor (HGF), also known as scatter factor, was identified independently as both a growth factor for hepatocytes and a fibroblast-derived cell motility factor. Subsequently, HGF was recognized as a multifunctional growth factor that is capable of acting as a potent mitogen, morphogen, motility factor, or angiogenic factor in a wide variety of organs. These complex biological functions occur through the receptor tyrosine kinase c-Met, which is a product of the c-met proto-oncogene and is composed of a 50-kDa extracellular α-subunit and a 145-kDa transmembrane β-subunit. The β-subunit contains tyrosine kinase domains, tyrosine phosphorylation sites, and tyrosine-docking sites. Binding of HGF with the receptor leads to autophosphorylation on several tyrosine residues, followed by the recruitment of the src homology 2 (SH2) domain-containing signaling molecules, including phosphatidylinositol 3-kinase (PI3K), a Ras nucleotide exchanger, phospholipase Cγ, Grb2, Grb2-associated binder-1 (Gab-1), Sos, and Shc adaptor. These signaling components are probably involved in diverse responses, which include the activation of the mitogen-activated protein kinase (MAPK) pathway.

In the present study, we have shown that HGF regulates cell proliferation in LECs and have identified the signaling pathways that contribute to HGF-induced proliferation. Our data demonstrate that HGF induces the proliferation of LECs through the induction of cyclin D1, which is regulated by the activation of the MAPK, (ERK and JNK/SAPK) and the PI3K/AKT pathways.
Materials and Methods

Antibodies and Reagents

Anti-phosphospecific JNK/SAPK (Thr183/Tyr185), p38 (Thr180/Tyr182), Elk1 (Ser385), ATF2 (Thr71), c-Jun (Ser63), AKT (Ser473), GSK3β (Ser9), anti-Elk1, ATF2, c-Jun, and AKT antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-phosphospecific ERK1/2, tyrosine (PY99), anti-ERK1/2, JNK/SAPK, p38, proliferating cell nuclear antigen (PCNA), and c-Met antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). GSK3β antibody was purchased from Transduction Laboratories (Palo Alto, CA), cyclin D1 antibody from BD Biosciences-PharMingen (Palo Alto, CA), and actin antibody from Sigma-Aldrich (St. Louis, MO). Human c-Met antibody for neutralization and c-HGF antibody were purchased from R&D Systems (Minneapolis, MN). Recombinant HGF was purchased from R&D Systems. The MAPK inhibitors U0126, SP600125, SB202190, and the PI3K inhibitor wortmannin were obtained from Calbiochem (La Jolla, CA).

Cell Culture

Human lens epithelial B3 cells (HLE B3) were kindly provided by Usha Andley (Washington University, St. Louis, MO). HLE B3 cells were cultured in Eagle’s minimum essential medium (MEM; Invitrogen-Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS; Invitrogen-Gibco) and 50 µg/mL gentamicin (Invitrogen-Gibco) at 37°C in a humidified 5% CO2 atmosphere. To obtain quiescent cells, the cells were incubated in MEM containing 1% FBS for 18 hours and then further cultured in the serum-free medium for 24 hours.

In Vitro Capsular Bags and Rat Lens Epithelial Explants

Capsular bags were prepared using porcine eyes, which were obtained from an abattoir. After removal of the cornea, the lens fiber mass was removed by hydrosuction. The resultant capsular bag was then dissected from the zonules, and a tension ring (CTR98A; Lucid Korea, Seoul, Korea) was inserted through the capsulorrhexis to retain the circular shape of capsular bag. Capsular bags were maintained in Dulbecco’s modified essential medium (DMEM; Invitrogen-Gibco) at 37°C in 5% CO2 atmosphere. At the end of the culture period, the explants were immunostained with 150 µL/mL of BrdU for 6 hours before the collection of explants.

Immunoprecipitation and Immunoblot Analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (0.05 M Tris-buffer [pH 7.2], 0.15M NaCl, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS) supplemented with protease inhibitor and phosphatase inhibitor. The protein extracts were quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). The samples containing 10 to 20 µg proteins were boiled in Laemmli sample buffer, separated on SDS polyacrylamide gel, electrophoretically transferred to nitrocellulose membrane (Amersham, Arlington Heights, IL) and blocked with the indicated primary antibodies. Proteins were visualized with the horseradish peroxidase-conjugated secondary antibodies (Zymed Laboratory, Inc., South San Francisco, CA) followed by chemiluminescence (ECL-Plus; Santa Cruz Biotechnology) detection.

Immunoprecipitation was performed on lysates generated from cells, which were treated with 10 ng/mL HGF for the indicated time. Lysates were incubated with antibody overnight at 4°C. Immunocomplexes were collected on protein A/G-plus agarose (Santa Cruz Bio-technology), washed with lysis buffer, resuspended in Laemmli sample buffer, and boiled for 5 minutes. Immunoprecipitates were then subjected to immunoblot analysis as described earlier.

Cell Proliferation Assay

The effect of HGF on proliferation of rat lens epithelial explants was evaluated by the incorporation of 5-bromo-2-deoxy-uridine (BrdU; Roche Diagnostics GmbH, Mannheim, Germany). Rat lens epithelial explants were exposed to 10 ng/mL HGF for 24 hours and then labeled with 150 µg/mL of BrdU for 6 hours before the collection of explants. At the end of the culture period, the explants were immunostained using a kit (5-Bromo-2’-deoxy-uridine Labeling and Detection Kit 1; Roche Diagnostics GmbH) according to the manufacturer’s protocol. The explants were mounted and examined using fluorescence microscopy. Nuclei were counterstained with Hoechst 33258 (Molecular Probes, Inc., Eugene, OR).

The proliferation of HLE B3 cells was examined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (Sigma-Aldrich). In brief, the cells were cultured as described earlier, except that the cells were seeded in 24-well tissue culture plates at a density of 5104 cells/well. The cells were treated with 0.5 mg/mL of the MTT labeling reagent and incubated at 37°C for 1 hour. A dark blue formazan product of MTT was extracted by the MTT lysing solution (40 mM HCl in isopropanol alcohol), transfer to 96-well microplates, and measured at an absorbance of 570 nm with an automatic ELISA reader (SpectraMax 250; Molecular Devices, Sunnyvale, CA).

Cell Cycle Analysis

The serum-starved cells were treated with HGF for 24 hours and stained with propidium iodide (Sigma-Aldrich) for determination of cellular DNA content. The percentage of cells for each different cycle phase was determined by flow cytometry (FACSVerse SE; BD Biosciences-Immunocytometry Systems, San Jose, CA).

RNA Extract and RT-PCR

Total RNA was extracted from HLE B3 cells (TRizol; Invitrogen-Gibco), according to the manufacturer’s protocol. Two micrograms of total RNA was reverse transcribed with AMV reverse transcriptase (Promega, Madison, WI). PCR was performed using a DNA thermal cycler (MJR Research, Waltham, MA). The sequences of PCR primers were as follows: cyclin A sense primer, 5’-GAGAGGCTATCCTGCTGGACG-3’; cyclin A antisense primer, 5’-AGCTCCGACAAATCTGATG-3’; cyclin B sense primer, 5’-GTTGATTGCTGGTTCAAGT-3’; cyclin B antisense primer, 5’-AGACCTTCTGAGAAAGCGA-3’; cyclin D1 sense primer, 5’-ACTACTTGGACGCTTCTG-3’; cyclin D1 antisense primer, 5’-CTCTGAGAGGAGAGCTGTG-3’; cyclin E sense primer, 5’-GAGCTCCCAGCAGGAGCATG-3’; cyclin E antisense primer, 5’-ATGATCAAGGGCCAGACG-3’; β-actin sense primer, 5’-AGGCAACCGGGAGAGATGAC-3’; and β-actin antisense primer, 5’-GAGGCGAGCGTCGAGACG-3’.

The thermocycler profile was as follows: denaturation at 94°C for 50 seconds. Annealing at 58°C for 45 seconds, and extension at 72°C for 50 seconds for 30 cycles. Twelve microliters of each PCR product were then electrophoresed on 0.8% agarose gels, and the intensities of the specific bands were analyzed.

Cyclin D1 Antisense Oligonucleotides

A 20-mer sense and antisense oligonucleotide was synthesized to the region around the translation start site of the human cyclin D1 mRNA and completely modified with phosphorothioate: For cyclin D1 sense, 5’-CCAGCCCATGGAACACACACG-3’; and antisense, 5’-GCTGTTTTCATGTCCTGGG-3’. The serum-starved cells were transfected with 800 nM oligonucleotide in 3 µL transfection reagent (CLONectin; BD Biosciences-Clontech) for 4 hours according to the manufacturer’s protocols. Cells were then washed and further cultured in serum-free medium supplemented with 10 ng/mL HGF for the indicated time.
Cells were lysed and processed either for Western blot analysis or for the proliferation assay.

**Statistical Analysis**

Results are expressed as the mean ± SE. Student’s t-test was used for statistical analysis and data at *P* < 0.05 were considered significantly different.

**RESULTS**

**Involvement of the Elevated HGF Expression in the Proliferation of LECs in Capsular Bag Culture**

The proliferation of LECs in capsular bag culture was examined by phase-contrast microscopy. The displacement of cells on the capsulorrhexis edge was observed at the outset of culture (Fig. 1Aa). After 3 days of culture, cells emerged from beneath the anterior capsule at the rhexis region (Fig. 1Ab). Subsequently, growth continued across the posterior capsule until cells on posterior capsule reached confluence (Figs. 1Ac, 1Ad). PCNA expression was determined by Western blot analysis using anti-PCNA antibody. The number of cells growing on the posterior capsule was determined by nuclear counts. The results are a compilation of three independent experiments using three separate fields.

**FIGURE 1.** The elevated HGF expression induced the proliferation of LECs in the capsular bag culture. (A) Phase-contrast micrograph of cells growing on the posterior capsule in capsular bag culture. Capsular bags were cultured for the indicated time. Cells were observed under the microscope and photographed (a–d). The cells on the anterior capsule are out of focus (b–d). (B) Capsular bags were cultured for the indicated time and then homogenized. The lysates were subjected to Western blot analysis with anti-PCNA antibody. Actin was used as the loading control. (C) Capsular bags were cultured in a serum-free medium for 14 days. The medium was sampled every 3 to 4 days and concentrated. The level of secreted HGF was determined by Western blot analysis using the anti-HGF antibody. (D) Phase micrograph of cells growing on the posterior capsule in capsular bags, which were cultured in the absence (a, c) or presence (b, d) of the neutralizing c-Met antibody (2 µg/mL) for 7 days. Cells were counterstained with Hoechst dye (c, d). The significant inhibition of cell proliferation was observed in the capsular bag treated with the neutralizing antibody. (E) Capsular bags were cultured in the absence or presence of the neutralizing c-Met antibody for 7 days and then counterstained with Hoechst dye. The number of cells growing on the posterior capsule was determined by nuclear counts. The results are a compilation of three independent experiments using three separate fields. (F) Capsular bags were cultured in the absence or presence of the neutralizing c-Met antibody for the indicated time, and then homogenized. The level of PCNA expression was determined by Western blot analysis using anti-PCNA antibody. The experiment was repeated three times with similar results. PC, posterior capsule; AC, anterior capsule. White asterisk: capsulorrhexis. *P* < 0.05 compared with control. Scale bar, 100 µm.
compared with that in the nontreated capsular bag (Figs. 1Da, 1Dc). Similarly, PCNA expression was significantly reduced by treatment with the neutralizing antibody, but not completely blocked (Fig. 1F).

**Effect of HGF on the Proliferation of LECs**

Next, we examined the effect of HGF on the proliferation of LECs using HLE B3, which expresses the HGF receptor.32 The receptor was rapidly tyrosine-phosphorylated when HLE B3 cells were stimulated with 10 ng/mL of HGF for the indicated period of times or with the various concentration of HGF (2.5–20 ng/mL) for 24 hours. Cell proliferation was evaluated using colorimetric MTT assay. (C) The serum-starved HLE B3 cells were stimulated with 10 ng/mL of HGF for the indicated time, and then cell lysates were immunoprecipitated with anti-PCNA antibody. (D) Rat lens epithelial explants were incubated in the absence or presence of 10 ng/mL HGF. After 24 hours culture, the explants were immunolabeled for BrdU-incorporation and counterstained with Hoechst dye. (E) The quiescent HLE B3 cells were stimulated with either 20% serum or 10 ng/mL of HGF for 24 hours and then stained with propidium iodide. The cells were subjected to flow cytometry. Data are the mean ± SE of triplicate determinations. *P < 0.05 compared with control. Scale bar: 50 μm.
observed (Fig. 2D). The increased proliferation of lens cells after treatment with HGF was further confirmed using flow cytometry. Serum-starved cells show a typical G1-arrest pattern, whereas the cells stimulated with HGF entered the cell cycle, with significantly increased S and G2/M rates, but the G0/G1 rate was reduced. A similar result was observed in cells stimulated with 20% serum (Fig. 2E).

**Stimulation of Cyclin D1 Expression by HGF in LECs**

To evaluate the role of HGF in the regulation of cell-cycle molecules, we examined whether HGF has any effect on the expression of cyclins. Reverse transcription–polymerase chain reaction (RT-PCR) products corresponding to the mRNAs of cyclins A, B, and E were detected in HLE B3 cells stimulated with HGF. Of interest, the expression of cyclin D1 mRNA increased in response to HGF (Fig. 3A). An immunoblot assay with anti-cyclin D1 antibodies showed that cyclin D1 protein was upregulated as early as 2 hours after treatment with HGF, and this increase was maintained for 16 hours, after which it returned to the basal level by 24 hours (Fig. 3B). In capsular bag culture, the expression of cyclin D1 also increased after 7 days of culture when the cells were highly proliferative. This increase in expression was significantly blocked by treatment with neutralizing antibodies for c-Met (Fig. 3C). To determine whether cyclin D1 is required for the cell-cycle progression stimulated by HGF, antisense cyclin D1 oligonucleotides were applied to quiescent HLE B3 cells to deplete cellular cyclin D1 protein. The HGF-stimulated induction of cyclin D1 protein expression was markedly blocked by treatment with antisense cyclin D1 oligonucleotides, whereas sense control oligonucleotides did not affect the level of cyclin D1 (Fig. 3D). Similarly, an MTT assay showed that the transfection of HLE B3 cells with the cyclin D1 antisense oligonucleotides led to significant inhibition of HGF-stimulated proliferation (Fig. 3E; \( P < 0.05 \)).

**HGF-Induced Activation of the MAPKs ERK and JNK/SAPK**

We undertook to identify the mechanism by which cyclin D1 expression is regulated in LECs. When HLE B3 cells were stimulated with HGF, the phosphorylated forms of ERK (p44 and p42) were increased. Western blot analysis showed that the maximum activation of ERK occurred 10 minutes after treatment and that this activation was maintained for up to 240 minutes, whereas there was no change in total ERK expression (Fig. 4A). The HGF-stimulated activation of ERK was completely inhibited by treatment with the MEK-specific inhibitor U0126 (Fig. 4B). Stimulation with HGF also resulted in a rapid increase in the phosphorylated forms of JNK/SAPK (p54 and p46). The activation of JNK/SAPK peaked 10 minutes after
Effect of the MAPKs, ERK and JNK/SAPK, on HGF-Induced Cyclin D1 Expression

The MAPKs activate several transcription factors, such as Elk1, ATF2, and c-Jun. We therefore examined the activation of these transcription factors by treatment with HGF for various periods. The activation of Elk1 increased as early as 15 minutes after the cells were stimulated with HGF (Fig. 5A), and addition of the MEK-specific inhibitor, U0126, significantly blocked the activation of Elk1 by HGF, whereas SP600125 did not (Fig. 5B). The activation of ATF2 and c-Jun peaked at 15 minutes and 30 minutes, respectively, and decreased thereafter (Fig. 5A). The JNK/SAPK-specific inhibitor, SP600125, blocked both ATF2 and c-Jun activation by HGF, but the addition of U0126 did not (Fig. 5B).

We investigated whether the HGF-stimulated induction of cyclin D1 in HLE B3 cells involves the MAP kinase cascade. When cells were pretreated with U0126 and SP600125 for 30 minutes followed by stimulation with HGF, cyclin D1 expression was partially inhibited by 58% and 52%, respectively, compared with DMSO treatment (P < 0.05), but there was no noticeable effect on cyclin D1 expression when cells were treated with the p38-specific inhibitor, SB202190 (Fig. 6). In contrast, when cells were treated with both U0126 and SP600125 for 30 minutes and then stimulated with HGF, the increased expression of cyclin D1 was completely inhibited (Fig. 6; P < 0.05).

DISCUSSION

The major goals of this study were to determine the effect of HGF in LECs and to identify the signaling pathway activated by HGF. Previous studies have reported that the expression of HGF is increased in cultures of human capsular bags, which is a useful model for studying the development of PCO, using enzyme-linked immunosorbent assays (Wormstone IM, et al. IOVS 1998;39:ARVO Abstract 983). Consistent with these reports, we observed in this study that the level of secreted HGF in the culture medium increased throughout the culture of capsular bags and that the expression of PCNA also increased during the same period, with an expression profile similar to that of HGF. This suggests that HGF is involved in the proliferation of LECs in capsular bag culture. Our data show that the cell growth and increase in PCNA expression were significantly, but not completely, blocked by the application of neutralizing antibodies directed against the HGF receptor, c-Met.
A

Inhibitors: DM U0 SB2 SP DM U0+SP

HGF: - + - - + +

Cyclin D1

Actin

B

FIGURE 6. The MAPKs ERK, and JNK/SAPK collaborated to regulate HGF-induced cyclin D1 expression. (A) The cell lysates from the quiescent HLE B3 cells treated with U0126, SB202190, SP600125 or both U0126 and SP600125 were immunoblotted with anti-cyclin D1 antibody. (B) The blots were scanned and analyzed by densitometry, and the results are represented in histogram as the average ± SE of three independent experiments. *p < 0.05 compared with DM+HGF(+). DM, DMSO; U0, U0126; SB2, SB202190; SP, SP600125.

providing direct evidence that HGF stimulates the proliferation of LECs. This partial blockade of proliferation may be because the antibody could not completely neutralize the receptor-ligand interaction. However, we have not ruled out the possibility that other cytokines, such as bFGF or EGF, are involved in the proliferation of LECs. Taken together, our results imply that HGF is upregulated in the LECs that remain after cataract surgery by unknown mechanisms and that secreted HGF may promote the proliferation of LECs through the activation of c-Met, thus contributing to the development of PCO.

Cell culture studies using HLE B3 cells have demonstrated that stimulation with HGF results in the induction of cyclin D1 mRNA and protein. It is worth noting that HGF stimulates the expression of cyclin D1 in LECs, because cyclin D1 is rate-limiting for progression through the G1 phase of the cell cycle.\(^{55-58}\) A previous study has demonstrated that cyclin D1 protein is readily detected in the proliferative anterior epithelial layer of the lens and to a lesser degree in differentiating lens fiber cells of the equatorial region. Moreover, ectopic expression of the D-type cyclins, but not cyclin E, sufficiently promotes exit from G1 in normally postmitotic lens fiber cells in which cyclin D1 is not detected.\(^{59}\) We also observed in the present study that the well-known mitogenic factors, bFGF and EGF, stimulated the expression of cyclin D1 (data not shown). Thus, the regulation of cyclin D1 may be a key event in lens epithelial cell proliferation. Our studies have shown that the inhibition of cyclin D1 protein using antisense oligonucleotides resulted in significant inhibition of HGF-induced proliferation, suggesting that HGF is a potent growth factor for LECs and that HGF-induced proliferation requires the upregulation of cyclin D1 expression.

How then does HGF regulate the expression of cyclin D1 in LECs? Extracellular mitogens promote cellular proliferation through receptor-mediated signaling circuitry that ultimately converges on the cell-cycle machinery. The D-type cyclins function as critical sensors of these signals. Receptor-dependent activation of Ras promotes transcription of the cyclin D1 gene through a kinase cascade involving Raf1, MEK, and ERK.\(^{40-44}\) Our data indicate that stimulation with HGF induces activation of ERK, which in turn promotes cyclin D1 expression. However, the MEK1-specific inhibitor, U0126, did not totally block HGF-stimulated cyclin D1 expression, indicating that cyclin D1 may also be regulated by other pathways. Recently, JNK/SAPK and p38 were shown to induce the upregulation of cyclin D1 and cell proliferation in different cell types.\(^{45,46}\) Indeed, we found that the stimulation with HGF induced the activation of JNK/SAPK and that HGF-stimulated cyclin D1 expression was partially inhibited by the specific JNK/SAPK inhibitor, SP600125, which completely abolished the HGF-stimulated phosphorylation of ATF2 and c-Jun. However, treatment with both U0126 and SP600125 led to the complete inhibition of cyclin D1 expression, suggesting that...
the HGF-stimulated induction of cyclin D1 requires collaborative interactions between the MEK-ERK and MKK-JNK/SAPK pathways in LECs. Moreover, our data show that the PI3K-specific inhibitor, wortmannin, blocked not only the activation of ERK and JNK/SAPK, but also the expression of cyclin D1, suggesting that PI3K acts upstream from these MAPKs and participates in the proliferation of LECs.

It is worth noting that stimulation with HGF induced the phosphorylation of GSK3β on Ser-9, which inactivates its kinase activity, which is mediated by the PI3K/AKT pathway. Previous studies have demonstrated that GSK3β catalyzes the phosphorylation of cyclin D1 on Thr-286, thereby regulating cyclin D1 turnover, which is mediated by ubiquitination and subsequent proteolytic destruction.\(^\text{47,48}\) Inactivation of GSK3β by enforced overexpression of a constitutively active form of AKT results in prolongation of the cyclin D1 half-life, as well as a decrease in D1 phosphorylation.\(^\text{47}\) Therefore, in LECs, HGF may promote the dephosphorylation and stabilization of cyclin D1 through the inactivation of GSK3β mediated by the PI3K/AKT pathway. This could enhance the activities of cyclin-dependent protein kinases 4 (CDK4) and 6 (CDK6), which in turn, both ERK and JNK/SAPK collaborate to induce the cyclin D1 expression, mediated by the activation of transcription factors, Elk1, ATF2, and c-Jun, which is required for the proliferation of LECs. In addition, HGF induces the inactivation of GSK3β by PI3K/AKT pathway. It may lead to dephosphorylation and stabilization of cyclin D1, and consequently enhance the ability of HGF to stimulate the proliferation of LECs.

**References**


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