Meloxicam suppresses hepatocellular carcinoma cell proliferation and migration by targeting COX-2/PGE2-regulated activation of the β-catenin signaling pathway

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Abstract. Recurrence and metastasis are the two leading causes of poor prognosis of hepatocellular carcinoma (HCC) patients. Cyclooxygenase (COX)-2 is overexpressed in many types of cancers including HCC and promotes its metastasis. Meloxicam is a selective COX-2 inhibitor that has been reported to exert an anti-proliferation and invasion/migration response in various tumors. In this study, we examined the role of meloxicam on HCC cell proliferation and migration and explored the molecular mechanisms underlying this effect. We found that meloxicam inhibited HCC cell proliferation and had a cell cycle arrest effect in human HCC cells. Furthermore, meloxicam suppressed the ability of HCC cells expressing higher levels of COX-2 and prostaglandin E2 (PGE2) to migration via potentiating expression of E-cadherin and alleviating expression of matrix metalloproteinase (MMP)-2 and -9. COX-2/PGE2 has been considered to activate the β-catenin signaling pathway which promotes cancer cell migration. We found that treatment with PGE2 significantly enhanced nuclear accumulation of β-catenin and the activation of GSK3β which could be reversed by meloxicam in HCC cells. We also observed that HCC cell migration and upregulation of the level of MMP-2/9 and downregulation of E-cadherin induced by PGE2 were suppressed by FH535, an inhibitor of β-catenin. Taken together, these findings provide a new treatment strategy against HCC proliferation and migration.

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Introduction

Hepatocellular carcinoma (HCC) ranks as the sixth most common malignancy as well as the third most common cause of cancer leading to death worldwide, and its incidence continues to rise (1,2). Hepatitis virus infection (hepatitis B or C viruses), alcohol-related liver cirrhosis, and non-alcoholic steatohepatitis have been recognized as the main risk factors for HCC (3). Despite improvements in surgical and medical treatments in the past decade, the outcome of patients with HCC remains unsatisfactory. Recurrence and metastasis are the two leading causes of poor prognosis of HCC patients (4). Therefore, there is an urgent need to understand the cellular mediators that contribute to the invasion and migration of HCC and explore new therapeutic strategies targeting these mediators.

Cyclooxygenase-2 (COX-2) has been considered a potential mediator of invasion and migration in a number of malignant diseases (5-8). It has also been reported to be associated with poor prognosis in many cancers (9,10). Overexpression of COX-2 enhances the extent of prostaglandin E2 (PGE2) which is the main metabolite of COX-2 and a ligand of G protein-coupled receptors including EP1, EP2, EP3, and EP4. Recently, several studies reported that COX-2/PGE2 stimulates AKT, NF-κB, and ERK1/2 signaling pathways to promote tumor angiogenesis, apoptosis, and invasiveness (8,11-13). Our previous studies demonstrated that meloxicam, a selective COX-2 inhibitor, suppresses HCC cell proliferation, migration, and invasion via regulating expression of matrix metallo-proteinase (MMP)-2 and E-cadherin in a COX-2-dependent manner (14). However, the precise mechanisms of migration and invasion of HCC are largely unknown.

Accumulating evidence has demonstrated that COX-2/PGE2 is associated with the β-catenin signaling pathway contributing to the growth of many cancers, such as skin (15), breast (16), neuroblastoma (17) and colorectal cancer (18). β-catenin is a 90 kD cytosolic protein and an important component of the Wnt signaling pathway. In the absence model of

Wnt signaling, β-catenin is recruited to the phosphorylation/destruction complex. Disturbance of the complex promotes the phosphorylation of β-catenin by glycogen synthase kinase-3β (GSK-3β) and casein kinase 1α (CK1α) leading to the proteasomal degradation of β-catenin (19). β-catenin accumulation eventually leads to its nuclear translocation and then it binds to members of the TCF/LEF family of transcription factors, thus regulating expression of various target genes which are associated with many cellular processes including cell survival, proliferation, and migration (20-22).

Meloxicam (Mel) is an NSAID that specifically inhibits COX-2. This selective COX-2 inhibitor has been demonstrated to exert an anti-invasion response in various tumors (5,23,24) including HCC cancer (14,25,26). However, whether meloxicam inhibits HCC cell invasion/migration by targeting COX-2/PGE2-regulated activation of the β-catenin signaling pathway remains unclear. In this study, we investigated the effects of meloxicam on the proliferation and migration potential of HCC cells and explored whether the antitumor effect of meloxicam is associated with the inactivation of the β-catenin signaling pathway and whether COX-2/PGE2 plays any part in this process.

Materials and methods

Cell culture. Of the 5 human HCC cell lines, HepG2, Bel-7402, and Huh-7 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and SMMC-7721 and SMMC-7402 were obtained from the Type Culture Collection Cell Bank, Chinese Academy of Science (Shanghai, China). The cells were routinely cultured in RPMI-1640 medium (Gibco)/DMEM (Hyclone) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotics at 37°C in 95% air and 5% CO_2.

Reagents and antibodies. Meloxicam was obtained from Merck Millipore (Darmstadt, Germany). PGE2 and FH535 were obtained from Sigma-Aldrich (San Diego, CA, USA). Primary antibodies to COX-2, p21, p27, MMP-2, and MMP-9 were obtained from Cell Signaling Technologies (Danvers, MA, USA). Antibodies to E-cadherin, β-catenin, GSK-3β, p-GSK-3β, Histone H3, and GAPDH were obtained from Abcam (Cambridge, UK).

Cell viability analysis. The effect of meloxicam on the cell viability of HCC cells was determined using the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assay as previously described (25).

Enzyme-linked immunosorbent assay (ELISA). The concentrations of PGE2 in supernatants of cell cultures were measured using the PGE2 ELISA Assay kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Cell cycle analysis. HepG2 and SMMC-7721 cells were grown in medium as mentioned above. At 50% confluency, cells were treated with meloxicam or not for 24 h. Cells were collected and processed for cell cycle analysis. Briefly, $5x10^4$ cells were suspended in 0.5 ml of PI solution, and incubated for 30 min in the dark according to the manufacturer's instruction. The cell cycle distribution was analyzed by FACS flow cytometry.

Cell migration analysis. The methods were previously described (14,25). In brief, $1x10^5$ cells in 300 μl of RPMI-1640 medium/DMEM (with 1% FBS) containing meloxicam or PGE2 alone or in combination were seeded into the upper chamber of a Transwell chamber (Corning, New York, USA). The bottom wells of the chambers were filled with 500 μl RPMI-1640 medium/DMEM containing 10% fetal bovine serum. After 48 h incubation, the chambers were fixed with 95% ethanol and then stained with 1% crystal violet. Images of three different fields (x100 magnification) were captured from each membrane, and the number of migrated cells counted.

Total RNA extraction and real-time PCR. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized by using a cDNA synthesis kit (Invitrogen). The reaction mixtures for quantitative RT-PCR were prepared as previously described (14). The primers targeting MMP-2 were (5'-TGA CGGTAAGGACGGACTC-3'; 5'-ATACTTCACACGGAC CACTTG-3'), MMP9 (5'-CCTCTGGAGGTTCGACGTGA-3'; 5'-TAGGCTTTCTCTCGGTACTGGAA-3'), E-cadherin (5'-TGCCCAGAAAATGAAAAAGG-3'; 5'-GGATGACAG CGTGAGAGA-3'), and GAPDH (5'-TTACTCCTTGGAGGC CATGTGGGC-3'; 5'-ACTGCCACCCAGAAGACTGTGGA TGG-3'). Expression levels were normalized to GAPDH. All protocols were carried out according to the manufacturer's instructions. Real-time PCR was performed using MX3000P Real-time PCR systems (Stratagene, Wilmington, DE, USA). Experiments were performed in triplicate, and the data were calculated by $\Delta\Delta Ct$ methods.

Western blot analysis. The method was previously described (27). After different treatments, protein concentrations in cell extracts were determined (Bio-Rad, Richmond, CA, USA). Equal amounts of each sample were resolved in SDS-PAGE gels, then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and probed with the primary antibodies described in reagents and antibodies.

siRNA transfection. β-catenin and COX-2 siRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and transfection was performed using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions.

Statistical analysis. Data are presented as the mean ± standard deviation (SD) and analyzed by one-way ANOVA followed by Dunnett's test with SPSS software (version 17.0, SPSS China, Shanghai, China), with values of $P<0.05$ considered statically significant.

Results

Effects of meloxicam on the PGE2 level and the proliferation potential of HCC cells in vitro. First, we examined expression of COX-2 protein in HCC cells by western blot analysis. As shown in Fig. 1A, HCC cell lines exhibited different levels
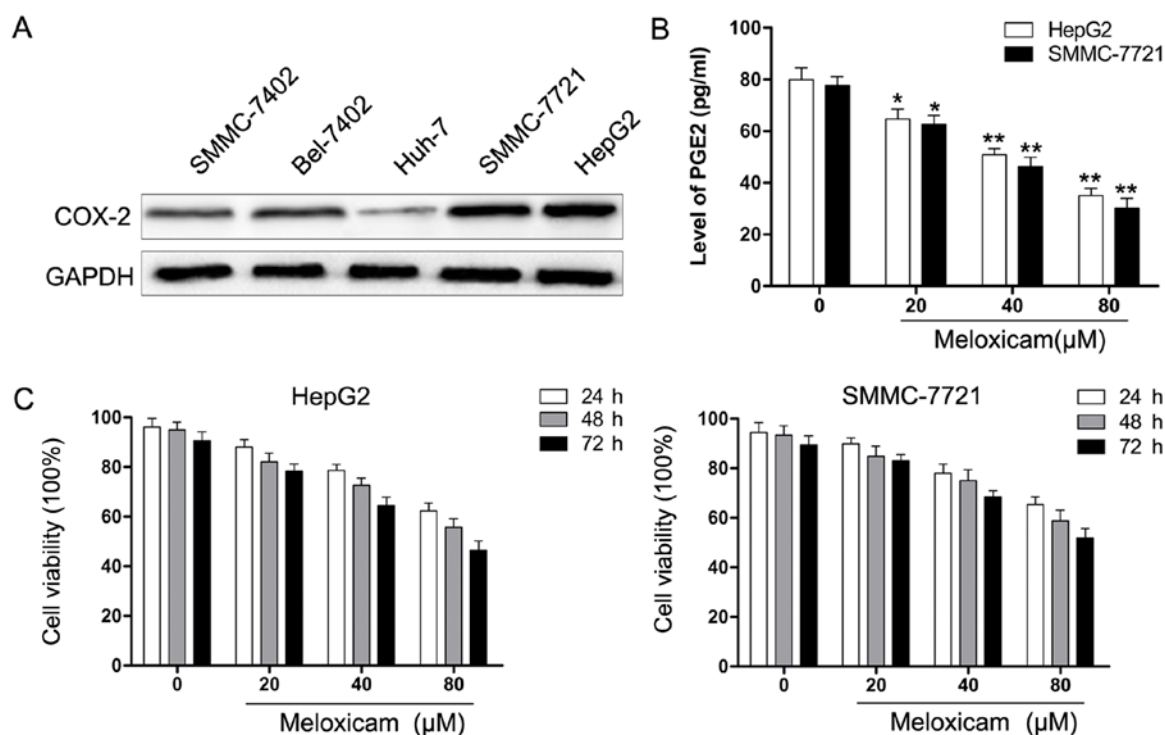
Figure 1. Effects of meloxicam on the PGE2 level and the proliferation potential of HCC cells *in vitro*. (A) Expression of COX-2 in HCC cell lines SMMC-7402, Bel-7402, Huh-7, SMMC-7721, and HepG2 was detected by western blotting. GAPDH was measured as the loading control. Results shown are representative of at least three independent experiments. (B) PGE2 levels of HCC cells were determined by ELISA following treatment with various concentration of meloxicam (0-80 μM). Data are presented as means $\pm$ SD of three independents experiments. *P<0.05, **P<0.01 vs. control. (C) Cell vitality was assessed by the CCK-8 assay. HepG2 and SMMC-7721 cells were exposed to various concentrations of meloxicam (0-80 μM) for 24, 48, or 72 h. Data are expressed as the percentage of control cells and are the means $\pm$ SD of three separate experiments.
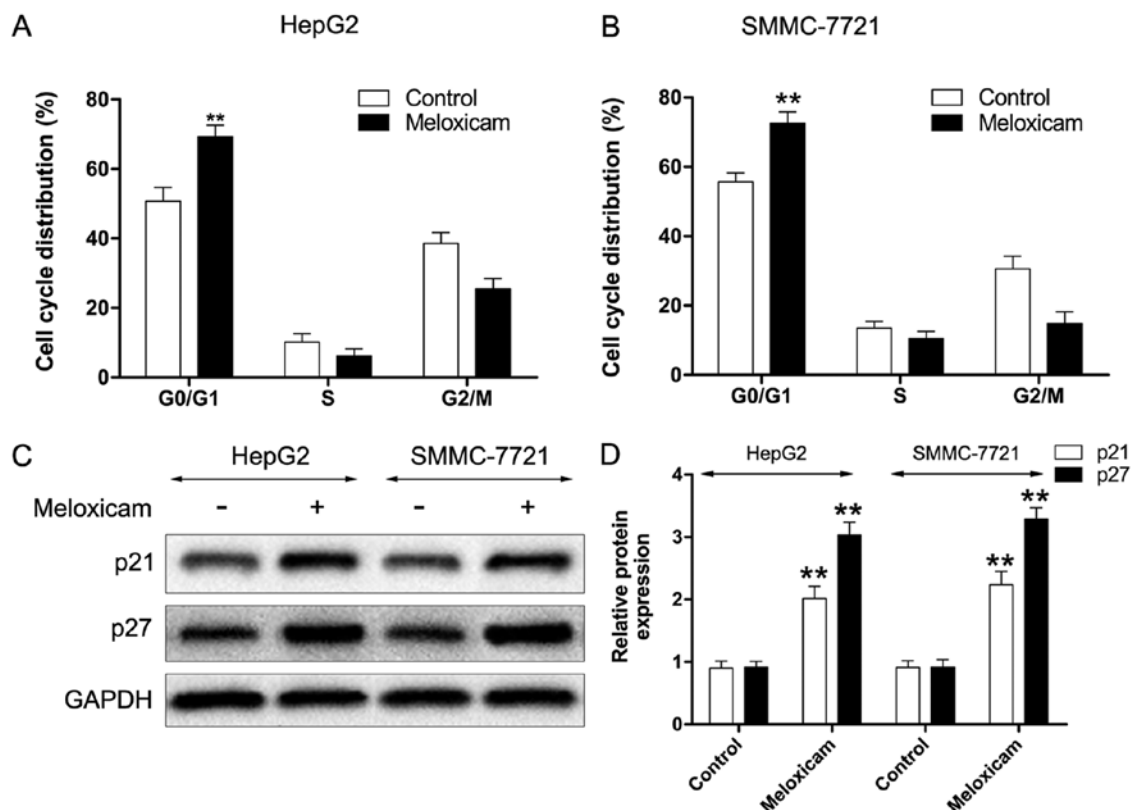

Figure 2. Meloxicam induces cell cycle arrest at the G1 phase in HepG2 and SMMC-7721 cells *in vitro*. (A and B) HepG2 and SMMC-7721 cells were treated with meloxicam (80 μM) or control for 24 h and then cell cycle analysis was measured as described in Materials and methods. Data are presented as means $\pm$ SD of three independents experiments. **P<0.01 vs. control. (C and D) Expression levels of p21 and p27 at 24 h after meloxicam treatment were analyzed by western blotting. GAPDH was measured as the loading control. Results shown are representative of at least three independent experiments. **P<0.01 vs. control.
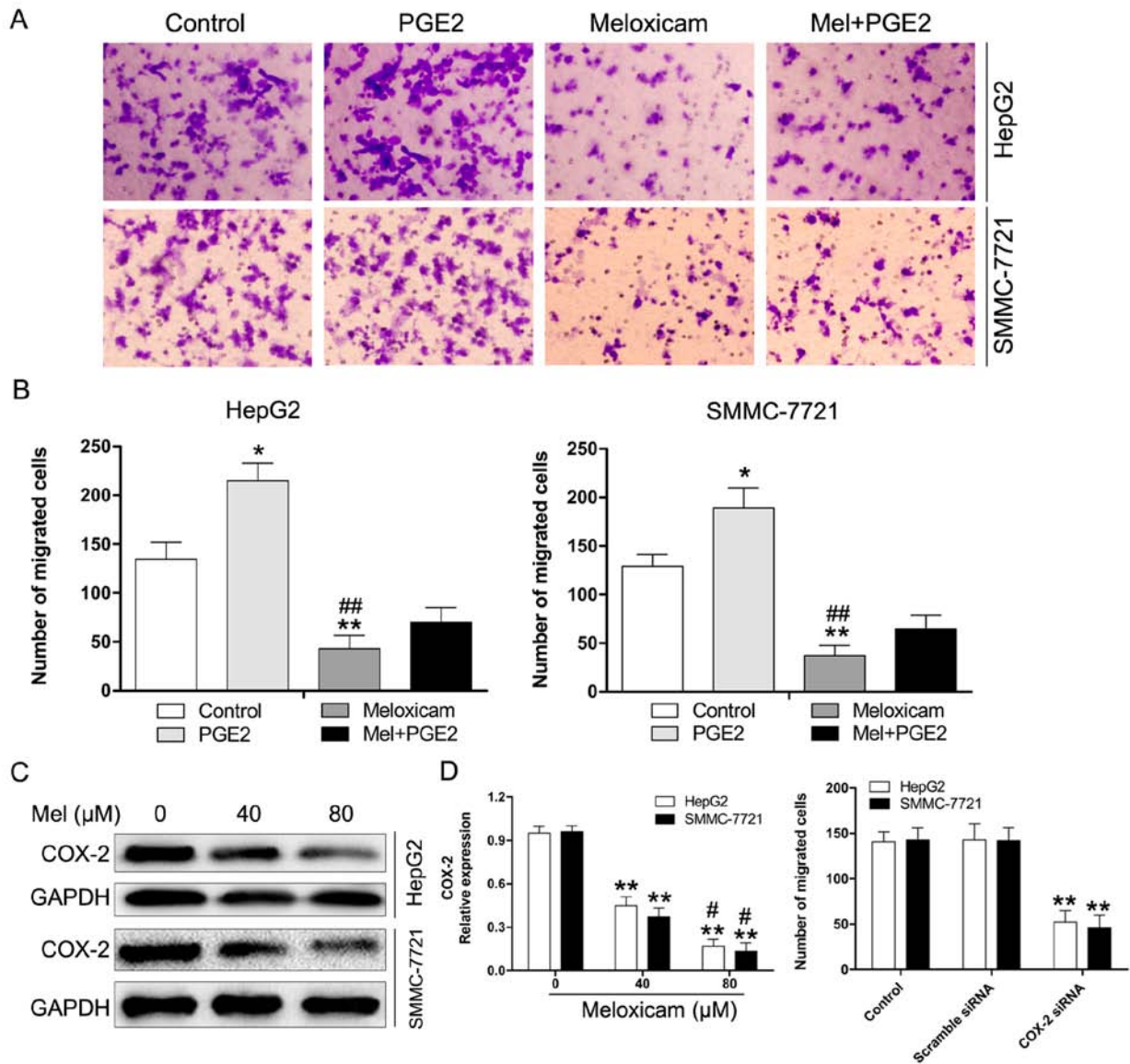
Figure 3. Effects of meloxicam on PGE2-induced HCC cell migration and endogenous expression of COX-2. (A and B) Representative images were taken from HepG2 and SMMC-7721 cells incubated with PGE2 (10 μM), meloxicam (80 μM), or a combination and subjected to cell migration assays as described in Materials and methods (magnification, x100). *P<0.05, **P<0.01 vs. control, ##P<0.01 vs. PGE2. (C) Cells were treated with various concentrations of meloxicam for 24 h and then harvested and cell lysates were subjected to western blot analysis for the measurement of COX-2 levels. GAPDH was measured as the loading control. Results shown are representative of at least three independent experiments. **P<0.01 vs. control, #P<0.05 vs. meloxicam (40 μM) group. (D) Transfection of HepG2 and SMMC-7721 cells with COX-2 siRNA significantly decreased cell migration. **P<0.01 vs. control.

of COX-2 and the results were consistent with our previous data (14). Based on the data, HepG2 and SMMC-7721 cells were chosen for the following experiments. Next, the PGE2 level of HepG2 and SMMC-7721 cells was determined by ELISA analysis. As shown in Fig. 1B, the level of PGE2 was significantly decreased with meloxicam treatment in a dose-dependent manner. HCC cells were exposed to various concentrations of meloxicam (0-80 μM) for 24, 48, or 72 h and cell viability was determined using the CCK-8 assay. As shown in Fig. 1C, the viability of HCC cells exposed to meloxicam was significantly reduced in a time- and concentration-dependent manner. This result showed the efficacy of meloxicam against HCC cell proliferation.

Effects of meloxicam on the cell cycle of HCC cells in vitro. In our previous study, we demonstrated that meloxicam has a cell cycle arrest effect in human HCC cells (25). In the current work, we further investigated the mechanism of meloxicam in regulating the cell cycle in HepG2 and SMMC-7721 cells. As shown in Fig. 2A and B, with meloxicam treatment both types of HCC cells were suppressed in the G1 phase after 24 h treatment. Furthermore, we found that expression levels of cyclin-dependent kinase (CDK) inhibitor proteins p21 and p27 in HepG2 and SMMC-7721 cells were significantly enhanced after treatment with meloxicam (Fig. 2C and D).

Effects of PGE2 and meloxicam on HCC cell migration in vitro. Previous studies reported that COX-2/PGE2 plays an important role in exerting pro-invasion/migration effects in many cancers (28-30). Here, we investigated the anti-migration effects of meloxicam on HCC cells with or without treatment with exogenous PGE2. As shown in Fig. 3A and B,
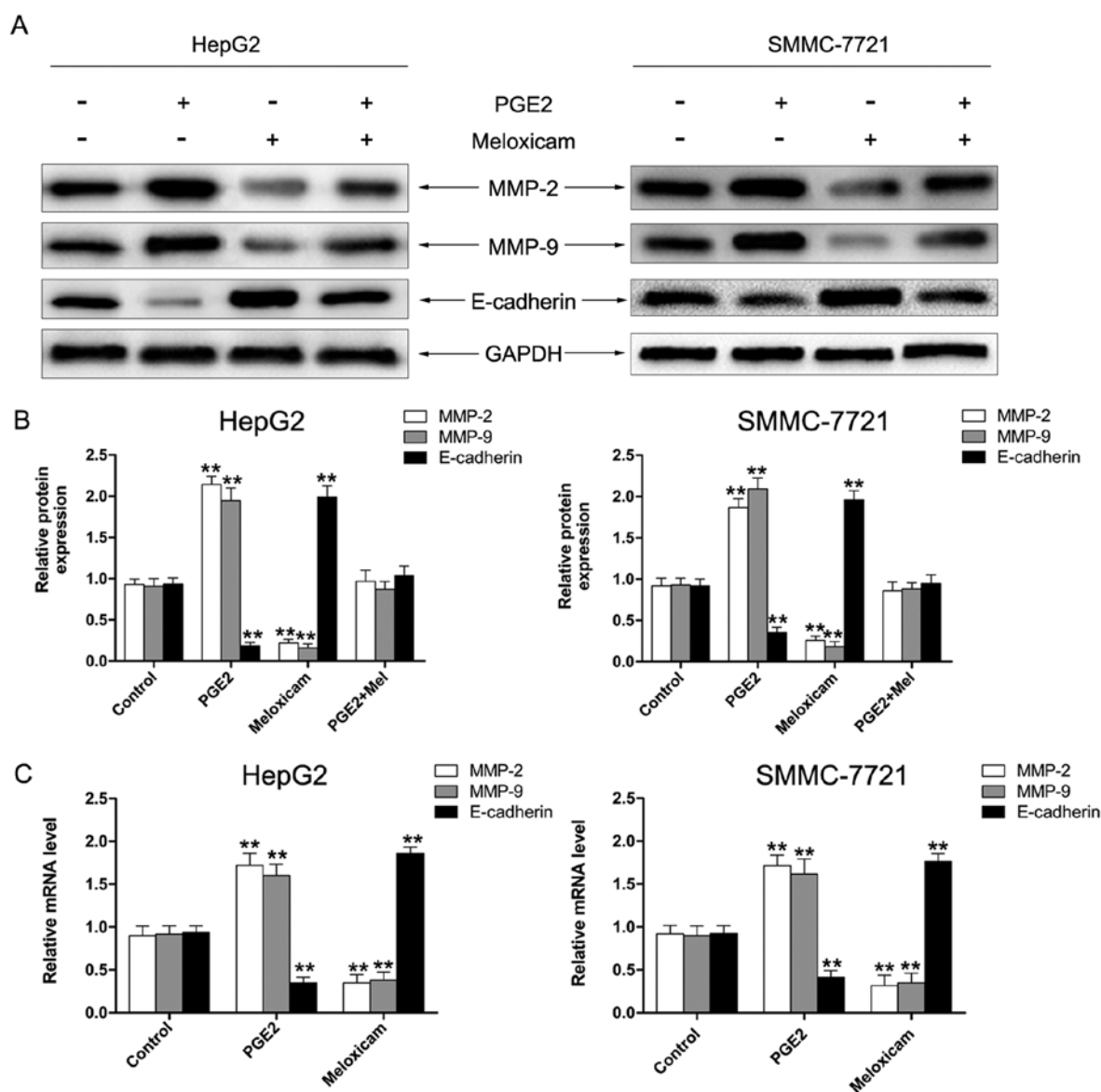
Figure 4. The effects of meloxicam on protein and mRNA expression of MMP-2, MMP-9, and E-cadherin in HCC cells *in vitro*. (A and B) HepG2 and SMMC-7721 cells were treated with PGE2 (10 μM), meloxicam (80 μM), or a combination for 24 h and then total protein was extracted. Western blotting was performed to detect protein levels of MMP-2, MMP-9, and E-cadherin. GAPDH was measured as the loading control. Results shown are representative of at least three independent experiments. **P<0.01 vs. control. (C) Cells were treated with PGE2 or meloxicam and total RNA was extracted. mRNA expression of MMP-2, MMP-9, and E-cadherin was determined by real-time quantitative RT-PCR. GAPDH served as an internal control. Data represent three independent experiments. **P<0.01 vs. control.

treatment with PGE2 significantly enhanced the migration of HepG2 and SMMC-7721 cells. However, this effect could be reversed by meloxicam. Moreover, we examined expression of COX-2 in HepG2 and SMMC-7721 cells treated with meloxicam or not using western blot analysis. As expected, we found that treatment of HepG2 and SMMC-7721 cells with meloxicam for 24 h induced a marked reduction of COX-2 expression in these cells (Fig. 3C). The effect of COX-2/PGE2 in HCC cell migration was further verified by downregulation of COX-2 by siRNA. As shown in Fig. 3D, transfection with COX-2 siRNA notably decreased the migration in HepG2 and SMMC-7721 cells. These results revealed that the suppression of endogenous levels of COX-2/ PGE2 expression is associated with the inhibition of HCC cell migration.

Effects of PGE2 and meloxicam on MMP-2/9 and E-cadherin expression in HCC cells. Since the downregulation of E-cadherin and upregulation of expression of MMP-2/9 is associated with enhancement in migration/invasion of cancer cells (31-34), we investigated protein expression and mRNA of MMP-2/9 and E-cadherin in HepG2 and SMMC-7721 cells. As shown in Fig. 4A and B, the level of MMP-2 and MMP-9 was significantly enhanced by PGE2 and reversed by meloxicam. Expression of E-cadherin was decreased by treatment with PGE2 whereas it was increased after being exposed to meloxicam. The results of RT-PCR showed similar effects in mRNA expression of MMP-2/9 and E-cadherin (Fig. 4C). These results suggested that meloxicam decreases MMP-2/9 activity and enhances the level of E-cadherin to inhibit the migration of HCC cells.
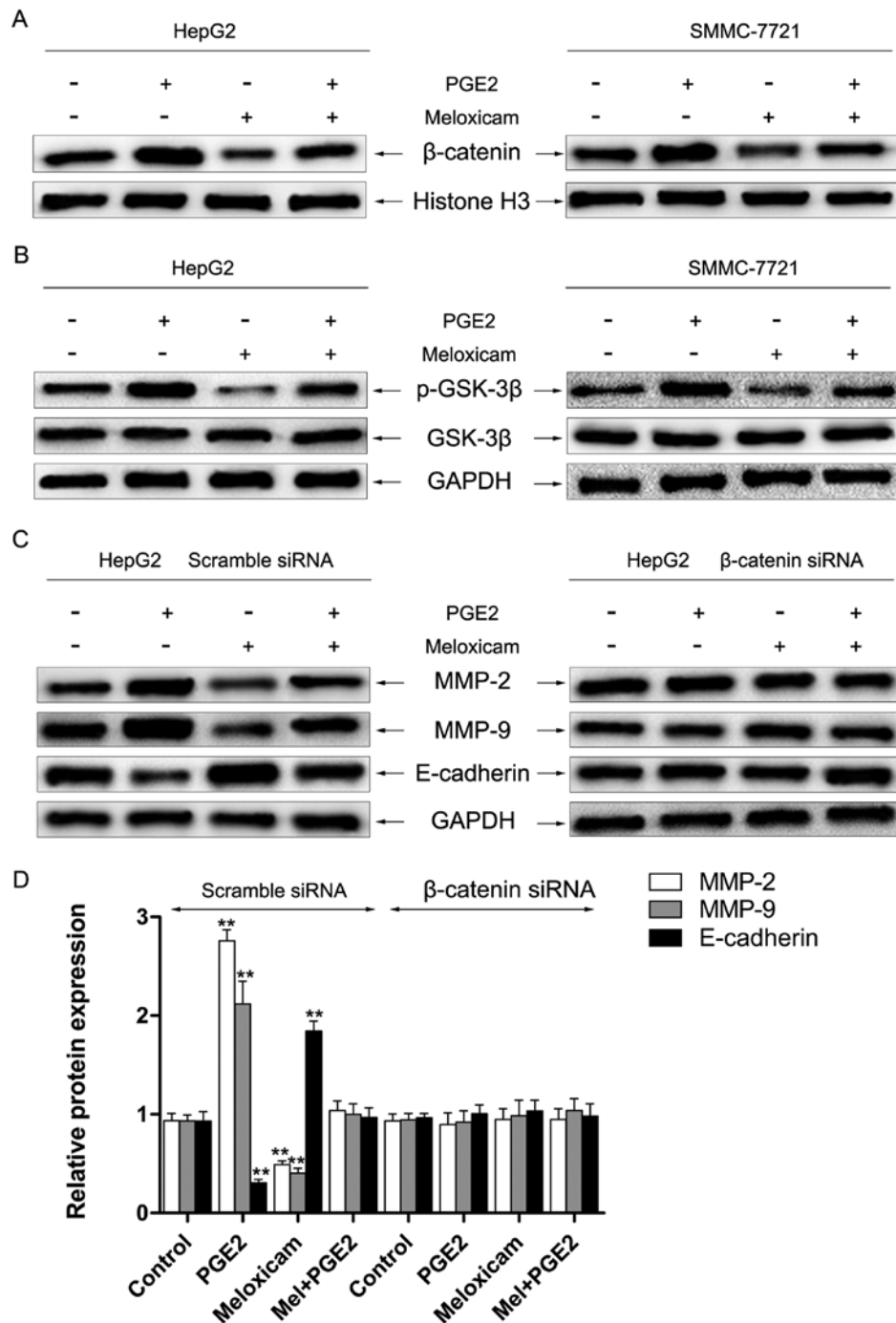
Figure 5. The effects of meloxicam on the β-catenin signaling pathway in HCC cells *in vitro*. (A) The effect of meloxicam (80 μM) on β-catenin expression in the nucleus of HepG2 and SMMC-7721 cells with or without PGE2 (10 μM). Histone H3 was measured as the loading control. (B) The effect of meloxicam (80 μM) on the GSK3β phosphorylation level in HCC cells with or without PGE2 (10 μM). GAPDH was measured as the loading control. (C and D) HepG2 cells were transfected with scramble or β-catenin siRNA and treated with PGE2 (10 μM) or meloxicam (80 μM) and total protein was extracted. Western blotting was performed to detect protein levels of MMP-2, MMP-9, and E-cadherin. GAPDH was measured as the loading control. Data represent three independent experiments. [**]$P<0.01$ vs. control.

Effects of PGE2 and meloxicam on the β-catenin signaling pathway in HCC cells in vitro. Accumulating evidence has demonstrated that PGE2 exerts a crucial role in promoting migration and regulating expression of MMP-2/9 and E-cadherin via the β-catenin signaling pathway (15,35). In this study, we investigated the role of meloxicam on the β-catenin signaling pathway. As depicted in Fig. 5A, treatment with PGE2 significantly enhanced nuclear accumulation of β-catenin which could be reversed by meloxicam in HepG2 and SMMC-7721

cells. Several studies reported that PGE2 can inactivate GSK3β and result in a consequent intracellular accumulation of β-catenin (36). Thus, we investigated the role of meloxicam on the level and activation of GSK3β. We found that treatment with meloxicam significantly inhibited the phosphorylation of GSK3β, however, expression of GSK3β was only slightly changed (Fig. 5B). Furthermore, knockdown of β-catenin by siRNA was utilized to examine expression of MMP-2/9 and E-cadherin. As shown in Fig. 5C and D, downregulation of
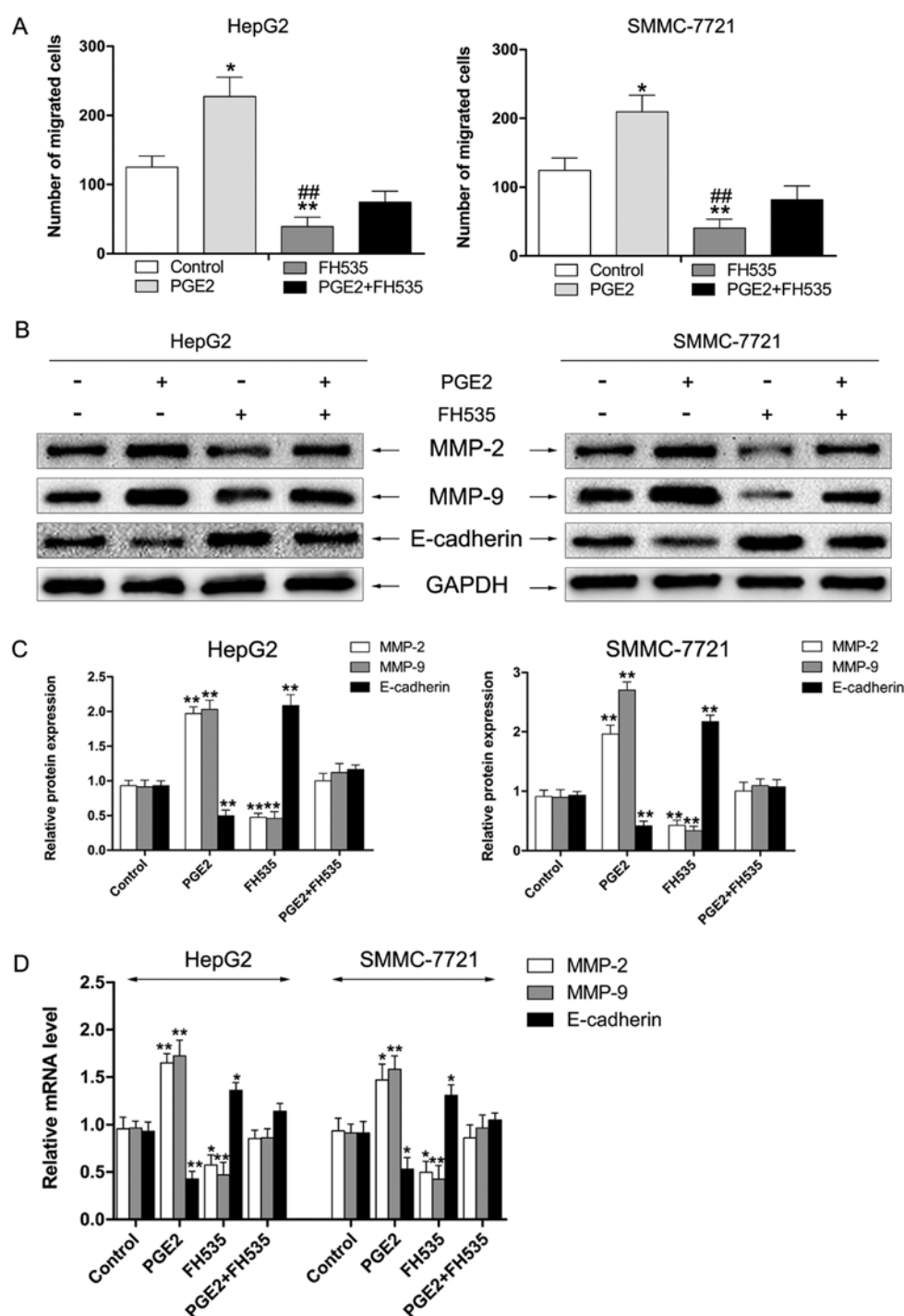
Figure 6. FH535 inhibits PGE2-enhanced cell migration of HCC cells *in vitro*. (A) FH535 suppresses PGE2-enhanced migration of HepG2 and SMMC-7721 cells. *P<0.05, **P<0.01 vs. control, ##P<0.01 vs. PGE2. (B and C) HCC cells were treated with PGE2 (10 μM), FH535 (5 μM), or a combination and total protein was extracted. Western blotting was performed to detect protein levels of MMP-2, MMP-9, and E-cadherin. GAPDH was measured as the loading control. Data represent three independent experiments. **P<0.01 vs. control. (D) HCC cells were treated with PGE2 (10 μM), FH535 (5 μM), or a combination and total RNA was extracted. mRNA expression of MMP-2, MMP-9, and E-cadherin was determined by RT-PCR. GAPDH served as an internal control. Data represent three independent experiments. *P<0.05, **P<0.01 vs. control.

β-catenin notably alleviated meloxicam-induced suppression of MMP-2/9 upregulation and E-cadherin downregulation by treatment of PGE2 in HepG2 cells. A similar result was also found in SMMC-7721 cells (data not shown).

FH535, an inhibitor of β-catenin, suppresses PGE2-induced cell migration of HCC cells in vitro. To further investigate whether activation of β-catenin and PGE2 has a role in migration of HCC cells, HepG2 and SMMC-7721 cells were exposed to PGE2 with or without treatment with FH535, an inhibitor of β-catenin. As shown in Fig. 6A, treatment with PGE2 significantly increased the migration ability of HepG2 and SMMC-7721 cells. However, treatment of cells with FH535 markedly suppressed PGE2-enhanced migration of HCC cells. The results of western blotting showed that the level of MMP-2/9 was reduced and E-cadherin was enhanced

by treatment with FH535 in HepG2 and SMMC-7721 cells (Fig. 6B and C). We also found similar effects in mRNA expression of MMP-2/9 and E-cadherin by RT-PCR assay (Fig. 6D).

Discussion

It was reported that COX-2 is overexpressed in various cancers and is associated with cancer cell migration/invasion. The selective COX-2 inhibitor has been recognized as exerting antitumor effects through suppression of PGE2 production. COX-2 overexpression has been considered to be linked to cancer cell-derived PGE2 which promotes tumor cell proliferation, invasion, and migration and reduces apoptosis (37). Our previous studies also demonstrated that meloxicam produces its antitumor effects against hepatocellular carcinoma in COX-2-dependent and -independent pathways (14,26). However, the exact anti-migration mechanism of meloxicam on the downstream pathway of PGE2 in HCC cells remains unknown. In this study, we found that HCC cell lines expressed different levels of COX-2 protein and that meloxicam exerted an anti-proliferation effect in HepG2 and SMMC-7721 cells via blocking the cell cycle in the G1 phase through regulating expression of CDK inhibitor proteins p21 and p27. Moreover, we observed that treatment with PGE2 significantly potentiated the migration potential whereas this effect was reversed after exposure to meloxicam, which suggested that meloxicam inhibition of the migration potential may be linked to the suppression of endogenous expression of COX-2 and production of PGE2 in HepG2 and SMMC-7721 cells. This concept is also supported by the evidence that transfection of HCC cells with COX-2 siRNA led to a marked reduction of cell migration in HepG2 and SMMC-7721 cells as compared to the migration of scramble siRNA-transfected HCC cells.

A large number of studies revealed that MMP-2/9 promote tumor metastasis and invasion by degrading extracellular matrix proteins (38,39). In addition, E-cadherin has been reported as a suppressor of invasion and metastasis in many cancers (40). In the present study, we explored the protein and mRNA MMP-2/9 and E-cadherin expression following exposure to meloxicam with or without PGE2. Our results showed that expression of MMP-2 and MMP-9 was increased by PGE2 and reversed by meloxicam and the extent of E-cadherin was decreased following treatment with PGE2 whereas it was increased after exposed to meloxicam. We also observed similar effects in mRNA expression of MMP-2/9 and E-cadherin by RT-PCR assay. These data demonstrated that meloxicam has an inhibitory role in the migration and invasion of HCC cells through mediating the level of MMP-2/9 and E-cadherin.

Various studies reported the role of β-catenin in cancer invasion/migration through regulating cell-to-cell adhesion (41). The constitutively active β-catenin signaling pathway results in disturbed cell-to-cell adhesion and consequent upregulation of the migration potential of tumor cells. Moreover, some studies demonstrated that PGE2 could have pro-oncogenic actions including proliferation and metastasis by stimulating β-catenin-mediated transcription in carcinogenesis (22,42). The data presented in the present study provide evidence that

meloxicam exerts its anti-migration effects through downregulation of nuclear accumulation of β-catenin and inhibiting the phosphorylation of GSK-3β. Knockdown of β-catenin by siRNA significantly reduced meloxicam-induced suppression of MMP-2/9 upregulation and E-cadherin downregulation by treatment of PGE2 in HepG2 and SMMC-7721 cells. To further explore the link between COX-2/PGE2 and β-catenin in HCC cell migration, we used FH535 (an inhibitor of β-catenin) to investigate whether PGE2-enhanced migration was dependent on the β-catenin signaling pathway. We observed that FH535 inhibited PGE2-enhanced migration of HCC cells.

In conclusion, the present study showed that meloxicam suppresses the migration of HCC cells by targeting PGE2-regulated activation of the GSK-3β/β-catenin signaling pathway. These findings suggest that meloxicam may be a potential therapeutic option for preventing HCC invasion/migration.

Acknowledgements

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