Digestive and Liver Disease 51 (2019) 263–274

Contents lists available at ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Liver, Pancreas and Biliary Tract

# Angiotensin II and tumor necrosis factor-α stimulate the growth, migration and invasion of BEL-7402 cells via down-regulation of GRK2 expression

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ARTICLE INFO

*Article history:*
Received 28 February 2018
Received in revised form 8 June 2018
Accepted 12 June 2018
Available online 20 June 2018

*Keywords:*
Bel-7402 cell
Cytokine
G protein-coupled receptor kinase 2
HCC

ABSTRACT

*Purpose:* To investigate the effects of angiotensin II (Ang II) and tumor necrosis factor-α (TNF-α) on the biological characteristics of hepatocellular carcinoma (HCC) cells and the associated changes in G protein-coupled receptor kinase 2 (GRK2) expression.
*Methods:* The mean serum levels of Ang II and TNF-α in normal subjects and patients with benign liver tumors (BLTs) and HCC were evaluated by enzyme-linked immunosorbent assay (ELISA), and liver samples from the patients with HCC and HCC mice were used to assess the protein levels of both cytokines, their major receptors and GRK2. In addition, the dynamics of Bel-7402 cells were determined with cell counting kit-8 (CCK-8) and Transwell experiments, while the levels of the primary cytokine receptors Ang II type-1 receptor (AT1R) and type-2 receptor (AT2R) as well as TNF receptor 1 (TNFR1) were detected by flow cytometry (FCM). The effects of Ang II and TNF-α on the GRK2 levels in Bel-7402 cells and on the dynamics of GRK2-knockdown HCC cells were also investigated.
*Results:* Both cytokines independently enhanced Bel-7402 cell growth, migration and invasion by decreasing the GRK2 level. In contrast, down-regulating the GRK2 level in Bel-7402 cells suppressed these effects. No synergistic effects were discovered when Ang II and TNF-α were administered together. Furthermore, increased AT1R and TNFR1 levels stimulated HCC initiation and progression, whereas AT2R overexpression produced the opposite effect.
*Conclusions:* The present results suggested that Ang II and TNF-α promote Bel-7402 cell growth, migration and invasion by down-regulating GRK2 expression, and that the associated receptors AT1R, AT2R and TNFR1 participate in HCC initiation and progression.


## 1. Introduction

Hepatocellular carcinoma (HCC) is widely distributed in China, particularly in the southeast coastal area. Indeed, China has the highest number of new HCC cases and HCC-associated deaths worldwide [1,2]. Additionally, HCC is the world's third leading cause of death related to cancer, and its global incidence continues to increase [3–5]. Currently, no specific treatments or effective adjuvant therapies are available for managing HCC because its pathogenesis remains to be elucidated [6,7]. For this reason, advanced HCC is primarily treated with surgical resection, and patients with HCC have a poor prognosis [8]. Therefore, investigating the involvement of cytokines in HCC cells is critical for understanding the initiation and progression of HCC and for developing new strategies to prevent and treat HCC.

The two cytokines angiotensin II (Ang II) and tumor necrosis factor-α (TNF-α) are related to the initiation and development of multiple tumors [9,10]. Ang II, which is a central element of the renin–angiotensin–aldosterone system (RAAS), stimulates the angiogenesis, inflammation and proliferation in HCC tissues through its primary receptor Ang II type 1 (AT1R), and Ang II promotes the opposite effects by activating Ang II type 2 receptor (AT2R) [11–14]. Moreover, TNF-α enhances the proliferation, metastasis and apoptosis of HCC cells through TNF receptor 1 (TNFR1), while TNF receptor 2 (TNFR2) is expressed in endothelial and immune cells [15]. Thus, TNFR1 is implicated in promoting both inflammation and apoptosis [16,17].

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https://doi.org/10.1016/j.dld.2018.06.007
1590-8658/

G protein-coupled receptor kinase 2 (GRK2) is distributed throughout various tissues [18] and induces the phosphorylation and subsequent desensitization of activated G protein-coupled receptors (GPCRs), thereby switching or terminating the GPCR-mediated signaling pathways. Several factors can regulate the activity and expression of GRK2 [19–21], which exerts diverse physiological and pathological functions and mediates various signaling pathways that are linked to the proliferative and metastatic potential of cancer cells [22]. Previous studies have verified that GRK2 significantly inhibits the growth and invasion of HCC cells, while different levels of reduced differentiation are associated with decreased GRK2 expression in HCC tissues [23,24]. Therefore, GRK2 negatively regulates the initiation and progression of HCC.

Ang II and TNF-α have been reported to control the GRK2 level in synoviocytes, endotheliocytes and cardiomyocytes, subsequently affecting the growth, inflammation and metastasis of histiocytes [25,26]. However, the links among Ang II, TNF-α and GRK2 have yet to be elucidated in HCC cells. Thus, we explored the effects of Ang II and TNF-α on Bel-7402 cells by assessing the associated changes in cell dynamics and the levels of GRK2, AT1R, AT2R and TNFR1.

## 2. Materials and methods

### 2.1. Patients and clinical samples

We recruited 24 patients with HCC and 24 patients with benign liver tumors (BLTs) who underwent hepatectomies between 2015 and 2016, without undergoing preoperative chemotherapy or radiotherapy (Hefei, China). Preoperative diagnoses were performed using appropriate imaging techniques. The cancerous and non-cancerous samples were histologically matched after the operation, and clinicopathological data were obtained for all the patients. Similarly, peripheral blood samples were collected from 36 patients with HCC and 28 patients with BLTs from 2014 to 2016. Moreover, peripheral blood samples from 50 normal subjects who underwent physical examinations were collected as the normal control sample. The Institutional Review Board of the First Affiliated Hospital of Anhui Medical University approved all aspects of the study. Ethical standards involving human experimentation and the Declaration of Helsinki were followed.

### 2.2. Enzyme-linked immunosorbent assay (ELISA)

Human peripheral blood was collected in a pro-coagulation tube and centrifuged at 1500 rpm for 15 min, and the collected supernatant served as the serum required for the experiment. Control wells and sample wells were preset in the enzyme-linked culture plates. The tested samples were added to the appropriate wells, and then horseradish peroxidase (HRP)-conjugated primary antibodies (BioFront Technologies; Tallahassee, FL, USA) against Ang II and TNF-α were applied for 1 h at 37 °C. Subsequently, each well was incubated with 100 μl of developing solution for 20 min in the dark. Finally, the absorbance of the culture supernatants was recorded at a wavelength of 450 nm using a spectrophotometer.

### 2.3. Mouse model of HCC

C57BL/6J mice (male, 2 weeks) were obtained from the Animal Experiment Center of Anhui Medical University (Hefei, China) and randomly assigned to a control or HCC model group. A single intraperitoneal injection of diethylnitrosamine (DEN, 20 mg/kg; Sigma; St. Louis, MO, USA) was applied to induce HCC, and the control mice received the same volume of saline. The mice were sacrificed at 0, 12, 24 or 36 weeks after injection, and liver samples were carefully dissected and weighed. Then, the liver specimens were divided for histopathological examinations (fixed with 10% formalin) and protein analysis studies (stored at −80 °C). All mice were bred and maintained at ambient temperature (22 ± 2 °C) under a 12-h cycle of daylight and darkness. The ethical committee of Anhui Medical University approved the studies involving animals, and the experiments were performed according to the instructions for laboratory animal experimentation.

### 2.4. Histopathology

After embedding in paraffin, the formalin-fixed liver specimens were sectioned (4 μm), dewaxed in absolute xylene and hydrated with graded ethanol solutions. Then, the liver sections were stained with hematoxylin and eosin (H&E) to observe the morphological changes. Finally, all of the liver samples were visualized using an Olympus BX53 video microscope (Olympus, Tokyo, Japan).

### 2.5. Immunohistochemistry (IHC)

IHC was used to detect the expression levels of Ang II, TNF-α, AT1R, AT2R, TNFR1 and GRK2 in the liver tissues of the HCC model mice and control mice. The liver tissue slices of the mice were dewaxed in an oven at 60 °C, immersed in a xylene solution, and hydrated in an ethanol gradient. Next, 3% hydrogen peroxide was added to the sections to block the activity of the endogenous peroxidases. After that, the sections were incubated with polyclonal rabbit anti-mouse primary antibodies (1:150; Abcam Plc; Cambridge, UK) overnight. On the next day, the sections were washed with distilled water and incubated for 30 min with an HRP-conjugated secondary antibody. Subsequently, a fresh 3,3′-diaminobenzidine solution and hematoxylin were applied to stain the specimens, and the expression levels of the target proteins were quantitatively analyzed by using an Olympus optical microscope (Tokyo, Japan) and Image-Pro Plus software system. At least six random fields (×200) were observed in each section.

### 2.6. Cell culture

Human Bel-7402 HCC cells from the Shanghai Cell Bank (Shanghai, China) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml) and fetal bovine serum (FBS, 10%; HyClone; Logan, UT, USA) at 37 °C in a 5% $CO_2$ atmosphere. Lipofectamine 3000 (Invitrogen; Carlsbad, CA, USA) was used to transfect the HCC cells with both GRK2 and scrambled siRNAs (Santa Cruz, CA, USA). The transfected Bel-7402 cells were grown for 48 h before further experiments to attain maximal GRK2 silencing.

### 2.7. Cell counting kit-8 (CCK-8) assay

The activities of the HCC cells were evaluated with CCK-8 experiments. Normal or transfected Bel-7402 cells in the exponential phase were seeded into culture medium containing single Ang II, single TNF-α or the two reagents combined (Sigma; St. Louis, MO, USA) at 5000 cells/well with three replicate wells. Administrated for 12, 24 or 48 h, each well was supplemented with CCK-8 solution (10 μl; Dojindo; Kumamoto, Japan) and maintained at 37 °C for 1 h. Subsequently, the absorbance of the culture supernatants was recorded at a wavelength of 450 nm using a spectrophotometer.

### 2.8. Transwell assay

A total of $1 \times 10^5$ normal or transfected Bel-7402 cells were seeded in the upper well of a Transwell chamber equipped with an 8-μm pore size membrane (Corning; NY, USA), and suspended in 100 μl of DMEM containing single Ang II, single TNF-α or the two reagents combined. Meanwhile, 500 μl of DMEM supplemented

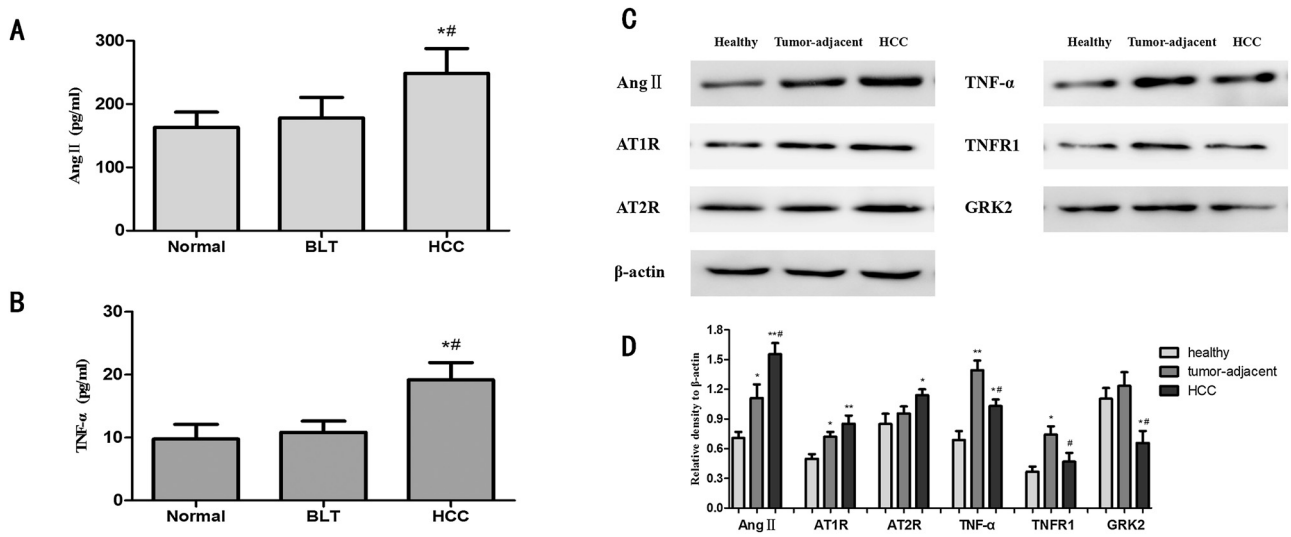


**Fig. 1.** Differential protein expression in human sera and liver tissues. (A–B) The mean serum levels of Ang II and TNF-α in the normal subjects and patients with BLT and HCC were evaluated using ELISAs. The numbers of normal subjects and patients with BLT and HCC were fifty, twenty-eight and thirty-six respectively. $^{*}P < 0.01$ compared with the normal subjects. $^{\#}P < 0.01$ compared with the BLT patients. (C–D) Representative western blots and semi-quantitative evaluations displaying the Ang II, TNF-α, AT1R, AT2R, TNFR1 and GRK2 levels in the HCC, tumor-adjacent and healthy liver tissues. The numbers of HCC, tumor-adjacent and healthy liver tissue samples were twenty-four. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the healthy liver tissue. $^{\#}P < 0.05$ compared with the tumor-adjacent tissue.

with 10% FBS was placed in the bottom well. The HCC cells were maintained as described above for 24 h, and then the cells in the upper well were cautiously wiped off, while the cells that had migrated to the bottom well were stained with crystal violet (0.1%). Finally, the fixed cells were air dried and counted in 6 random fields for each chamber (×200) under an Olympus optical microscope (Tokyo, Japan).

### 2.9. Flow cytometry (FCM)

Bel-7402 cells were planted into 6-well culture plates at $1 \times 10^6$ cells/well and incubated with single Ang II, single TNF-α or the two reagents combined for 48 h. Next, the cells were harvested and incubated with primary rabbit anti-human antibodies (Santa Cruz Biotechnology) against AT1R, AT2R and TNFR1 at 37 °C for 1 h. After 30 min of incubation with a fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit secondary antibody (ZSGB-BIO) in the dark, the ratios of AT1R-, AT2R- and TNFR1-positive Bel-7402 cells were discerned using flow cytometry (FC500; Beckman Coulter).

### 2.10. Western blotting

RIPA buffer (Beyotime, Shanghai, China) was utilized to extract proteins from the Bel-7402 cells or liver tissues (human and mouse liver samples), and the Lorry assay was performed to determine the protein concentrations [27]. The protein extracts were diluted in 5× sample buffer and boiled for 10 min. Subsequently, the samples were separated by SDS-PAGE and immuno-blotted onto a polyvinylidene fluoride membrane. After blocking the membranes with 5% bovine serum albumin at room temperature for 1 h, polyclonal rabbit anti-human primary antibodies (1:1000; Proteintech Group Inc.) against Ang II, TNF-α, AT1R, AT2R, TNFR1 or GRK2 were applied overnight. Next, the membranes were incubated with HRP-conjugated secondary antibodies for 2 h. Finally, the proteins were imaged using an enhanced chemiluminescence kit (Pierce Biotechnology Inc.) and quantified by an ImageQuant LAS 4000 mini system.

### 2.11. Reverse transcription PCR (RT-PCR) and real-time PCR

Total RNA was extracted from Bel-7402 cells using TRIzol reagent (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions, and complementary DNAs (cDNAs) were synthesized using a reverse transcription kit (Invitrogen Life Technologies). The primer pairs for the PCR amplification of GRK2 and β-actin were as follows: GRK2-forward: 5′-GTCTCCACTCCTGACTCTGAA-3′, GRK2-reverse: 5′-TAGGGTACAAATGACCTCAGC-3′; β-actin-forward: 5′-TTCTGTGGCATCCACGAAACT-3′, β-actin-reverse: GAAGCATTTGCGGTGGACGAT-3′, and the lengths of PCR products for GRK2 and β-actin were 160 and 312 bp respectively. Finally, the amplified RT-PCR products were separated on 1.5% agarose gel and visualized with ethidium bromide staining, and the real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method.

### 2.12. Statistical analysis

SPSS version 19.0 (Chicago, IL, USA) was used for the statistical analyses, and the data were analyzed by applying Student's *t*-tests or one-way ANOVAs. The results are presented as mean ± standard deviations (SD) and were considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1. Differential expression of proteins in human sera and liver tissues

The ELISA results demonstrated that the mean serum levels of Ang II and TNF-α in the HCC patients were obviously higher than those in the BLT patients and normal subjects, and there were no evident differences in the mean serum levels of Ang II and TNF-α between the BLT patients and normal subjects (Fig. 1A and B). Furthermore, Fig. 1C depicts the results of the western blot analyses of 24 matched liver samples. Based on a semi-quantitative assessment, the Ang II, AT1R and AT2R expression levels were obviously higher in the HCC tissue than those in the healthy liver tissue. In addition, the tumor-adjacent tissue exhibited increased Ang II and

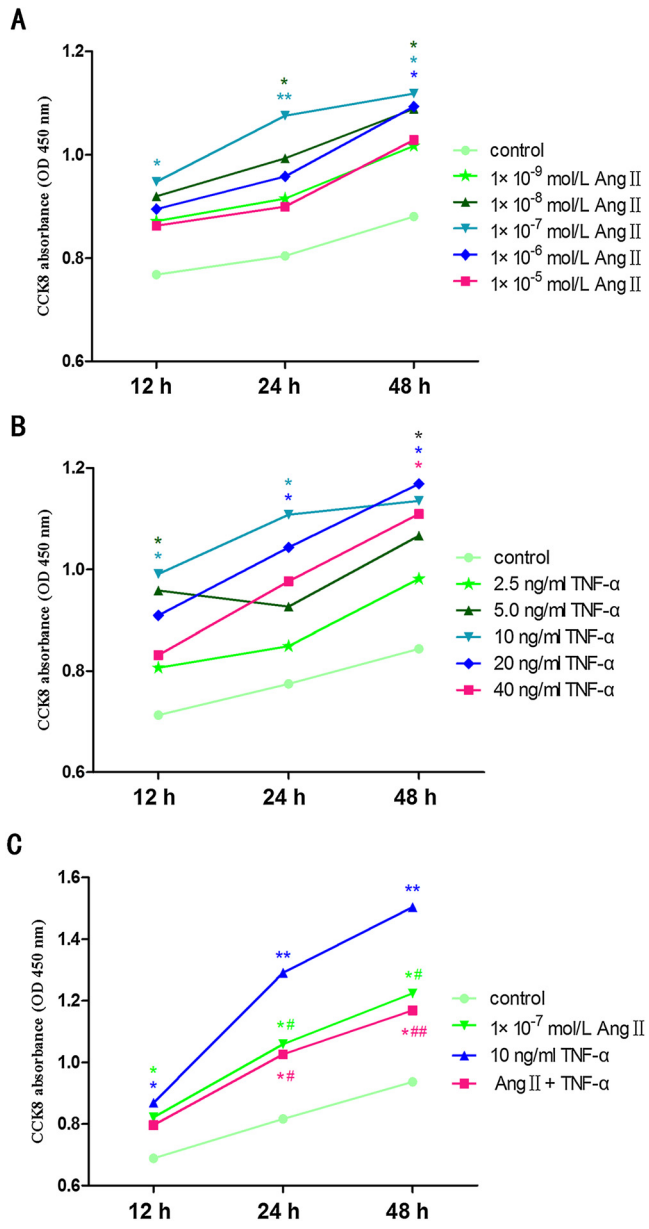


**Fig. 2.** The influences of Ang II and TNF-α on Bel-7402 cell growth. (A–B) The CCK-8 absorbance values of Bel-7402 cells that were incubated with varying concentrations of Ang II and TNF-α for 12, 24 and 48 h. (C) The CCK-8 absorbance values of Bel-7402 cells that were incubated with single Ang II, single TNF-α or the two reagents combined for 12, 24 and 48 h. The data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the single TNF-α group.

AT1R levels compared with those in the healthy liver tissue. Meantime, the TNF-α and TNFR1 contents were also increased in the tumor-adjacent tissue compared with the levels in the HCC and healthy liver tissues. Moreover, compared with that in the healthy liver tissue, TNF-α expression was elevated in the HCC tissue, but no significant differences in TNFR1 expression were found. Additionally, the GRK2 protein level in the HCC tissue was evidently lower than that in the tumor-adjacent and healthy liver tissues, whereas no obvious differences in GRK2 expression were observed between the tumor-adjacent and healthy liver tissues (Fig. 1D).

### 3.2. Cell growth

To ascertain the influences of Ang II and TNF-α on HCC cell growth, Bel-7402 cells underwent treatment for 12, 24 and 48 h with varying concentrations of Ang II and TNF-α (Fig. 2A and B). The data suggested the sensitivity of the cells to $1 \times 10^{-7}$ mol/l Ang II and 10 ng/ml TNF-α, and these concentrations were used in all subsequent experiments. The proliferative activity of the Bel-7402 cells in the single Ang II, single TNF-α and two reagents combined groups was evidently higher than that in the control group, and these effects were time-dependent (Fig. 2C). In addition, the proliferation of the Bel-7402 cells in the single TNF-α group was obviously increased compared with that in the single Ang II and two reagents combined groups, but no significant differences were found between the single Ang II and two reagents combined groups.

### 3.3. Cell migration and invasion

Considering that the motility potential of tumor cells is typically associated with metastasis, we investigated the effects of treatments with single Ang II, single TNF-α and the two reagents

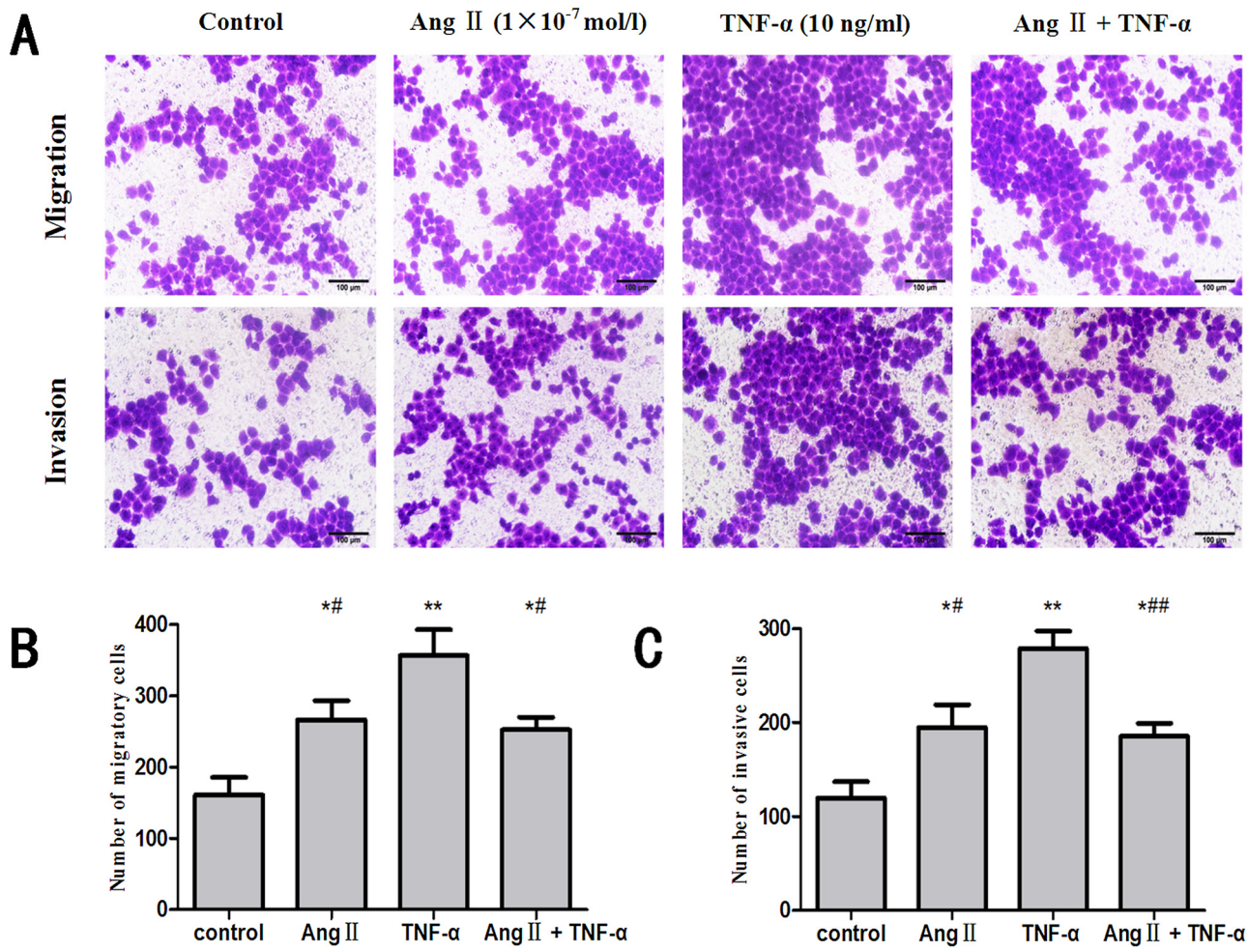


**Fig. 3.** The influences of Ang II and TNF-α on Bel-7402 cell motility. (A) Representative images of the Transwell assays showing the migration and invasion of Bel-7402 cells (magnification, ×200). (B–C) Quantitative analysis of the Bel-7402 cell migration and invasion induced by treatment with single Ang II, single TNF-α or the two reagents combined for 24 h. The data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the single TNF-α group.

combined for 24 h on Bel-7402 cell migration and invasion using Transwell assays (Fig. 3A). The results demonstrated that the three different treatments significantly promoted Bel-7402 cell migration and invasion, and the administration of single TNF-α resulted in greater migration and invasion abilities of the HCC cells than did treatment with single Ang II or the two reagents combined. Moreover, no obvious differences were found between the single Ang II and two reagents combined groups (Fig. 3B and C).

### 3.4. Changes in the expression of the relevant receptors on HCC cells

After treatment of the Bel-7402 cells for 48 h with single Ang II, single TNF-α or the two reagents combined, the ratios of AT1R-, AT2R- and TNFR1-positive HCC cells were compared to those of the control group using FCM assays (Fig. 4A). The data suggested that the incubation of the Bel-7402 cells with single Ang II or the two reagents combined obviously increased the AT1R level compared with that in the single TNF-α and control groups, and no differences were found between the single Ang II and two reagents combined groups or between the single TNF-α and control groups. Meanwhile, a significantly higher AT2R level was observed in the two reagents combined group than in the other treatment groups. Moreover, compared to that in the single TNF-α and control groups, incremental AT2R expression was found in the single Ang II group, while the single TNF-α and control groups showed no difference. On the other hand, the single TNF-α treatment evidently up-regulated the TNFR1 level compared with that in the other three groups. Similarly, TNFR1 expression was higher in the two reagents combined group than in the single Ang II and control groups, and the single Ang II and control groups exhibited no differences (Fig. 4B).

### 3.5. Modulation of GRK2 expression in HCC cells

The Bel-7402 cells were stimulated with single Ang II, single TNF-α or the two reagents combined, and the GRK2 level relative to that in the control group was measured by western blotting (Fig. 5A). The results indicated no obvious differences among the four experimental groups after treatment for 12 h. However, a significant reduction in the GRK2 level was found in the single TNF-α group versus the two reagents combined and control groups when the cells were cultured for 24 h, while no differences were found among the single Ang II, two reagents combined and control groups or between the single Ang II and single TNF-α groups. Additionally, the GRK2 level was decreased in all treatment groups compared with that in the control group after 48 h of administration. Moreover, treatment with single TNF-α led to lower GRK2 expression than that in the two reagents combined group, and no differences were observed between the single Ang II and single TNF-α groups or between the single Ang II and two reagents combined groups

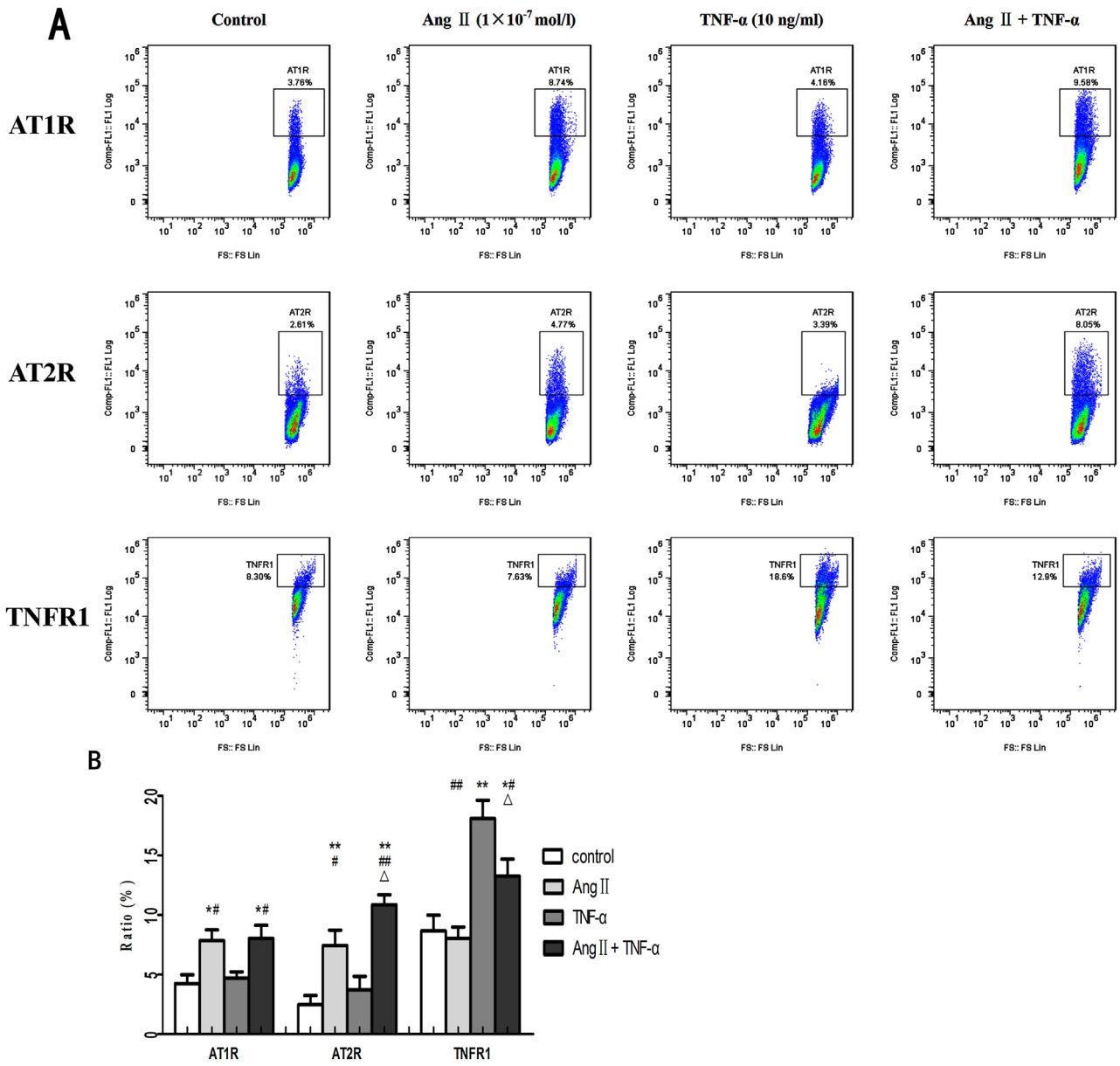


**Fig. 4.** The effects of Ang II and TNF-α on the AT1R, AT2R and TNFR1 expression levels in Bel-7402 cells. (A) Representative FCM plots displaying the expression of AT1R, AT2R and TNFR1 on the surface of Bel-7402 cells. (B) The ratios of AT1R-, AT2R- and TNFR1-positive Bel-7402 cells were recorded after treatment with single Ang II, single TNF-α or the two reagents combined for 48 h. The data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the single TNF-α group. $^{\triangle}P < 0.05$ compared with the single Ang II group.

(Fig. 5B). The results of the RT-PCR and real-time PCR experiments further demonstrated the conclusions obtained from the western blot analyses (Fig. 5C and D).

### 3.6. GRK2-related modulation in HCC cells

To corroborate the involvement of GRK2 in the modulation of Bel-7402 cells' growth, migration and invasion potentials, the cells underwent transfection with GRK2 siRNA to decrease the GRK2 level (Fig. 6A and B). After incubation for 12, 24 and 48 h, the proliferative activity of the GRK2 siRNA-transfected HCC cells was significantly increased compared with that of the scrambled siRNA-transfected HCC cells, as verified by the CCK-8 assay (Fig. 6C). Furthermore, following another 24 h of incubation in Transwell chambers, the GRK2 siRNA transfected group exhibited stronger migration and invasion potentials than the scrambled siRNA-transfected group (Fig. 6D–F).

### 3.7. Related modulatory effects of Ang II and TNF-α on GRK2-knockdown HCC cells

The GRK2-knockdown Bel-7402 cells were treated with single Ang II, single TNF-α or the two reagents combined for 12 and 24 h. Subsequent CCK-8 assays revealed no significant differences in the proliferative activity compared with that of the control group. Additionally, the administration of single TNF-α for 48 h resulted in a more prominent effect on cell growth than that observed in the control group, and no differences were found among the single Ang II, two reagents combined and control groups or among the single Ang II, single TNF-α and two reagents combined groups (Fig. 7A). Furthermore, the GRK2-knockdown Bel-7402 cells were planted into Transwell chambers and then incubated with single Ang II, single

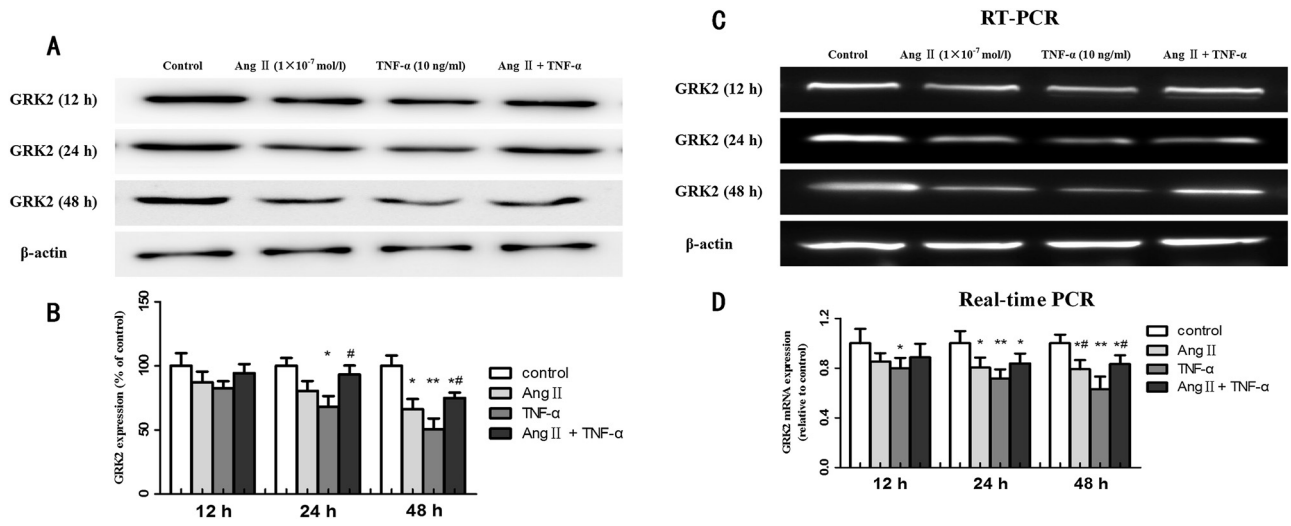


**Fig. 5.** The effects of Ang II and TNF-α on the GRK2 expression level in Bel-7402 cells. (A–B) Representative western blots and semi-quantitative evaluations showing the GRK2 protein level in Bel-7402 cells that were incubated with single Ang II, single TNF-α or the two reagents combined for 12, 24 and 48 h. (C–D) RT-PCR and real-time PCR were performed to assess the GRK2 mRNA level in Bel-7402 cells that were incubated with single Ang II, single TNF-α or the two reagents combined for 12, 24 and 48 h. The data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group. $^{\#}P < 0.05$ compared with the single TNF-α group.

TNF-α or the two reagents combined for 24 h (Fig. 7B). Our results suggested that the migration and invasion potentials of the HCC cells treated with single TNF-α were evidently higher than those of the control group. Moreover, the invasion potential of the HCC cells was enhanced in the single Ang II group compared with that in the control group, and no differences were observed among the single Ang II, single TNF-α and two reagents combined groups or between the two reagents combined and control groups (Fig. 7C and D).

### *3.8. Ang II, TNF-α, AT1R, AT2R, TNFR1 and GRK2 expression levels in a DEN-induced HCC mouse model*

In the HCC model mice, the livers exhibited mild swelling and partial proliferation of the fibrous tissue at 12 weeks after DEN injection. When tumor growth was induced for 24 weeks, the livers became markedly hardened, and tumor nodules of different sizes on the surface and damage to the liver lobules were observed. Thirty-six weeks later, the livers and tumor nodules were further enlarged, with the hepatocytes showing different sizes and shapes, and the liver lobules were obviously damaged and presented with necrosis (Fig. 8A and B). Compared with that of the control group, the liver index of the HCC model group was clearly increased (Fig. 8C).

We further investigated the Ang II, TNF-α, AT1R, AT2R, TNFR1 and GRK2 expression levels in the liver tissues of the HCC mice at different stages (0, 12, 24 or 36 weeks) of HCC formation. Western blot analyses suggested that the Ang II, TNF-α, AT1R, TNFR1 and GRK2 levels in the control group did not evidently change over time, whereas the AT2R level obviously decreased over time (Fig. 8D and E). Meantime, the Ang II, TNF-α, AT1R and TNFR1 levels in the HCC model group gradually increased over time, while the AT2R level exhibited no significant change over time, and the GRK2 level gradually decreased over time (Fig. 8F and G). Furthermore, the IHC results indicated that the expression levels of Ang II, TNF-α, AT1R and TNFR1 in the HCC model group were clearly increased compared with those in the control group after 36 weeks of HCC formation, and there was no significant difference in AT2R expression between the HCC model and control groups. In addition, the HCC model group exhibited lower expression of GRK2 than the control group (Fig. 8H and I).

## 4. Discussion

The HCC syndrome influences the digestive system and causes serious physical and mental impairments, but the relevant etiology of HCC has yet to be confirmed [28–30]. As a central player in the RAAS, Ang II promotes the initiation and growth of tumor vessels in HCC tissues by regulating platelet-derived growth factor, vascular endothelial growth factor and other associated molecules [31]. Currently, it is believed that the effects of TNF-α on the bioactivities of HCC cells are dose-dependent, i.e., high TNF-α concentrations can inhibit proliferation and induce apoptosis, but low TNF-α concentrations mainly promote growth and invasion [32]. In this study, the expression levels of Ang II and TNF-α in the sera and liver tissues from the HCC patients and HCC mice were significantly increased, and $1 \times 10^{-7}$ mol/l Ang II and 10 ng/ml TNF-α obviously enhanced Bel-7402 cell growth, migration and invasion. These findings revealed the involvement of these two cytokines in the induction and development of HCC.

Ang II and TNF-α have been confirmed to interact to influence the inflammation, proliferation and metastasis of multiple histiocytes [33,34]. Accordingly, we aimed to unravel the nature of the interaction between Ang II and TNF-α in the initiation and development of HCC. Based on the results, Ang II and TNF-α potentiated Bel-7402 cell growth, migration and invasion, but did not have synergistic effects when combined. Additionally, the AT1R, AT2R and TNFR1 levels were obviously different in the diverse liver samples from the HCC patients and HCC mice. Similarly, the expression of the abovementioned proteins on the surfaces of the Bel-7402 cells were clearly changed after administration of single Ang II, single TNF-α or the two reagents combined. These results indicated that the over-expression of AT1R and TNFR1 may promote HCC cell growth, migration and invasion, whereas a high AT2R level elicited the opposite effects.

GPCRs are 7-transmembrane helical proteins that function as signal transducers, and the signal pathways mediated by GPCRs can be phosphorylated and terminated by GRK2, which impacts the growth, metastasis and apoptosis of ovarian carcinoma cells, pancreatic carcinoma cells and other tumor cells [35–37]. Indeed, GRK2 is an important signaling molecule that can influence multiple biological activities in HCC cells. For example, GRK2 inhibits the proliferative and migratory abilities of HCC cells by suppressing

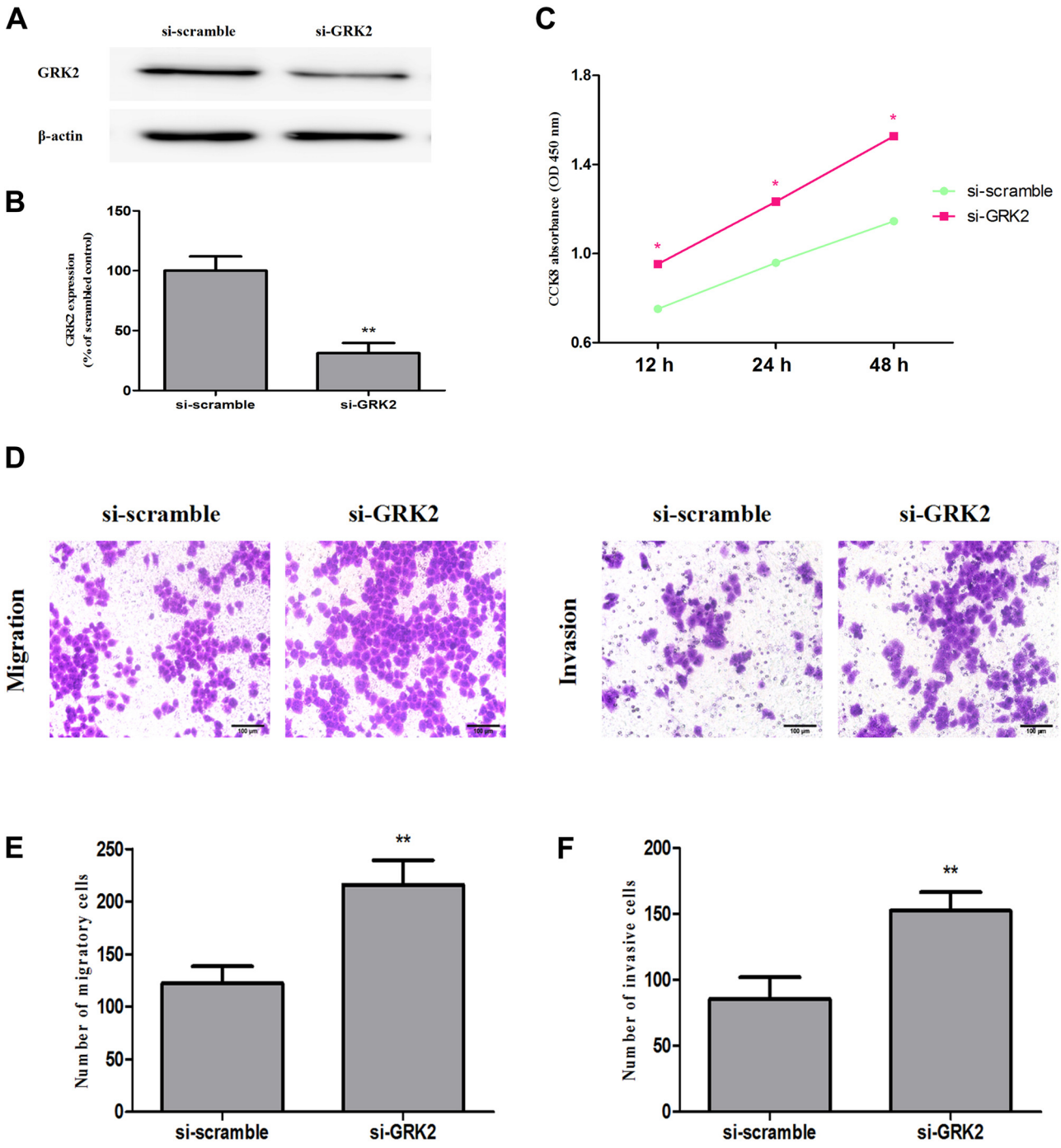


**Fig. 6.** The influences of GRK2 on Bel-7402 cell growth, migration and invasion. (A–B) Representative western blots and semi-quantitative evaluations showing the GRK2 level in the siRNAs-transfected Bel-7402 cells. (C) The CCK-8 absorbance values of siRNAs-transfected Bel-7402 cells that were incubated for 12, 24 and 48 h. (D) Representative images of the Transwell assays showing the migration and invasion of siRNAs-transfected Bel-7402 cells (magnification, ×200). (E–F) Quantitative analysis of the siRNAs-transfected Bel-7402 cell migration and invasion after incubation for 24 h. The data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the scrambled control group.

the expression levels of insulin-like growth factor 1 receptor and early growth response-1 [38]. Based on our experimental results, the GRK2 levels in the liver tissues from the HCC patients and HCC mice were significantly decreased, and the down-regulation of GRK2 expression in the Bel-7402 cells promoted the growth-related characteristics of the HCC cells. These findings suggested that GRK2 suppresses the initiation and progression of HCC.

In HCC cells, the Ang II, TNF-α and GRK2 levels are associated with cell growth, migration and invasion, and thus influence the prognosis of HCC patients [39,40]. To further explore the mechanisms of action of different cytokines on the initiation and progression of HCC, investigation of the relationships among Ang II, TNF-α and GRK2 in HCC cells is important. The present results confirmed that treatment with single Ang II, single TNF-α or the two reagents combined significantly reduced GRK2 expression in the Bel-7402 cells in a time-dependent manner. Moreover, the inhibitory effects of each experimental treatment on the GRK2 levels in HCC cells were consistent with their potential to stimulate cell growth and invasion. Meanwhile, the down-regulation of GRK2 expression in the Bel-7402 cells obviously inhibited the effects of all

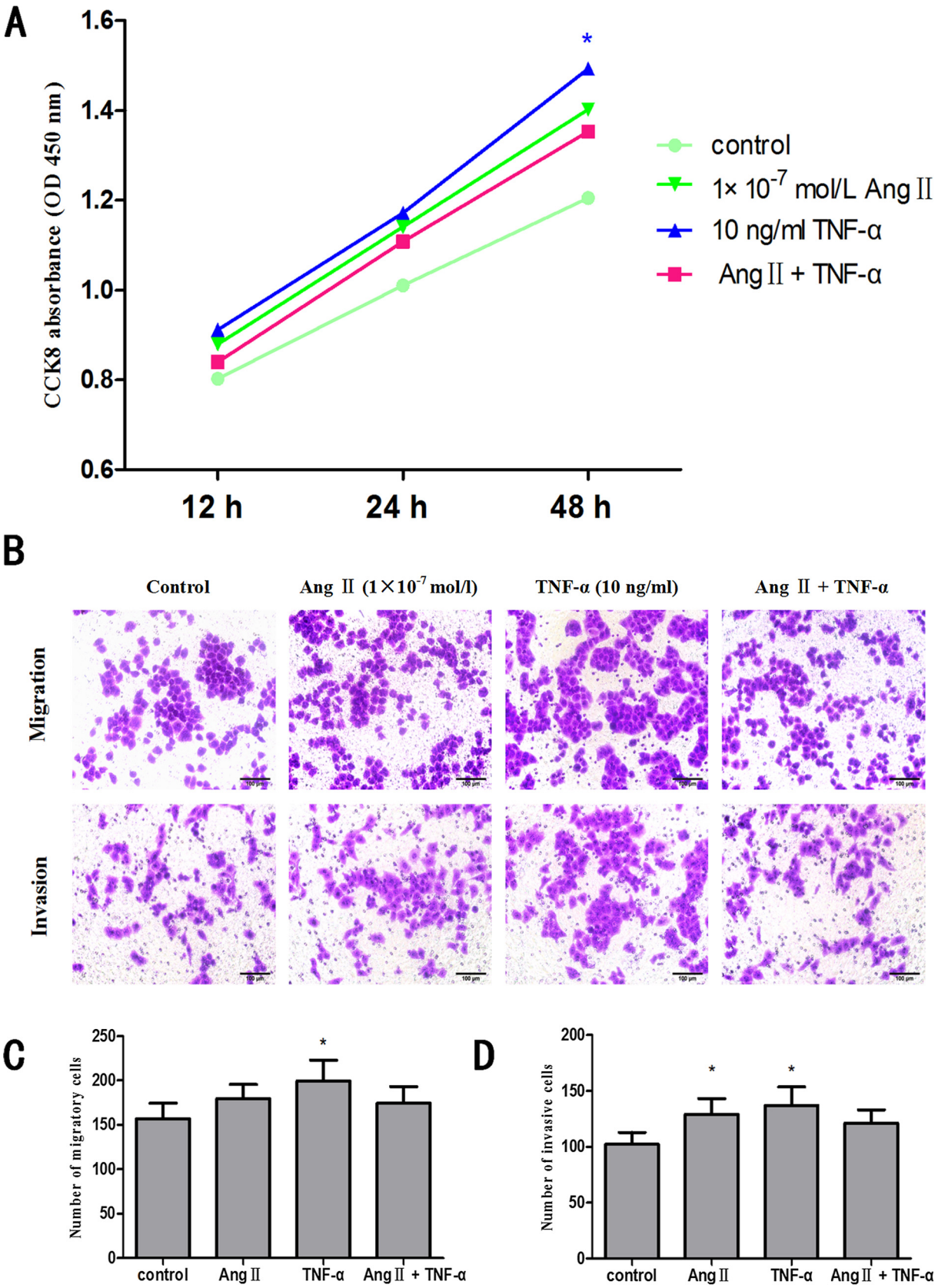


**Fig. 7.** The influences of Ang II and TNF-α on the growth, migration and invasion of GRK2-knockdown Bel-7402 cells. (A) The CCK-8 absorbance values of GRK2-knockdown Bel-7402 cells that were incubated with single Ang II, single TNF-α or the two reagents combined for 12, 24 and 48 h. (B) Representative images of the Transwell assays showing the migration and invasion of GRK2-knockdown Bel-7402 cells (magnification, ×200). (C–D) Quantitative analysis of the GRK2-knockdown Bel-7402 cell migration and invasion induced by treatment with single Ang II, single TNF-α or the two reagents combined for 24 h. The data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$ compared with the control group.

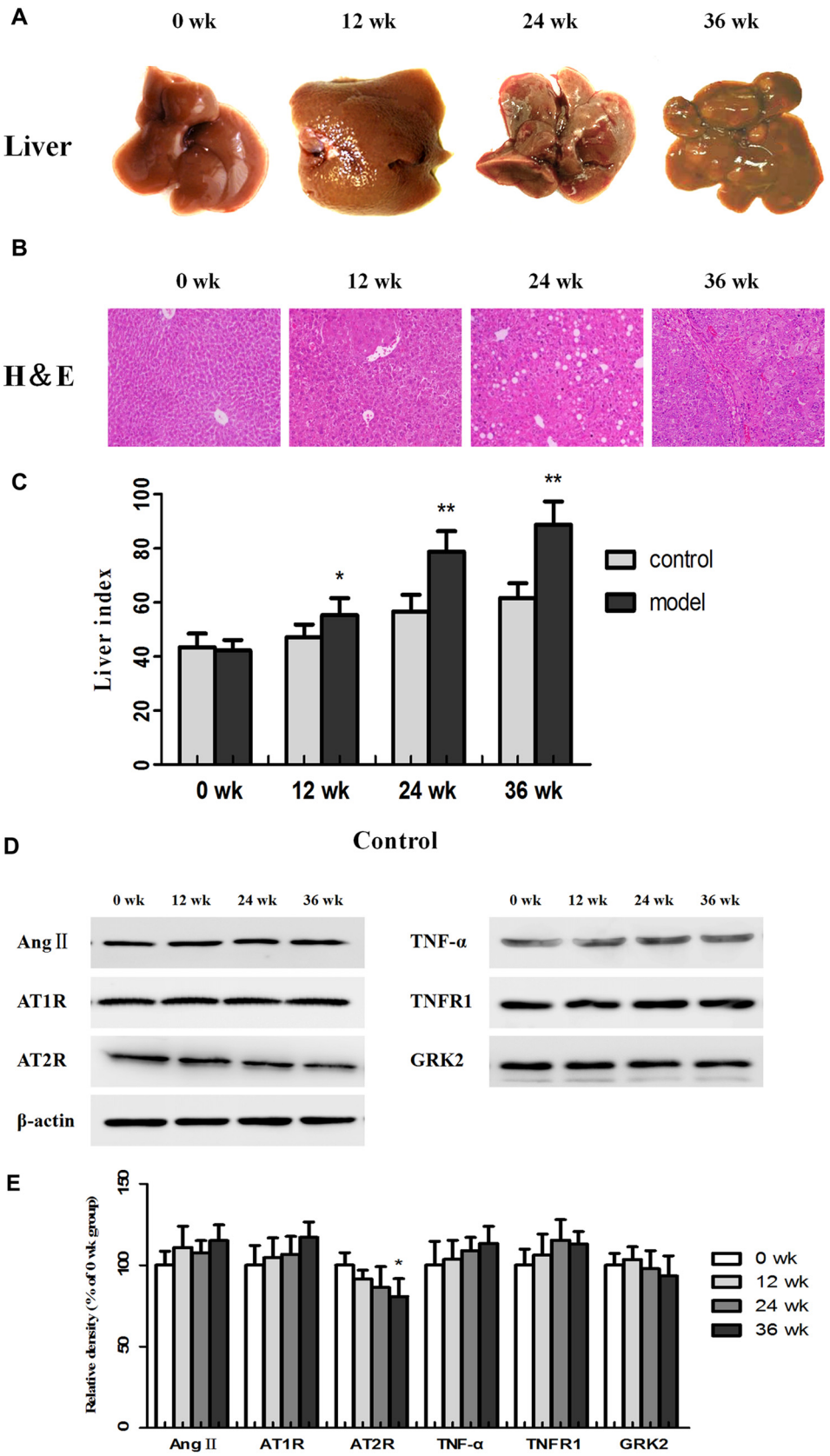


**Fig. 8.** The expression levels of Ang II, TNF-α, AT1R, AT2R, TNFR1 and GRK2 in the DEN-induced HCC mouse model. (A–B) Morphological changes in the livers of the DEN-induced HCC model mice (H&E staining; magnification, ×100). (C) The changes of the liver index in the DEN-induced HCC mouse model. Liver index = liver weight (mg)/body weight (g). (D–G) The Ang II, TNF-α, AT1R, AT2R, TNFR1 and GRK2 expression levels in the liver tissues of mice were evaluated by western blot analysis. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the 0 wk group. (H–I) The levels of Ang II, TNF-α, AT1R, AT2R, TNFR1 and GRK2 proteins in mouse liver tissues were estimated by IHC analysis (magnification, ×200). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group. The number of mice in each experimental group was six.

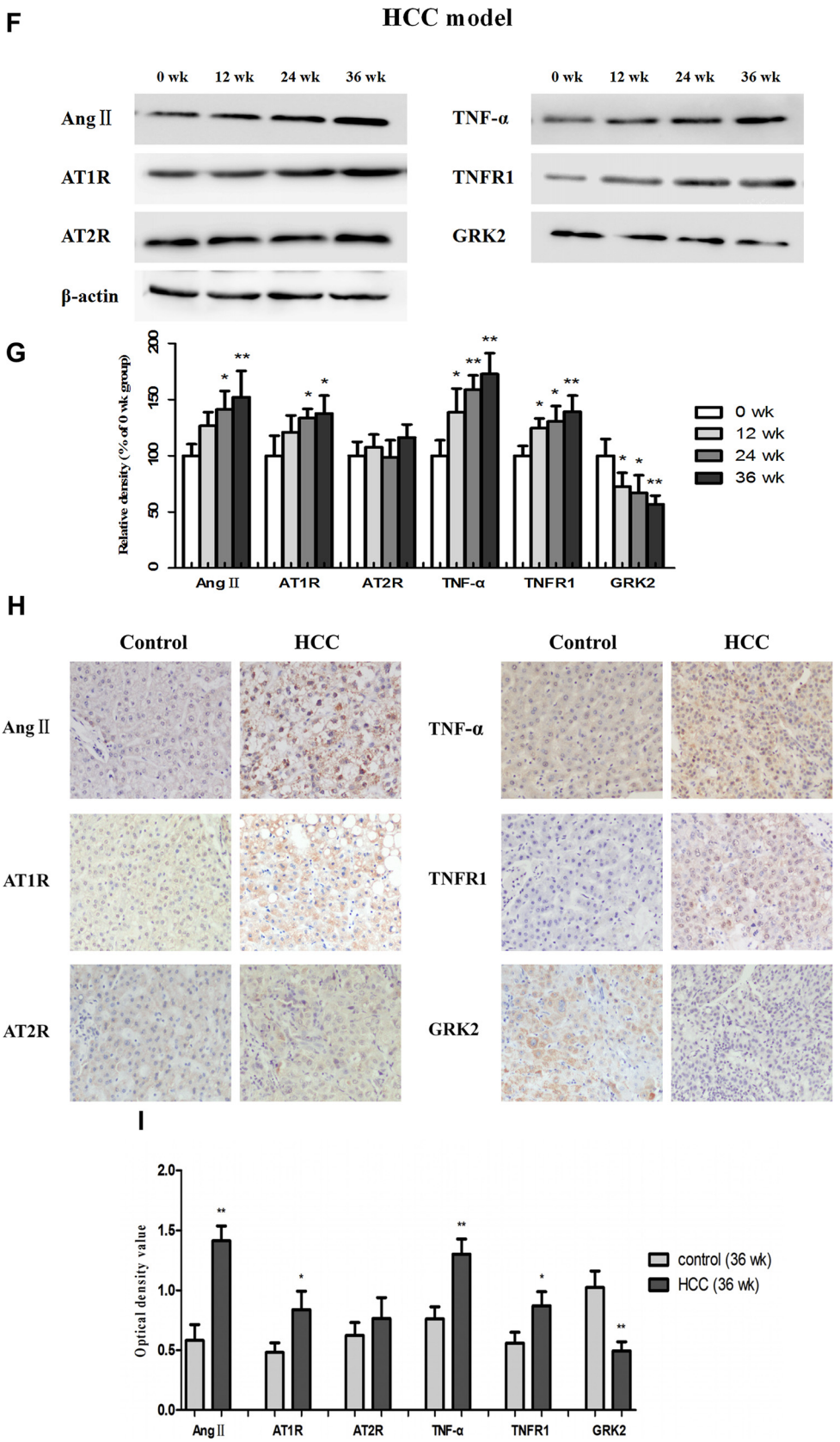


**Fig. 8.** (*Continued*)

the experimental treatments on the growth- and invasion-related biological activities of HCC cells. These data indicated that Ang II and TNF-α promoted HCC initiation and progression by suppressing the GRK2 level.

In conclusion, Ang II and TNF-α can affect the growth and invasion of HCC cells by modulating the levels of related receptors and GRK2. Therefore, through exploration of the latent mechanisms and correlations among different cytokines in HCC initiation and progression, this paper provides novel ideas and potential therapeutic approaches for HCC.

## Conflicts of interest

None declared.

## Funding

This work was funded by the Natural Science Foundation of China (Nos. 81330081, 81673444) and the Natural Science Foundation of Anhui Province (No. 1508085MH182).

## Acknowledgements

The authors are thankful to all staff members of Institute of Clinical Pharmacology, Anhui Medical University for their assistance with the study.

## References

[1] Zhong JH, Peng NF, You XM, et al. Tumor stage and primary treatment of hepatocellular carcinoma at a large tertiary hospital in China: a real-world study. Oncotarget 2017;8:18296–302.
[2] Yi PS, Zhang M, Zhao JT, et al. Liver resection for intermediate hepatocellular carcinoma. World J Hepatol 2016;8:607–15.
[3] Kim DW, Talati C, Kim R. Hepatocellular carcinoma (HCC): beyond sorafenib-chemotherapy. J Gastrointest Oncol 2017;8:256–65.
[4] Rao CV, Asch AS, Yamada HY. Frequently mutated genes/pathways and genomic instability as prevention targets in liver cancer. Carcinogenesis 2017;38:2–11.
[5] Trojan J, Zangos S, Schnitzbauer AA. Diagnostics and treatment of hepatocellular carcinoma in 2016: standards and developments. Visc Med 2016;32:116–20.
[6] Donadon M, Solbiati L, Dawson L, et al. Hepatocellular carcinoma: the role of interventional oncology. Liver Cancer 2016;6:34–43.
[7] Colagrande S, Inghilesi AL, Aburas S, et al. Challenges of advanced hepatocellular carcinoma. World J Gastroenterol 2016;22:7645–59.
[8] Samonakis DN, Kouroumalis EA. Systemic treatment for hepatocellular carcinoma: still unmet expectations. World J Hepatol 2017;9:80–90.
[9] Radin DP, Krebs A, Maqsudlu A, et al. Our ACE in the hole: justifying the use of angiotensin-converting enzyme inhibitors as adjuvants to standard chemotherapy. Anticancer Res 2018;38:45–9.
[10] Huang BP, Lin CS, Wang CJ, et al. Upregulation of heat shock protein 70 and the differential protein expression induced by tumor necrosis factor-alpha enhances migration and inhibits apoptosis of hepatocellular carcinoma cell HepG2. Int J Med Sci 2017;14:284–93.
[11] Pinter M, Weinmann A, Wörns MA, et al. Use of inhibitors of the renin–angiotensin system is associated with longer survival in patients with hepatocellular carcinoma. U Eur Gastroenterol J 2017;5:987–96.
[12] Ji Y, Wang Z, Li Z, et al. Angiotensin II enhances proliferation and inflammation through AT1/PKC/NF-κB signaling pathway in hepatocellular carcinoma cells. Cell Physiol Biochem 2016;39:13–32.
[13] Carey RM. Update on angiotensin AT2 receptors. Curr Opin Nephrol Hypertens 2017;26:91–6.
[14] Sun L, Shi J. Advance in research of Angiotensin II and its receptor and malignant tumor. Zhongguo Fei Ai Za Zhi 2016;19:615–9.
[15] Verwoerd A, Hijdra D, Vorselaars AD, et al. Infliximab therapy balances regulatory T cells, tumour necrosis factor receptor 2 (TNFR2) expression and soluble TNFR2 in sarcoidosis. Clin Exp Immunol 2016;185:263–70.
[16] Al-Lamki RS, Mayadas TN. TNF receptors: signaling pathways and contribution to renal dysfunction. Kidney Int 2015;87:281–96.
[17] Zhang H, Xiao W. TNFR1 and TNFR2 differentially mediate TNF-α-induced inflammatory responses in rheumatoid arthritis fibroblast-like synoviocytes. Cell Biol Int 2017;41:415–22.
[18] Nogués L, Reglero C, Rivas V, et al. G-protein-coupled receptor kinase 2 as a potential modulator of the hallmarks of cancer. Mol Pharmacol 2017;91:220–8.
[19] Dela Paz NG, Melchior B, Frangos JA. Shear stress induces Gαq/11 activation independently of G protein-coupled receptor activation in endothelial cells. Am J Physiol Cell Physiol 2017;312:C428–37.
[20] Han CC, Ma Y, Li Y, et al. Regulatory effects of GRK2 on GPCRs and non-GPCRs and possible use as a drug target (review). Int J Mol Med 2016;38:987–94.
[21] Cannavo A, Komici K, Bencivenga L, et al. GRK2 as a therapeutic target for heart failure. Expert Opin Ther Targets 2018;22:75–83.
[22] Penela P, Nogués L, Mayor Jr F. Role of G protein-coupled receptor kinases in cell migration. Curr Opin Cell Biol 2014;27:10–7.
[23] Xu ZW, Yan SX, Wu HX, et al. The influence of TNF-α and Ang II on the proliferation, migration and invasion of HepG2 cells by regulating the expression of GRK2. Cancer Chemother Pharmacol 2017;79:747–58.
[24] Wei Z, Hurtt R, Ciccarelli M, et al. Growth inhibition of human hepatocellular carcinoma cells by overexpression of G-protein-coupled receptor kinase 2. J Cell Physiol 2012;227:2371–7.
[25] Taguchi K, Kobayashi T, Takenouchi Y, et al. Angiotensin II causes endothelial dysfunction via the GRK2/Akt/eNOS pathway in aortas from a murine type 2 diabetic model. Pharmacol Res 2011;64:535–46.
[26] Steury MD, Lucas PC, McCabe LR, et al. G-protein-coupled receptor kinase-2 is a critical regulator of TNF-α signaling in colon epithelial cells. Biochem J 2017;474:2301–13.
[27] Ieyasu A, Ishida R, Kimura T, et al. An all-recombinant protein-based culture system specifically identifies hematopoietic stem cell maintenance factors. Stem Cell Rep 2017;8:500–8.
[28] Zamor PJ, deLemos AS, Russo MW. Viral hepatitis and hepatocellular carcinoma: etiology and management. J Gastrointest Oncol 2017;8:229–42.
[29] Akamatsu N, Cillo U, Cucchetti A, et al. Surgery and hepatocellular carcinoma. Liver Cancer 2016;6:44–50.
[30] Sia D, Villanueva A, Friedman SL, et al. Liver cancer cell of origin, molecular class, and effects on patient prognosis. Gastroenterology 2017;152:745–61.
[31] Bocca C, Novo E, Miglietta A, et al. Angiogenesis and fibrogenesis in chronic liver diseases. Cell Mol Gastroenterol Hepatol 2015;1:477–88.
[32] Wang H, Liu J, Hu X, et al. Prognostic and therapeutic values of tumor necrosis factor-alpha in hepatocellular carcinoma. Med Sci Monit 2016;22:3694–704.
[33] Justin Rucker A, Crowley SD. The role of macrophages in hypertension and its complications. Pflugers Arch 2017;469:419–30.
[34] Zhu L, Carretero OA, Xu J, et al. Activation of angiotensin II type 2 receptor suppresses TNF-α-induced ICAM-1 via NF-κB: possible role of ACE2. Am J Physiol Heart Circ Physiol 2015;309:H827–34.
[35] Haakenson J, Zhang X. HDAC6 and ovarian cancer. Int J Mol Sci 2013;14:9514–35.
[36] Zhou L, Wang MY, Liang ZY, et al. G-protein-coupled receptor kinase 2 in pancreatic cancer: clinicopathologic and prognostic significance. Hum Pathol 2016;56:171–7.
[37] Wang ZQ, He CY, Hu L, et al. Long noncoding RNA UCA1 promotes tumour metastasis by inducing GRK2 degradation in gastric cancer. Cancer Lett 2017;408:10–21.
[38] Ma Y, Han CC, Huang Q, et al. GRK2 overexpression inhibits IGF1-induced proliferation and migration of human hepatocellular carcinoma cells by down-regulating EGR1. Oncol Rep 2016;35:3068–74.
[39] Facciorusso A, Del Prete V, Crucinio N, et al. Angiotensin receptor blockers improve survival outcomes after radiofrequency ablation in hepatocarcinoma patients. J Gastroenterol Hepatol 2015;30:1643–50.
[40] Cha H, Lee EJ, Seong J. Multi-analyte analysis of cytokines that predict outcomes in patients with hepatocellular carcinoma treated with radiotherapy. World J Gastroenterol 2017;23:2077–85.