RESEARCH ARTICLE

PKM2 Regulates Hepatocellular Carcinoma Cell Epithelial-mesenchymal Transition and Migration upon EGFR Activation

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Abstract

Pyruvate kinase isozyme type M2 (PKM2) was first found in hepatocellular carcinoma (HCC), and its expression has been thought to correlate with prognosis. A large number of studies have demonstrated that epithelial-mesenchymal transition (EMT) is a crucial event in hepatocellular carcinoma (HCC) and associated metastasis, resulting in enhanced malignancy of HCC. However, the roles of PKM2 in HCC EMT and metastasis remain largely unknown. The present study aimed to determine the effects of PKM2 in EGF-induced HCC EMT and elucidate the molecular mechanisms in vitro. Our results showed that EGF promoted EMT in HCC cell lines as evidenced by altered morphology, expression of EMT-associated markers, and enhanced invasion capacity. Furthermore, the present study also revealed that nuclear translocation of PKM2, which is regulated by the ERK pathway, regulated β-catenin-TCF/LEF-1 transcriptional activity and associated EMT in HCC cell lines. These discoveries provide evidence of novel roles of PKM2 in the progression of HCC and potential therapeutic target for advanced cases.

Keywords: Epithelial growth factor receptor - PKM2 - β-catenin - epithelial-mesenchymal transition - HCC

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer and the second leading cause of cancer-related death in the world (Altekruse et al., 2012; Wiangnon et al., 2012; Norsa'adah et al., 2013). Surgical resection, providing a long-term survival of HCC patients, is regarded as one of the standard treatments of HCC if the tumor is resectable (Personeni et al., 2012; Zhou et al., 2012b). However, approximately 30-50% patients develop HCC recurrence during the first year after surgery, and in general, the recurrent HCCs are more diffuse and hardly treatable (Li et al., 2013; Zhu et al., 2013). Therefore, prevention of HCC recurrence is the key to improve the survival rate of the patients. However, unfortunately, the molecular mechanisms and the biomarkers for HCC recurrence remain largely unknown.

EMT is a process in which cells with epithelial phenotypes acquire mesenchymal characteristics. This transition is characterized by combined loss of epithelial cell junction proteins such as E-cadherin and by concomitant gain of mesenchymal markers such as Vimentin and N-cadherin (Eastham et al., 2007). Recently, it is widely accepted that EMT is the initiate step of intra-hepatic and extra-hepatic, Which were necessary for recurrence of HCC after resection (Tsuji et al., 2009). However, the mechanisms of EMT in HCC remain largely unknown.

Pyruvate kinase isozyme type M2 (PKM2) is expressed in cells with a high rate of nucleic acid synthesis, such as normal proliferating cells, embryonic cells, and especially tumor cells (Tamada et al., 2012). PKM2 catalyzes phosphoenolpyruvate to pyruvate and is responsible for net ATP production within glycolysis (Macintyre et al., 2011). PKM2 contains an inducible nuclear translocation signal in its C-domain (Yang et al., 2012). It has been described that nuclear PKM2 is pro-proliferative or pro-apoptotic, depending on environmental conditions (Spoden et al., 2009). Recently, it has been found that PKM2 inhibits or promotes EMT in gastric cancer cells, depending on differentiation status of the cells (Wang et al., 2013). Furthermore, expression of PKM2 mRNA is significantly correlated with the progression of malignant phenotypes in HCC (Tanaka et al., 2013). However, the

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role of PKM2 in EMT of HCC remains to be determined. Dysregulation of signal transduction pathways of growth factors, including transforming growth factor beta (TGF-β)/Smad, platelet-derived growth factor (PDGF)/PDGF receptor and hepatocyte growth factor (HGF)/c-Met, contributes to EMT of HCC (Fischer et al., 2007; Ogunwobi et al., 2011; Xu et al., 2003). Activation of epidermal growth factor (EGF) signaling is initiated by induction of the phosphorylation of EGF receptor (EGFR). It has been shown that EGFR is activated in 40-70% of HCC, and the activation of EGFR correlates with pathogenesis and prognosis of HCC (Buckley et al., 2008). It had been reported that EGFR signal was necessary for PKM2 promoting tumor progression. Here, the present study was to investigate the role of PKM2 in induction of HCC EMT and disclose the underlying mechanism.

Materials and Methods

Reagents and antibodies

Recombinant human EGF was purchased from Peprotech (Rocky Hill, New Jersey, USA) and was dissolved in 0.1% bovine serum albumin (BSA; Shanghai, China). Chemicals of LY294002, rapamycin, U0126, and SKI-1 were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies used for Western blotting analyses were against p-β-catenin (Ser552), ERK1/2, p-ERK1/2 (Thr202/Tyr204), LRP, p-LRP (Ser1490), Axin1, SRC, p-SRC (Tyr416), Snail, Twist, Slug, EGFR, vimentin, E-cadherin (Cell Signaling Technology Inc, Danvers, MA, USA), β-catenin, GSK3β, p-GSK3β (Ser9), EGFR, p-EGFR (Thr 669), p-mTOR (Ser2448), lamin A/C (Epitomics, San Francisco, CA, USA), mTOR, p-β-catenin (Tyr333), AKT, p-AKT (Ser473), PKM2 (Signalway Antibody, Baltimore, USA), c-Myc, cyclin D1 (GeneTex, Irvine, CA, USA), and GAPDH (Sigma, St Louis, MO, USA). All horseradish peroxidase-conjugated secondary antibodies and the fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were commercially obtained from Cell Signaling Technology Inc. (Danvers, MA, USA).

Cell lines and culture

HepG2, SMMC7721 and MHCC-97-H cell lines were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and were cultured in Dulbecco's modified eagle medium (DMEM) or RPMI-1640 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 μg/ml streptomycin, and grown in a 95% air and 5% CO_2 humidified atmosphere at 37°C.

Cell proliferation assays

The effect of EGF on cell proliferation was determined using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI, USA). Briefly, HCC cells, seeded in 96-well plates (3×10^4 cells/well), were serum-starved in DMEM for 24 h, and then treated with EGF (0-100 ng/ml) for 24 h, followed by incubation with the MTS reagent (20 μl/well) for 4 h. Finally, the optical density (OD) was measured at 490 nm using a SynergyTM 2 Multi-function Microplate Reader (Bio-Tek Instruments, Winooski, Vermont, USA).

Wound healing assays

Cells were seeded in 6-well plates and were then subjected to various treatments as indicated. After reaching 90% confluence, the cell monolayers were wounded with a sterile plastic tip and were cultured in serum-free DMEM in the presence of EGF (0-100 ng/ml). Migration of cells into wounded areas was evaluated and photographed under an inverted microscope equipped with a digital camera.

Transwell cell motility assays

Cell motility was also assessed using an 8-μm pore size Transwell system (Corning, NY, USA). Briefly, serum-starved cells were trypsinized, resuspended in DMEM, and added to the upper chamber in 24-well plates (1×10^5 cells/well) in the absence or presence of EGF and/or the inhibitors at indicated concentrations, while the lower chamber contained 600 μl of DMEM with 10% FBS. After incubation for 24 h, cells that migrated to the lower surface of the filter membrane were fixed in ethanol and stained with crystal violet. Migrated cells were counted and photographed under an inverted microscope equipped with a digital camera.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized with 1 μg of total RNA using a PrimeScript RT reagent kit (TakaraBio, Tokyo, Japan). The qRT-PCR was performed using IQTM SYBR Green supermix and the iQ5 real-time detection system (Bio-Rad Laboratories, Hercules, CA, USA). Primers used for qRT-PCR were as described in the following (Zhang et al., 2013): E-cadherin: (forward) 5'-TCCCATCAGCTGCCCAGAAA-3', (reverse) 3'-TGACTCCTGTGTTCCTGTTA-5'; Vimentin: (forward) 5'-ATGAGTACCGGAGACAGGTGCAGA-3'; (reverse) 5'-ATAGCAGCTTCAACGGCAAAGTTCA-3'; Snail: (forward) 5'-TTCTTCTGCGCTACTGCTGCG-3'; (reverse) 3'-GGGCAGGTATGGAGAGGAAGA-5'; GAPDH: (forward) 5'-TGGTATCGTGGAAGGACTCAT GAC-3'; (reverse) 3'-ATGCCAGTGAGCTTCCCGTTCA GC-5'.

Reaction mixtures contained 7.5 μl of SYBR Green I dye master mix (Quanta), 2 pM of forward and reverse primers. Thermocycle conditions included initial denaturation at 50°C and 95°C (10 min each), followed by 40 cycles at 95°C (15 s) and 60°C (1 min). mRNA abundance was determined by $2^{-\Delta\Delta CT}$ method. In the mean time, the PCR products were size-fractionated on 1.5% agarose gels and visualized on a UV transilluminator.

Western blotting analyses

Total lysates from treated cells were prepared with RIPA buffer [50 mM Tris, pH 7.2; 150 mM NaCl; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 1% Nonidet P-40; 10 mM NaF; 1 mM Na_3VO_4; protease

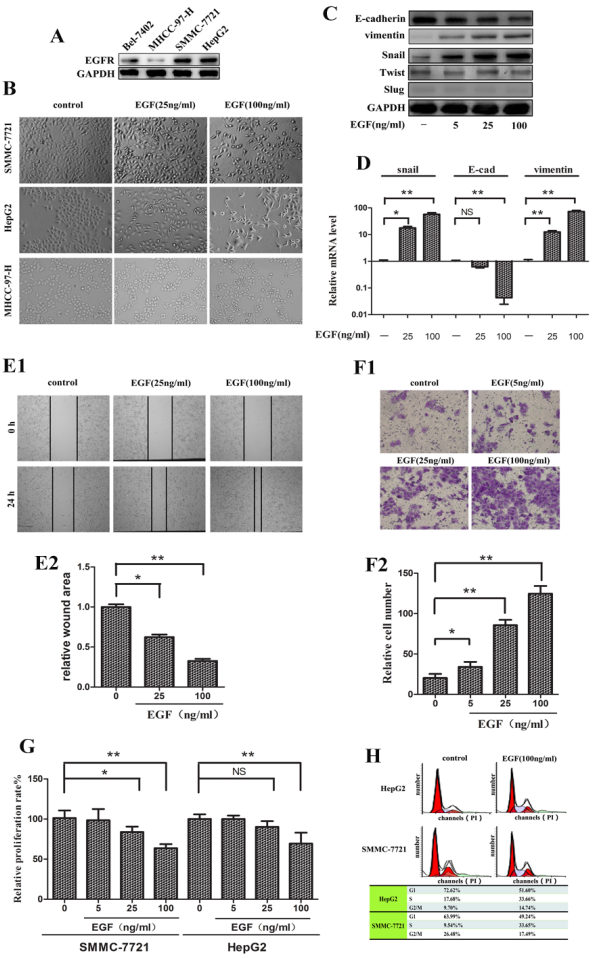

Figure 1. Activation of EGF Signaling Stimulates the Expression of EMT Markers and Cell Motility of HCC Cells. Serum-starved HCC cells were treated with EGF at indicated concentrations for 24 h. (A) Expression of EGFR was evaluated in HCC cells lines of Bel-7402, MHCC-97-H, SMMC-7721 and HepG2 by Western blotting analyses; (B) Cell morphology was assessed in HepG2 and SMMC-7721 cells; Expression of EMT markers in SMMC-7721 were receptively analyzed by Western blotting analyses (C) and by q-RT-PCR (D); HCC cell invasion was assessed in SMMC-7721 by wound healing (E1 and E2) and by transwell invasion (F1 and F2); (G) EGF inhibited HCC cells proliferation significantly in a dose dependent manner in 24 hours. (H) flow cytometric cell cycle analysis showed EGF treated for 24 hours dramatically decreased the proportion of cells in the S or G2 phase. GAPDH was used in Western blotting analyses as an invariant control for equal loading. Representatives were from three independent experiments. Values in assays were expressed as mean ± SD. *$P \leq 0.05$, **$P \leq 0.01$. NS=non-significant

inhibitor cocktail (1:1000, Sigma, St. Louis, MO)]. Lysates were sonicated for 10 s and centrifuged at 14, 000 rpm for 10 min at 4°C. Nuclei were isolated using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA) following the manufacturer's protocol. Protein concentration was determined by bicinchoninic acid assay with BSA as a standard (Pierce, Rockford, IL, USA). Equivalent amounts of protein (50 μg/lane) were separated on 7.5%-12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were incubated with PBS containing 0.05% Tween 20 and 5% nonfat dry milk to block nonspecific binding and were incubated with primary antibodies, then with appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized by using Renaissance chemiluminescence reagent (Perkin-Elmer Life Science, Boston, MA, USA).

Knock-down protein expression by small interfering RNA (siRNA)

Cells were transfected with β-catenin siRNA, Snail siRNA, PKM2 siRNA or non-specific control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using the opti-MEM plus X-tremeGENE siRNA transfection reagent (Roche, Basel, Switzerland) prepared in antibiotic-free complete culture medium. After 24 h, the cells were used for experiments. The effect of each siRNA was confirmed by Western blot analyses using corresponding antibodies.

Dual luciferase reporter gene assays

Cells in 96-well plates were transiently transfected with 0.1 μg/well of the TCF/LEF-responsive luciferase construct (Luc2P/TCF-LEF/Hygro, Promega, Madison, WI, USA) for 24 h. Transfection efficiencies were normalized with renilla luciferase reporter plasmid. Relative promoter activity was measured by dual-luciferase reporter assay system using the Glomax 96 Microplate Luminometer (Promega, Madison, WI, USA)

Immunofluorescence (IF) staining

Cells plated on glass coverslips in 6-well plates were incubated with 100 ng/ml EGF and/or inhibitors at indicated concentrations for 24 h and were then fixed in 4% paraformaldehyde for 15 min. After being blocked for 1 h at room temperature with 1% BSA, the cells were incubated overnight at 4°C with mouse monoclonal antibody to β-catenin (1:500). Following washing with PBS for 3 times at room temperature, the cells were further incubated with FITC-conjugated goat anti-mouse IgG and Hoechst 33342 (Sigma) for 1 h at room temperature. The coverslips were mounted to glass slides, and the immunofluorescence staining was visualized and photographed using a Zeiss inverted fluorescence microscope. Negative control staining was performed by omitting the primary antibody.

Flow cytometry assay

Cells treated with or without EGF for 24 h were detached with trypsin and washed in cold phosphate buffered saline (PBS). For cell cycle analysis, precipitated cells were fixed by cold 70% ethanol of 500 μl overnight at -20°C and washed in cold PBS. Fixed cells were then incubated with RNase at 37°C for 30 min and stained with propidium iodide for 15 min at room temperature in dark and immediately analyzed by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA).

Statistical analysis

Data were presented as mean ± SD, and results were analyzed using SPSS16.0 software. The significance of difference was determined by one-way ANOVA with

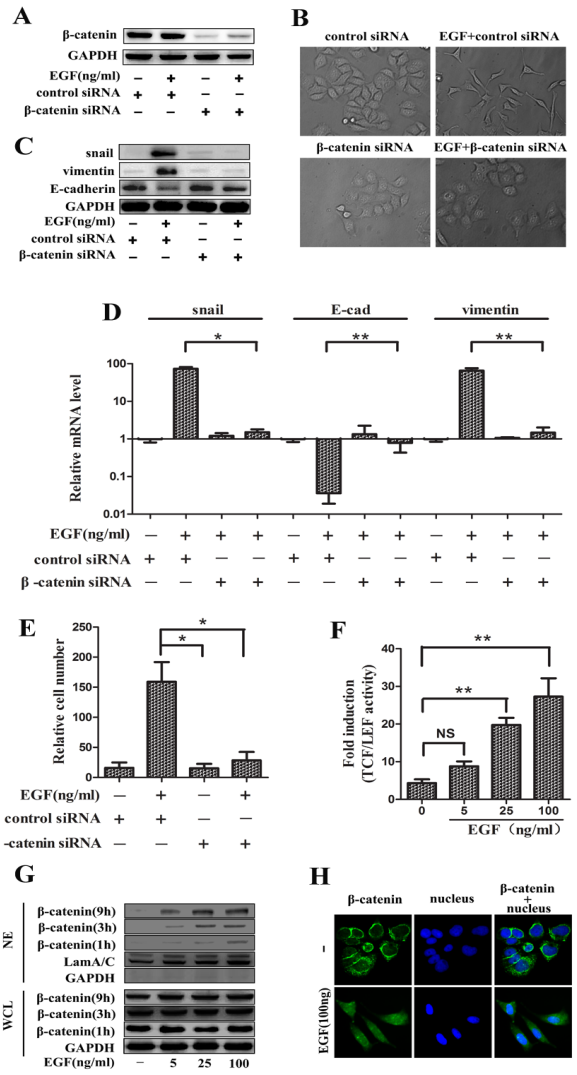

Figure 2. β-Catenin Mediates EGF-induced EMT and Cell Motility in HCC Cells. For inhibition of β-catenin expression by siRNA, cells were first transfected with β-catenin siRNA or nc-siRNA. After 24 h recovery, cells were treated with EGF at indicated concentrations for an additional 24 h. GAPDH or Lam A/C was used in Western blotting analyses as an invariant control for equal loading. Representatives were from three independent experiments. Values in assays were expressed as mean ± SD. *$P \leq 0.05$, **$P \leq 0.01$. NS=non-significant. (A) Western blotting analyses of β-catenin expression in HCC cells after transient transfection with β-catenin siRNA or nc-siRNA; (B) Evaluation of effects of EGF on cell morphology (×400) after transient transfection with β-catenin siRNA or nc-siRNA; Determination of the role of EGF in regulation of expression of protein markers of EMT after transient transfection with β-catenin siRNA by Western blotting analyses (C) and q-RT-PCR assays (D); (E) Evaluation of effects of EGF on cell invasion of SMMC-7721 cells by transwell invasion assays after transient transfection with β-catenin siRNA; (F) SMMC-7721 cells were first transfected with β-catenin siRNA or nc-siRNA, pGL4.49[luc2p /TCF -LEF RE/Hygro] and internal control plasmid for 24 h, then cells were treated with EGF at indicated concentrations for additional 24 h, dual luciferase reporter gene was used to address the effect of EGF on inducting of β-catenin transcriptional activity in HCC cells. SMMC-7721 cells were treated with EGF at indicated concentrations for indicated time,(G) Western blot and (H) IF were used to explore the effect of EGF signaling on inducing β-catenin nuclear translocation in HCC cell lines. Representatives were from three independent experiments. NE: nuclear extracts, WCL: whole-cell lysates

the post-hoc Dunnett's test. Values of $P < 0.05$ were considered to be statistically significant.

Results

EGF promotes EMT and cell migration in HCC cell lines

EMT describes the dedifferentiation switch between polarized epithelial cancer cells and contractile and motile mesenchymal (invasive) cells during cancer progression and metastasis (Guarino, 2007). We first examined EGFR expression in four HCC cell lines, including Bel-7402, MHC-97-H, SMMC-7721 and HepG2. It appeared that HepG2 and SMMC-7721 cells were expressing much more EGFR than MHCC-97 and Bel-7402 cells (Figure 1A). Of interest, treatment with EGF (25 and 100 ng/ml) for 24 h induced a remarkable morphological change (from cobble-stone-like appearance to elongated leading-trailing mesenchymal shape, indicating EMT) in HepG2 and SMMC-7721 cells, and a weak morphological change in MHCC-97 cells (Figure 1B). This observation suggested that higher expression of EGFR in HCC cells might be better correlated with the development of EMT. To further explore the role of the activation of EGF signaling in the development of EMT, the expression of a group of markers for EMT were examined in SMMC-7721 cells after treatment with EGF for 24 h. Results from Western blotting analyses showed that the epithelial marker E-cadherin was markedly down-regulated, while the mesenchymal markers vimentin and Snail were dramatically up-regulated after treatment with EGF in the cells in a concentration-dependent manner (Figure 1C), though the expression of Twist and Slug was not obviously altered. Similarly, EGF also up-regulated the mRNA levels of vimentin and Snail, and down-regulated the mRNA level of E-cadherin, as detected by qRT-PCR (Figure 1D).

Additional experiments were conducted to evaluate the role of EGF in regulating cell motility, another major phenotype of EMT. It was observed that both wound healing assays (Figure 1E1 and E2) and Transwell assays (Figure 1F1 and F2) consistently demonstrated that treatment with EGF for 24 h stimulated cell motility in a concentration-dependent manner in SMMC-7721 cells. Taken together, these results indicated that activation of EGF signaling stimulated the expression of the markers of EMT and cell motility of HCC cells.

It is known that EGF can stimulate cell proliferation. However, our current data showed that EGF did not increase HCC cell proliferation significantly (Figure 1G). Further flow cytometry analyses showed that EGF induced cell cycle arrest at S phase in HCC cell lines (Figure 1H). Taken together, these results indicated that EGF induced EMT and enhanced cell motility within 24 h in HCC cell lines, while it can not promote HCC cell proliferation in the same stimulating time.

β-Catenin mediates EGF-induced EMT and cell motility in SMMC-7721

β-Catenin has been found to be co-activated with EGFR in HCC (Llovet et al., 2008). To determine whether β-catenin mediates EMT and cell motility, expression

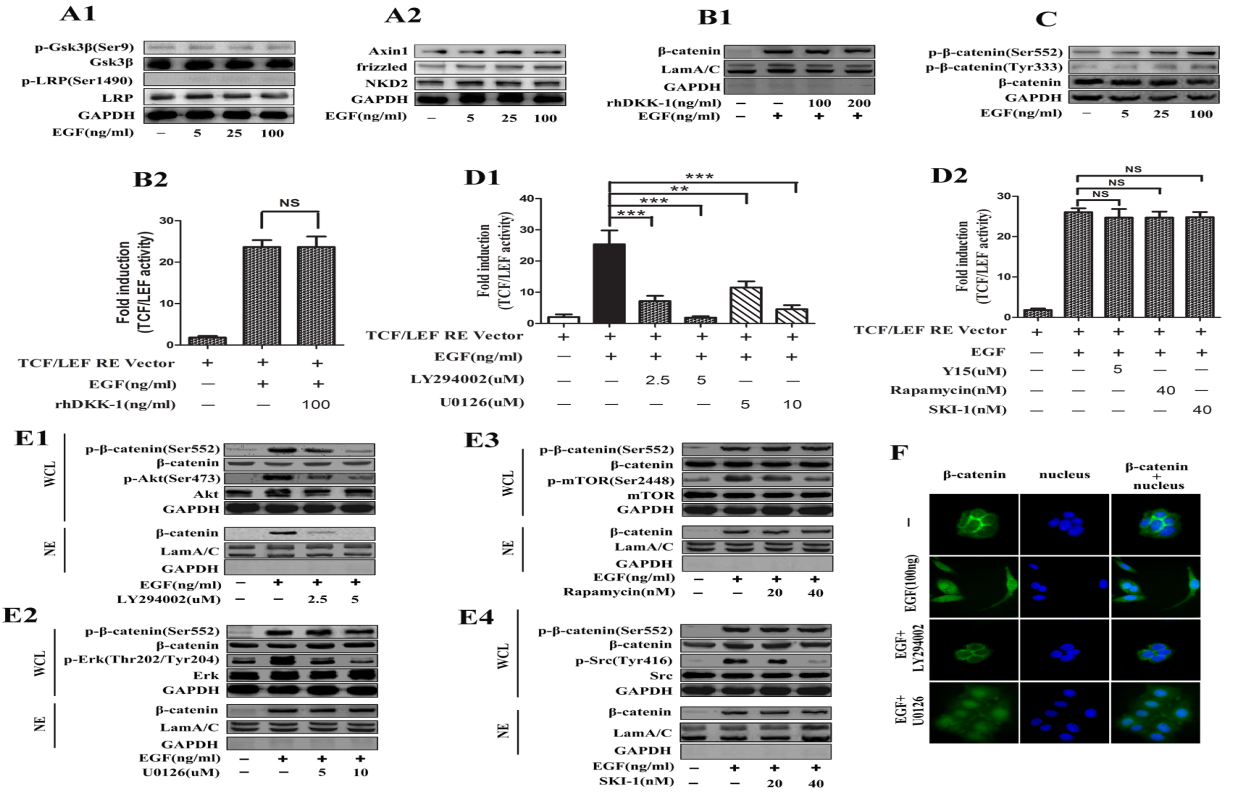

Figure 3. Both PI3K and ERK Pathways are Involved in EGF-induced β-catenin-TCF-LEF Transactivation. For Dual luciferase reporter assay, cells were first transfected with pGL4.49[luc2p/TCF-LEF RE/Hygro] and internal control plasmid for 24 h, then cells were subjected to different treatments described in each panels below. (A1 and A2) SMMC-7721 cells was treated with EGF at indicated concentrations for 24 h, Western blot was used to investigate the effect of EGF on the expression of related Wnt pathway proteins. SMMC-7721 cells were pretreated with Wnt inhibitor (rhDKK) at indicated concentrations for 30 min, then treated with EGF at indicated concentrations for 24 h, (B1) Western blot and (B2) dual luciferase reporter gene assayes were used to investigate the effect of Wnt inhibitor on EGF-induced β-catenin transactivation. (C) SMMC-7721 cells were treated with EGF at indicated concentrations for 9 h, Western blot was used to investigate the effect of EGF on phosphorylation level of β-catenin at Tyr 333 and Ser 552. (D1 and D2) SMMC-7721 cells were pretreated with specific inhibitors of PI3K (LY294002), mTOR (rapamycin), SRC (SKI-1), FAK (Y15) and ERK (U0126) at indicated concentrations for 30 min, respectively, then treated with EGF at indicated concentrations for 24 h, Luciferase reporter gene assay was used to address the role of above inhibitors in EGF-induced β-catenin transactivation. SMMC-7721 cells were pretreated with specific inhibitors of PI3K (LY294002), and ERK (U0126) at indicated concentrations for 30 min, respectively, then treated with EGF at indicated concentrations for 24 h, (E1, E2, E3 and E4) Western blot and (F) IF assays were used to explore the role of PI3K and ERK signals in EGF-induced β-catenin nuclear translocation. GAPDH and LamA/C are loading controls. Representatives were from three independent experiments. NE: nuclear extracts, WCL: whole-cell lysates. Data are expressed as mean ± SD. $*P \le 0.05$, $**P \le 0.01$, $***P \le 0.001$, NS=non-significant

of β-catenin was silenced by siRNA in SMMC-7721 cells. It was observed that siRNA significantly and dose-dependently inhibited the expression of β-catenin in SMMC-7721 cells (Figure 2A). The down-regulation of β-catenin by siRNA effectively abrogated EGF-induced EMT morphological change in the cells (Figure 2B). Further experiments observed that EGF failed to alter the expression of the EMT markers at protein and mRNA levels in β-catenin-silenced HCC cells demonstrated by Western blotting analyses (Figure 2C) and qRT-PCR analyses (Figure 2D). Consistently, the down-regulation of β-catenin by siRNA dramatically suppressed EGF-stimulated cell motility (Figure 2E). These observations collectively indicated that β-catenin played a critical role in mediating EGF-induced EMT and motility in HCC cell. As β-catenin regulates EMT and tumor metastasis usually by enhancing transcriptional activity of the transcription factor TCF-LEF (Fang et al., 2007; Ji et al., 2009; Lu et al., 2003), we investigated it is, therefore, plausible to elucidate whether EGF up-regulates TCF-LEF transcriptional activity in HCC cells. SMMC-7721 cells were transfected with a β-catenin trans-activation activity luciferase reporter plasmid Luc2P/TCF-LEF/Hygro. After recovery, cells were treated with EGF at various concentrations for 24 h. As shown in (Figure 2F) by luciferase assays, EGF dose-dependently increased β-catenin-TCF-LEF trans-activation demonstrated by elevating luciferase activities in SMMC-7721 cells. Further experiments confirmed that EGF induced the accumulation of nuclear β-catenin in the cells after treatment with EGF at indicated concentrations, indicated by Western blotting analyses (Figure 2G) and by immuno-fluorescent staining (Figure 2H). Taken together, our results indicated that β-Catenin mediated EGF-induced EMT and cell motility in HCC cells.

Both PI3K and ERK pathways are involved in EGF-induced β-catenin-TCF-LEF transactivation, EMT and cell motility

Wnt-dependent and -independent mechanisms have

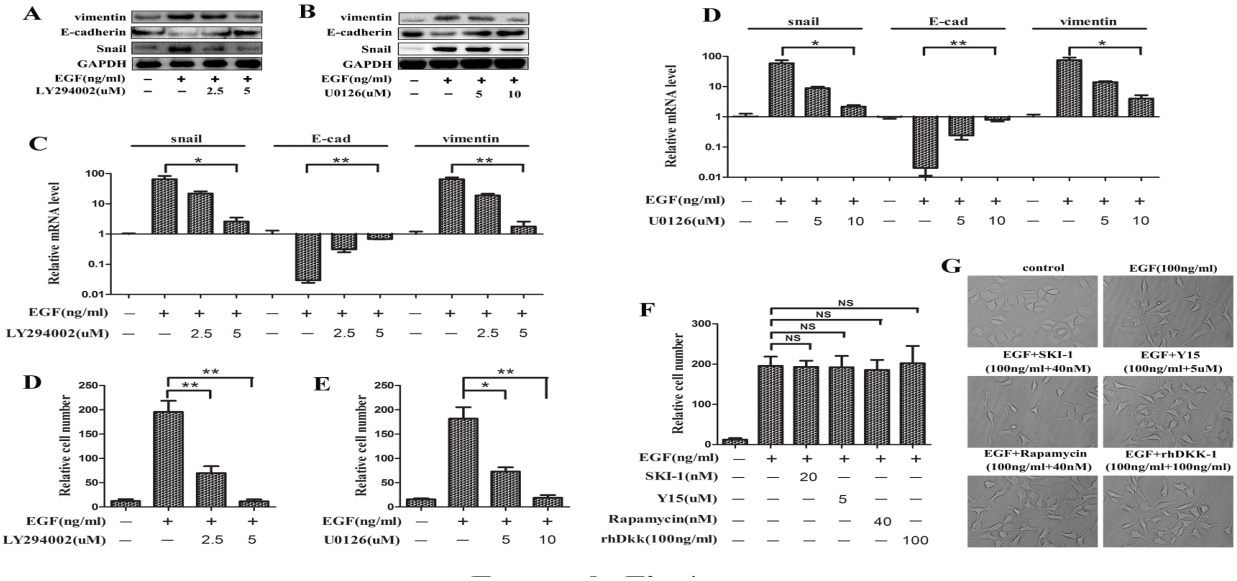

Figure 4. Both PI3K and ERK Pathways are Involved in EGF-induced EMT and Cell Motility. SMMC-7721 cells were pretreated with specific inhibitors of PI3K (LY294002), mTOR (rapamycin), SRC (SKI-1), FAK (Y15) or ERK (U0126) at indicated concentrations for 30 min, respectively, followed by the treatment with EGF at indicated concentrations for 24 h. (A and B) Western blotting analyses, (C) q-RT-PCR and (G) cell morphology (×400) assays were used to investigate the effect of above inhibitors on EGF-induced HCC EMT; (D, E and F) transwell assays were used to address the effect of above inhibitors on EGF-induced HCC cell motility. GAPDH was used in Western blotting analyses as an invariant control for equal loading. Representatives were from three independent experiments. Values in assays were expressed as mean ± SD. *$P \le 0.05$, **$P \le 0.01$, ***$P \le 0.001$, NS = non- significant

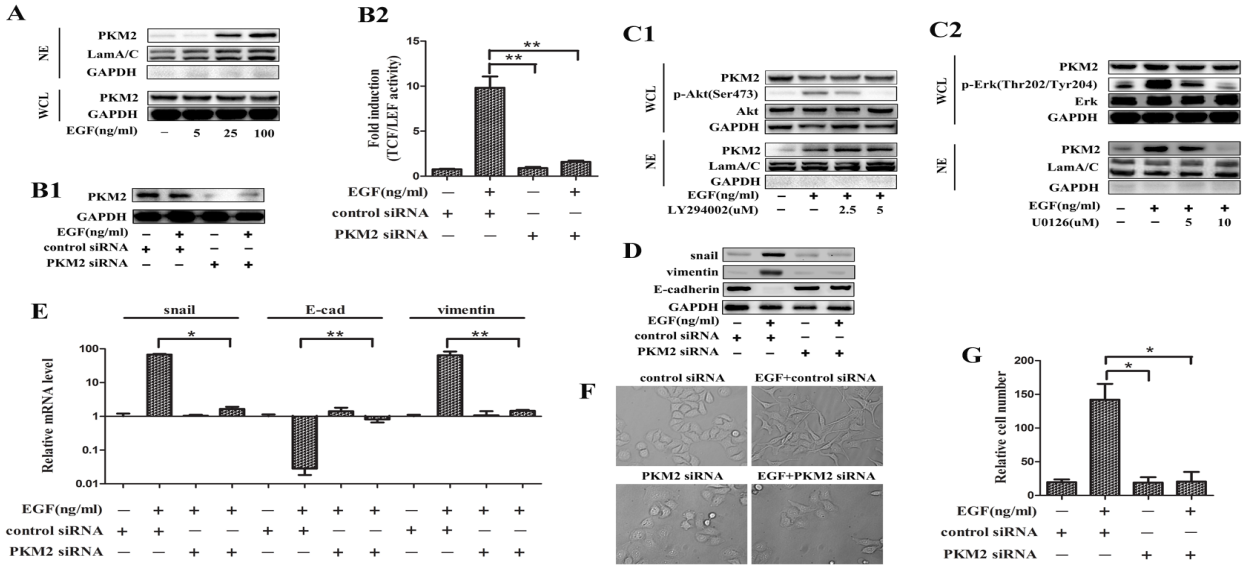

Figure 5. ERK1/2-dependent Nuclear Translocation of PKM2 Regulates β-catenin-TCF- LEF Transcriptional Activity, EMT and Motility in HCC Cells. For transient transfection with siRNAs, cells were first transfected with 160 nM PKM2 siRNA or 160 nM nc-siRNA for 24 h, then cells were subjected to different treatments described in each panels below. (A) SMMC-7721 cells were treated with EGF at indicated concentrations for 9 h, Western blot assay was used to address the effects of EGF on the expression of PKM2. (B1) Western blot analysis showed that PKM2 was knocked down in HCC cells. (B2) For Luciferase assay, SMMC-7721 cells were co-transfected with pGL4.49 [luc2p /TCF -LEF RE/Hygro] and internal control plasmid for 24 h, then cells were treated with EGF at indicated concentrations for additional 24 h, Luciferase reporter gene assay was used to explore the role of PKM2 in EGF-induced TCF-LEF transcriptional activity in HCC cells. (C1 and C2) SMMC-7721 cells were pretreated with PI3K, ERK inhibitors for 30 min, respectively, then treated with EGF at indicated concentrations for 9 h. Western blot was used to investigate the role of PI3K and ERK signals in EGF-induced nuclear translocation of PKM2 in HCC cells. (D) Western blot, (E) q-PCR, (F) cell morphology (×400) assays were used to investigate the role of PKM2 in EGF-induced EMT in HCC cells; (G) Transwell analysis was used to investigate the role of PKM2 in EGF-induced motility in HCC cells. Representatives were from three independent experiments. GAPDH and LamA/C are loading controls. NE: nuclear extracts, WCL: whole-cell lysates. Data are expressed as mean ± SD. *$P \le 0.05$, **$P \le 0.01$, ***$P \le 0.001$, NS=non-significant

been observed to be involved in EGF-induced β-catenin-TCF-LEF transactivation (Hu et al., 2010). However, the role of Wnt pathway in HCC cells is unclear. To address this issue, SMMC-7721 cells were treated with EGF for

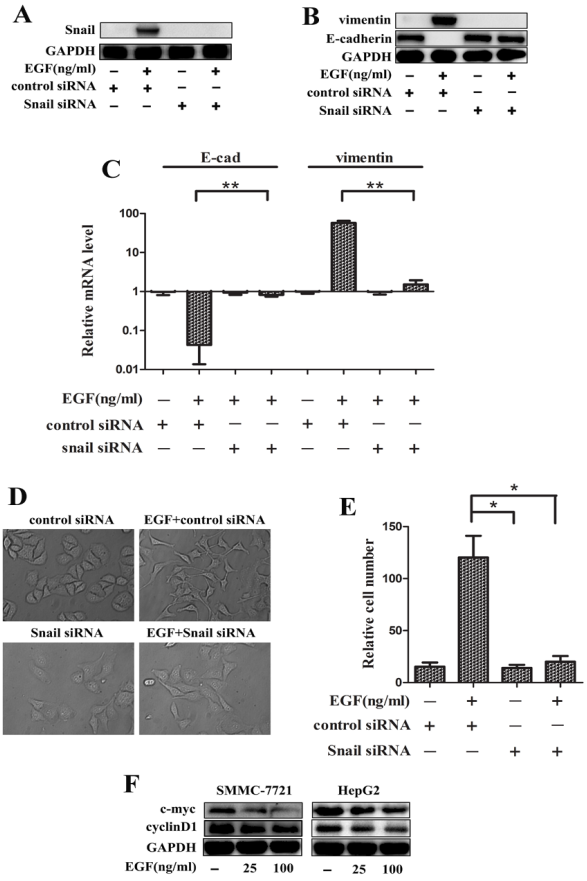

Figure 6. Snail is Critical for EGF-induced EMT and Motility in HCC Cells. For inhibition of Snail expression by siRNA, SMMC-7721 cells were first transfected with Snail siRNA or nc-siRNA. After 24 h recovery, cells were treated with EGF (100 ng/ml) for an additional 24 h. (A) Western blotting analyses showed that Snail was knocked down in HCC cells; (B) Western blotting analyses and (C) q-RT-PCR and (D) cell morphology (×400) assays were used to investigate the role of Snail in EGF-induced EMT in HCC cells; (E) Transwell assay was used to address the role of Snail in EGF-induced motility in HCC cells. (F) Western blot analyses showed that c-myc and cyclinD1 was decreased dose-dependently after treating with indicated concentrations of EGF in SMMC-7721 and HepG2 cell lines. GAPDH was used in Western blotting analyses as an invariant control for equal loading. Representatives were from three independent experiments. Values in assays were expressed as mean ± SD. $*P \leq 0.05$, $**P \leq 0.01$. NS=non-significant

24 h, followed by Western blotting analyses and TCF-LEF luciferase reporter gene analyses. We found that EGF did not obviously alter the expression of Axin1, frizzled, NKD2, p-GSK3β, GSK3β, p-LRP and LRP in the cells (Figure 3A1 and A2). Furthermore, treatment with rhDKK-1 (100-200 ng/ml), a selective Wnt inhibitor, for 24 h, did not prevent EGF-induced β-catenin nuclear translocation (Figure 3B1) and TCF-LEF transcriptional activity (Figure 3B2). The results suggested that EGF up-regulated β-catenin transcriptional activity via a Wnt-independent mechanism.

Next, we asked whether EGF affected phosphorylation of β-catenin, which has been shown to be positively correlated with its nuclear translocation and independent of Wnt signaling (Fang et al., 2007; Yang et al., 2011). Interestingly, treatment with EGF for 24 h induced phosphorylation of β-catenin at Ser552 and Tyr 333 in a concentration-dependent manner in SMMC-7721 cells (Figure 3C). This observation supported the hypothesis that the activation of EGFR resulted in phosphorylation of β-catenin, leading to Wnt-independent nuclear translocation of β-catenin in HCC cell.

Several signaling pathways, including PI3K/AKT, mTOR, SRC, FAK, and ERK pathways, have been found to be involved in β-catenin-TCF-LEF transactivation (Fang et al., 2007; Ji et al., 2009; Yang et al., 2011). To define which pathway contributes to EGF-induced transcriptional activity of β-catenin, specific inhibitors of PI3K (LY294002), mTOR (rapamycin), SRC (SKI-1), FAK (Y15) and ERK (U0126) were utilized. We found that treatment with LY294002 (2.5-5 μM) and U0126 (5-2.5 μM), but not SKI-1 (20-40 nM), rapamycin (20-40 nM), or Y15 (5 μM), for 24 h, inhibited EGF-induced TCF-LEF luciferase activity in a concentration-dependent way (Figure 3D1-D2). Furthermore, treatment with LY294002 for 9 h concentration-dependently also attenuated EGF-induced nuclear translocation and phosphorylation of β-catenin (Ser552) (Figure 3E1). To our surprise, treatment with U0126, like SKI-1 and rapamycin, did not obviously affect EGF-induced nuclear translocation and phosphorylation of β-catenin (Ser552) (Figure 3E2-E4). Moreover, immunofluorescence staining showed that LY294002 not only prevented EGF-induced β-catenin nuclear translocation, but also blocked EGF-induced morphological change in SMMC-7721 cells (Figure 3F). Unexpectedly, U0126 blocked EGF-induced morphological change, but did not affect EGF-induced β-catenin nuclear translocation (Figure 3F), suggesting that another molecule regulated by ERK, together with β-catenin, might co-regulate EGF-induced TCF-LEF transcriptional activity in HCC cells. The findings revealed that PI3K and ERK, but not SRC, mTOR and FAK pathways, mediated EGF-induced β-catenin-TCF-LEF transactivation in HCC cells.

To further demonstrate the role of PI3K and ERK pathways in EGF-induced EMT and cell motility, SMMC-7721 cells were pretreated with LY294002 and U0126 for 30 min, followed by stimulating with EGF for 24 h. As shown in Figure 4, EGF up-regulated expression of vimentin and Snail and down-regulated expression of E-cadherin in the cells at both protein and mRNA levels (Figure 4A-C). Pretreatment with LY294002 or U0126 attenuated the effect of EGF on the expression of vimentin, Snail and E-cadherin (Figure 4A-C). Similarly, LY294002 or U0126 also attenuated EGF-stimulated cell motility (Figure 4D and 4E). In addition, we noticed that the inhibitors of SRC, FAK, mTOR or Wnt under the same condition did not obviously affect EGF-induced cell morphological change (Figure 4G) and cell motility (Figure 4F). Collectively, our observations indicated that EGF increased β-catenin transcriptional activity, and induced EMT and cell motility by activating PI3K and ERK pathways in HCC cells.

ERK1/2-dependent nuclear translocation of PKM2 regulates β-catenin-TCF-LEF transcriptional activity, EMT and motility in SMMC-7721

To test whether ERK1/2-dependent nuclear

translocation of PKM2 could regulate EGF-induced β-catenin transactivation in HCC cells, SMMC-7721 cells were treated with EGF (5-100 ng/ml) for 9 h, followed by nuclear extraction. We found that EGF elevated nuclear PKM2 level in a concentration-dependent manner, but did not obviously affect the total cellular level of PKM2 (Figure 5A). At 25 ng/ml, EGF was able to induce significant expression of PKM2 in nuclei.

To validate whether PKM2 was really involved in EGF-induced TCF-LEF transactivation, PKM2 was silenced by using siRNA in the HCC cells. We found that down-regulation of PKM2 by siRNA almost completely abolished EGF-induced TCF-LEF transcriptional activity (Figure 5B1-B2). Interestingly, U0126, but not LY294002, prevented EGF-induced nuclear translocation of PKM2 (Figure 5C1-C2). The results supported the conclusion that ERK, but not PI3K, regulated EGF-induced PKM2 nuclear translocation and β-catenin-TCF-LEF transactivation in HCC cells.

In addition, we found that the down-regulation of PKM2 by siRNA potently inhibited EGF-induced changes of EMT markers, as detected by Western blotting analyses (Figure 5D) and qRT-PCR (Figure 5E), as well as cell morphology in SMMC-7721 cells (Figure 5F). In line with the above findings, silencing PKM2 also blocked EGF-stimulated cell motility (Figure 5G). Therefore, the results collectively indicated that PKM2 was essential for EGF-induced EMT and cell motility in HCC cells.

Snail is critical for EGF-induced EMT and motility in SMMC-7721

Snail is a transcription factor that controls EMT by inhibiting E-cadherin expression (Cano et al., 2000). To explore the role of Snail in EGF-induced EMT and motility in HCC cells, Snail was silenced by siRNA in SMMC-7721 cells. We found that the down-regulation of Snail by siRNA almost completely abrogated EGF-induced up-regulation of vimentin and down-regulation of E-cadherin at both protein (Figure 6A and B) and mRNA levels (Figure 6C). Furthermore, silencing Snail also prevented EGF-induced morphological change (Figure 6D), as well as EGF-stimulated motility in the cells (Figure 6E). The results supported the notion that Snail played a crucial role for EGF-induced EMT and cell motility in HCC cells.

Discussion

Advanced and metastatic HCC is a highly devastating disease with limited and largely ineffective treatment options (Eggert et al., 2013; Mittal et al., 2013). Consequently, there is still a great need for a better understanding of the underlying biology of advanced HCC and the efforts towards finding effective molecular-targeted therapy should be intensified. HCC is associated with a high potential for vascular invasion, metastasis and recurrence even after surgical resection, leading to poor prognosis (Altekruse et al., 2012). An important biological process that has been shown to underlie tumor progression and metastasis is EMT (Guarino, 2007). Recently, it is widely accepted that EMT is the initiate step of intra-hepatic and extra-hepatic (Maheswaran et al., 2012). Dysregulation of growth factor pathways has been thought to be correlated with the poor prognosis of HCC. The growth factors reported to promote EMT in HCC include TGF-β, PDGF and HGF (Xu et al., 2003; Fischer et al., 2007; Ogunwobi et al., 2011). Recent evidence shows that EGFR is overexpressed in 40-70% of HCC, and its activation is involved in HCC pathogenesis and prognosis (Buckley et al., 2008), but few reports are seen to address the role of EGFR signaling in induction of EMT in HCC cells. Thus, our present investigations were designed to explore whether EGFR activation could induce EMT in HCC cell lines and to uncover the underlying mechanism.

EGFR is a RTK that is overexpressed in a wide variety of human carcinomas, including non-small cell lung, breast, head and neck, bladder, ovarian, prostate cancer, and HCC, and it has been associated with a number of advanced diseases and poor prognosis (Liu et al., 2002). In addition, it is well-known that EGFR is not only important for cell proliferation, but also for a number of varied processes likely to be significant for carcinomas progression such as cell adhesion, migration and invasion, which are major steps in the EMT event (Al Moustafa et al., 2012). Our present data showed that EGF and resultant EGFR activation could promote EMT and associated migration in HCC cell lines. These results were consistent with the previous reports that EGF could induce EMT and migration in some other cancer cells. Interestingly, the proliferative property of EGF was not observed in our experiments. In contrast, we found that EGF caused cell cycle arrest in tested HCC cell lines. Actually, these observations have been recaptured in the EMT process induced by hypoxia and TGF-β in HCC. However, the differences are that hypoxia and TGF-β induced cell cycle arrest at G1phase in HCC, while EGF caused cell cycle arrest at S phase. This implied that EGF was likely to have mechanisms different from hypoxia and TGF-β in induction of cell cycle arrest in HCC cell lines, which is awaited to be revealed further.

Previous reports demonstrated that EGF could lead to disruption of cell-cell junctions and promotion of invasiveness via the phosphorylation of the E-cadherin/β-catenin complex, thus resulting in dissociation of the latter and functional loss of E-cadherin. These events could in turn release β-catenin and into the cytoplasm and then into the nucleus, where it acts as a co-activator of T-cell and lymphoid enhancer factors (TCF/LEF) in the transcriptional activation of target genes (such as c-myc, snail-family members, cyclinD1 and Vimentin) involved in proliferation, invasion and tumor progression (Fang et al., 2007; Ji et al., 2009). Recent studies showed that EGF promoted glioblastoma cancer cell proliferation by increasing the expression of c-myc and cyclin D1, Which were regulated by the level of β-catenin phosphorylation at Tyr 333 residue (Yang et al., 2011). Our current results suggested that PI3K could phosphorylate β-catenin at Ser552 residue in a GSK3β-independent way upon EGFR activation, which upregulated the expression of snail and Vimentin, leading to HCC cell EMT and migration. Interestingly, decreased expression of c-myc and cyclin

D1 were also observed in present study (Figure 6F). This reason may be that different dosages and treating time of have different effects on the proliferation of some cell lines,

PKM2 is a key glycolytic enzyme that regulates the Warburg effect and is necessary for tumor growth (Wong et al., 2013). Recent studies indicated that nuclear PKM2 could activate gene transcription and cell proliferation in human glioblastoma, prostate cancer and breast cancer cells (Gao et al., 2012; Lee et al., 2008; Luo et al., 2011a; Luo et al., 2011b). Here we observed its role in EGF-induced EMT and cell motility in HCC cells. Recent clinical investigations have demonstrated that PKM2 is up-regulated in colorectal cancer and correlated with colon cancer metastasis (Zhou et al., 2012a), but the mechanism is unknown. The findings from this study and others strongly suggest that PKM2 plays an important role not only in cell proliferation but also in EMT/cell motility, contributing to tumor metastasis.

Previous studies have documented that PDGF signaling could result in the nuclear accumulation of β-catenin, leading to EMT and cell invasion in HCC cells (Fischer et al., 2007). Besides, HGF/c-met pathway was found to promote EMT via upregulation of cyclooxygenase 2 and PI3K (Pan et al., 2010). Recently, it has been reported that Wnt/β-catenin signaling could enhance hypoxia-induced EMT in HCC via crosstalk with hypoxia-inducible factor-1α (HIF-1α) (Zhang et al., 2013). In the present study, we demonstrated that EGF induced EMT by activating PI3K and ERK pathways in HCC cell lines. Our results also indicated that PI3K and ERK pathways could upregulate nuclear levels of β-catenin and PKM2, respectively, and that the two pathways could co-regulate β-catenin-TCF/LEF transcriptional activity and EMT in HCC cell lines. Therefore, β-catenin, HIF-1α and PKM2 could be key molecules involved in the EMT process in HCC. Interestingly, there have been many reports demonstrating the interactions between these molecules, which were important for tumor proliferation, invasion and progression (Luo et al., 2011a; Yang et al., 2011; Zhang et al., 2013). It could be postulated that the EMT process induced by different factors in HCC could be attributed to some common mechanisms involving β-catenin, HIF-1α and PKM2 simultaneously. In addition, our current studies might indicate that β-catenin, HIF-1α and PKM2 could be markers of EMT and prognostic factor in HCC. Intervention of their interactions might be a new therapeutic strategy for HCC.

Clinical evidence has revealed that EGFR could be a molecular target for tumor therapy, and that about one-third of patients with advaced HCC exhibited activation of Wnt pathway, mostly as a result of β-catenin transactivation (Guan et al., 2012; Nejak-Bowen et al., 2011). In addition, co-activation of EGFR and β-catenin is commonly seen in patients with HCC (Llovet et al., 2008). Some reports have demonstrated that Wnt/β-catenin activation could induce the EGFR activation in HCC (Tan et al., 2005). Interestingly, in the present study, we showed that EGFR activation induced β-catenin activation in HCC cell lines, highlighting the crosstalk between EGFR and Wnt pathways, which could be important for HCC tumorigenesis, progression and metastasis. Previous studies indicated that there could be some potential convergent points between Wnt and EGFR pathways, presumably including Frizzled, β-catenin and NKD2 (Hu et al., 2010). Herein we showed that EGFR signaling crosstalked with Wnt pathway via PI3K phosphorylation of β-catenin in HCC cells. It therefore could be postulated that inhibition of these convergent points or combination disruption of Wnt and EGFR pathways could be more effective for therapeutic options for HCC.

In summary, the present study revealed that nuclear translocation of PKM2, regulated HCC cells EMT and migration in vitro. These discoveries provided novel role of PKM2 in the progression of HCC and potential therapeutic target for advanced HCC.

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