Proliferation of Hepatic Oval Cells via Cyclooxygenase-2 and Extracellular Matrix Protein Signaling during Liver Regeneration Following 2-AAF/Partial Hepatectomy in Rats

Si Hyun Bae*, Seh Hoon Oh[†], Seung Kew Yoon*, Joung Ah Park[‡], Gi Dae Kim[§], Wonhee Hur[‡], Jong Young Choi*, Il Hoan Oh[‡], and Kun Ho Yoon*

Department of Internal Medicine, The Catholic University of Korea, College of Medicine, Seoul, Korea, [†] Wake Forest Institute for Regenerative Medicine, Wake Forest University School of Medicine, Winston-Salem, NC, USA, [‡] High Performance Cell Therapy Center, The Catholic University of Korea College of Medicine, Seoul, Korea, and [§] The Seoul National University, College of Pharmacy, Seoul, Korea

Background/Aims: In the 2-acetylaminofluorene (2-AAF)/ 70% partial hepatectomy (PHx) model, the mechanism underlying the differentiation of activated hepatic oval cells (HOCs) into hepatocytes and bile ductile cells is unclear. We investigated the role of cyclooxygenase-2 (COX-2) in HOCs and the relationship between COX-2 and extracellular matrix proteins in cellular proliferation. **Methods:** Reverse transcription-polymerase chain reaction, immunohistochemical staining, and Western blotting were used to assess COX-2 expression. The co-localization of COX-2 with Thy1, c-Met, epithelial cell adhesion molecule, and α-smooth muscle actin was also examined. Additionally, we investigated whether connective tissue growth factor (CTGF), fibronectin (FN), extracellular signal-regulated kinase 1/2 (P-ERK1/2), and AKT were expressed in HOCs. **Results:** The expression of COX-2, prostaglandin E2 receptors, and c-Met was upregulated in HOCs. However, HOCs treated with the COX-2 inhibitor NS398 showed decreased COX-2, CTGF, FN, and AKT expression, whereas P-ERK1/2 was unaffected. Additionally, NS398 inhibited HOC proliferation, but not the proliferation of HOCs cultured on FN-coated dishes. Furthermore, the proliferative response of HOCs treated with NS398 was reversed by hepatic growth factor treatment. **Conclusions:** These results suggest that HOC proliferation is mediated through COX-2, extracellular FN expression, and AKT activation. Thus, COX-2 plays an important role in HOC proliferation following acute injury. **(Gut Liver 2011;5:367-376)**

Key Words: Hepatic oval cells; Cyclooxygenase-2; Liver generation

INTRODUCTION

The proliferative liver responds to traumatic, chemical, metabolic, infectious, and other injuries by activating a complex mixture of cytokines and chemokines from remnant tissue.[1] In hepatic injury animal models, the administration of 2-acetylaminofluorene (2-AAF) before and during hepatic injury is associated with increased numbers of hepatic oval cells (HOCs) and increased differentiation to hepatocytes and bile ductular cells during liver regeneration, as the impaired hepatocyte response-to-growth signal suppresses hepatocyte proliferation.[2-5] HOCs display distinct phenotypic markers, including CD34, c-kit, c-Met, alpha-fetoprotein (AFP), and Thy1 surface antigen.[6-8] Among them, Thy1, which is expressed on bone marrow cells and stem cells in the fetal liver, plays an important role in hematopoiesis and hepatic development.[9-11] In an animal model of HOC activation, Thy1^{+} HOCs express high levels of connective tissue growth factor (CTGF), which is critical for HOC activation during liver regeneration.[12] HOCs that possess epithelial cell adhesion molecule (EpCAM) (EpCAM[+] HOCs) are potential adult hepatic epithelial progenitors, but they do not express the previously reported hematopoietic stem cell markers CD34, c-kit, or Thy1.[13]

The conversion of arachidonic acid to prostaglandins (PGs), which is the irreversible step in prostanoid biosynthesis, is mediated by two cyclooxygenases (COXs), COX-1 and COX-2, which are encoded by unique genes located on different chromosomes.[14,15] Between them, COX-2, which was initially identified as an immediate early growth-response gene, is induced by a wide variety of stimuli such as cytokines, hormones, mito-

gens, and growth factors,[16-20] and is frequently overexpressed in various tumor cells.[21] The effects of COX-2 and PG-dependent signaling on HOC activation have been reported in liver cancer. The COX-2 inhibitor-induced reduction in HOC number may be due to the COX-2-dependent inhibition of Akt phosphorylation and the induction of apoptosis.[22]

During liver remodeling, including hepatic fibrosis and liver regeneration, several growth factors affect HOC proliferation and differentiation.[1,23] Among them, hepatocyte growth factor (HGF) has a variety of activities in various cells, exerting mitogenic[24] and morpho-organogenic[25] effects by activating c-Met, a tyrosine kinase receptor.[26] When HGF binds the c-Met receptor, signaling molecules such as extracellular signal-regulated kinase (ERK)[27] and AKT protein kinase[28] are activated. Among them, the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway is dedicated to a variety of system-related biological effects of HGF, including protection against apoptosis in primary hepatocytes.[29]

Hepatic stellate cells (HSCs) influence the growth and development of HOCs. HSCs in the periportal regions increase in response to liver injury[30] and produce fibronectin (FN), a component of the extracellular matrix (ECM), which is crucial for liver regeneration. CTGF on an FN-concentrated provisional matrix facilitates HOC activation, and FN is critical for HOC proliferative functions.[31] A positive correlation has been reported between FN concentration and COX-2 expression in human cancer cells.[32-34] However, the precise mechanism linking FN and *COX-2* expression during liver regeneration is unclear.

Herein, we explored COX-2 expression during HOC activation and liver regeneration following 2-AAF/70% partial hepatectomy (PHx), and the nature of the signals that modulate COX-2 expression. The co-localization of c-Met and EpCAM with COX-2 clearly demonstrated that COX-2 is expressed in HOCs. Our results show that an increasing level of COX-2 and c-Met expression in HOCs may occur through PG signaling during liver regeneration in the 2-AAF/PHx model. Moreover, FN was expressed in HOCs. The relationships between COX-2 and proliferation-related signals during liver regeneration were also investigated. The COX-2 inhibitor NS398 decreased HOC proliferation and significantly suppressed CTGF, FN, and AKT signaling, but did not affect phosphorylated ERK 1/2 (P-ERK 1/2). Interestingly, NS398 did not impair HOC proliferation in FN-coated dishes, indicating that FN induces HOC proliferation through COX-2 signaling. COX-2 expression increased when HOCs were challenged with a COX-2 inhibitor, followed by a change to an HGF-containing medium.

MATERIALS AND METHODS

1. Animals and experimental groups

Male Fischer rats weighing 120 to 150 g were divided into 3 equal groups (20 rats each). All animal experiments were conducted according to protocols approved by the Animal Care and Use Committee of the Catholic University of Korea. Time-released 2-AAF (35 mg/pellet over 14 days) treatment was achieved using a product supplied by Innovative Research (Sarasota, FL, USA). The pellets were inserted subcutaneously 7 days before PHx. Three rats from each group were killed, and their livers were collected. All time points are indicated as days after PHx treatment.

2. HOC culture

A Thy1-specific antibody in conjunction with magnetic-activated cell sorting (MACS) was used to isolate HOCs, with yields averaging 3×10^6 cells per animal on day 9. Thy1^{+} cells were isolated by MACS, as described previously.[5] HOCs were cultured in Iscove's MDM solution containing 10% fetal bovine serum (FBS), 5 μg/mL insulin, 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies, Grand Island, NY, USA).

3. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of COX-2 and prostaglandin E2 receptor mRNA expression

RT-PCR analysis was conducted on total RNA isolated from tissues from normal, PHx and 2-AAF/PHx livers, as well as isolated HOCs, using an RNeasy Kit (Qiagen, Valencia, CA, USA). Two micrograms of RNA were used for each round of cDNA synthesis. RT was performed using a GeneAmp RNA PCR Kit and a DNA thermal cycler (Perkin Elmer, Norwalk, CT, USA), which were also used for PCR. The primers used for *COX-2*, *EP1*, *EP2*, *EP3*, and *EP4* were: 5′-AAG CCT CGG CCA GAT GCC AT-3′ forward and 5′-GTA GTA CTG TGG GAT TGA TAT C-3′ reverse (*COX-2*, 340 bp), 5′-ACG TGG GTC ACT ACG AGC TAC-3′ forward and 5′-GCT GTG GTT GAA GTG ATG GAT C-3′ reverse (*EP1*, 301 bp), 5′-GGG TCT CCT TGC TCT TCT GTT-3′ forward and 5′-CTT TCG GGA AGA GGT TTC ATC C-3′ reverse (*EP2*, 392 bp), 5′-GTA TGC CAG CCA CAT GAA GAC-3′ forward and 5′-GAT GTG CCC CAT AAG CTG AT AGC-3′ reverse (*EP3*, 370 bp), and 5′-GCC AAG TGT GGT GAA AGA CAT-3′ forward and 5′-CTC TCT GGC TCC CAC TAA CCT -3′ reverse (*EP4*, 482 bp). Briefly, mRNA was reverse-transcribed and the cDNA was subjected to 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and an extension period of 72°C for 1 minute. The amplified products were subjected to 1% agarose gel electrophoresis and stained with ethidium bromide. The mRNA levels were normalized using *GAPDH* as a housekeeping gene.

4. Immunohistochemical staining

Double-immunofluorescence staining for Thy1.1 (BD Biosciences, San Jose, CA, USA), c-Met (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and EpCAM (Abcam, Cambridge, MA, USA) with COX-2 (Transduction Lab, Franklin Lakes, NJ, USA) was performed to verify the identity of the HOCs. Addition-

ally, immunofluorescence staining of α-smooth muscle actin (SMA) (Abcam) with COX-2 was performed, using a previously described cytochemical method, to exclude myofibroblasts as the COX-2 source.[35] Immunostaining for OV-6 (R & D Systems, Minneapolis, MN, USA), COX-2, Thy1.1, albumin (DakoCytomation, Carpinteria, CA, USA), AFP (DakoCytomation), FN, and

CD45 (BD Biosciences) was performed on isolated Thy1^{+} HOCs. Signals were detected using a Vector ABC Kit (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine tetrahydrochloride (DakoCytomation). Finally, BrdU (DakoCytomation) staining was conducted for the HOC proliferation assay on FN-coated culture dishes following NS398 treatment, as described
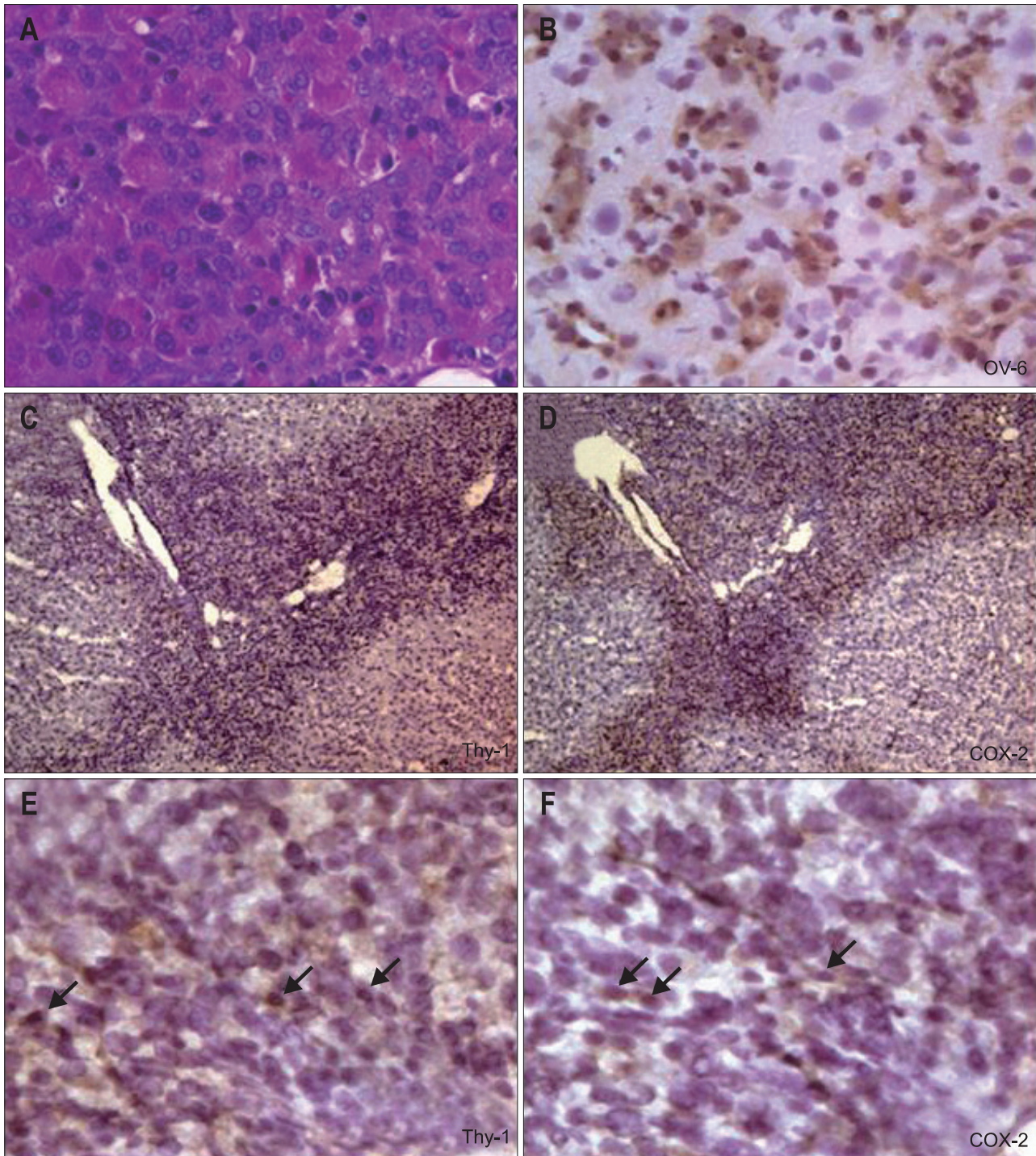
Fig. 1. Proliferation of hepatic oval cells (HOCs) on day 9 after induction of the 2-acetylaminofluorene (2-AAF)/70% partial hepatectomy (PHx) model. Immunohistochemical (IHC) staining is conducted to detect OV-6, thymus cell antigen1 (Thy1), and cyclooxygenase (COX)-2. (A) Hematoxylin and eosin staining (magnification, ×400). (B) OV-6 IHC staining in activated HOCs. OV-6 is a hepatic oval cell marker (magnification, ×400). (C, E) Thy1 and (D, F) COX-2 IHC staining in activated HOCs (magnification, ×100 and ×400, respectively).

previously.[36] Cells (4.5×10^4) in 6-well culture dishes were inoculated on FN-coated or uncoated dishes with 10% FBS supplemented with Iscove's MDM for 24 hours, after which a medium containing 100 μM NS398 was used in some cases. After 1 hour of incubation, 100 μM BrdU cell-labeling reagent was added. The cells were fixed after 24 hours, and the percentage of BrdU-labeled nuclei was determined by counting >100 nuclei in three random fields (×400 magnification).

5. Western blot analysis

Enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham Life Science (Arlington Heights, IL, USA), and a monoclonal mouse anti-COX-2 antibody was purchased from PharMingen (San Diego, CA, USA). Polyclonal rabbit anti-c-Met, -CTGF, -FN, -AKT, -p-AKT, -ERK 1/2, -p-ERK 1/2, and -β-actin antibodies were purchased from Santa Cruz Biotechnology. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

6. Regulation of COX-2 expression by NS398 and HGF

HOCs were selected to evaluate the inhibitory growth effect of the COX-2 inhibitor NS398 (Cayman Chemicals, Ann Arbor, MI, USA). COX-2 expression was inhibited in HOC cultures treated with 100 μM NS398, as described previously.[37] Cells (3×10^5) were grown in Iscove's MDM supplemented with 10% FBS, 100 μg/mL penicillin, 20 μM HGF, and 0.25 μg/mL streptomycin and then stimulated with 20 μM HGF for various time periods.

7. Statistical analysis

All experiments were repeated a minimum of three times. All RT-PCR, BrdU staining, and Western blot data are expressed as the mean±SD. The data in some figures are from a representative experiment, which was qualitatively similar to replicate experiments. Statistical significance was determined by comparing the two groups using a two-tailed Student's t-test. Asterisks in the figures indicate significant differences ($p \leq 0.05$) between experimental groups and the corresponding control.

RESULTS

1. COX-2 gene and protein expression in activated HOCs

HOCs appeared as dark blue areas in hematoxylin and eosin-stained liver sections, due to their large nuclei and small amount of cytoplasm, with numerous HOCs in the periportal region on day 9 after 2-AAF/PHx treatment (Fig. 1A). HOCs were confirmed by labeling with OV-6 (Fig. 1B). An additional immunomarker widely utilized in HOC labeling, Thy1.1, was also used (Fig. 1C and E). These results confirm that the 2-AAF/PHx model can be used effectively to activate HOCs in periportal regions during liver regeneration, consistent with previous observations.[38] Additionally, COX-2-expressing cells were evident around the periportal region (Fig. 1D and F), and COX-

2 staining in the HOCs was detected in the cytoplasm (Fig. 1F, 2D, 2G, 2J, and 2M). Double-immunofluorescence staining was performed on serial liver sections to verify COX-2 expression in the HOCs and to assess whether the HOCs co-expressed Thy1, c-Met, EpCAM, and α-SMA with COX-2 (Fig. 2). Fig. 2F and I show that in some HOCs COX-2 (Fig. 2D and G) was colocalized with the HOC markers Thy1 (Fig. 2E) and c-Met (Fig. 2H), whereas the co-localization of COX-2 (Fig. 2J) and EpCAM (Fig. 2K) did not occur in the peri-bile ductile or hepatic lobule area (Fig. 2L). Moreover, the co-localization of COX-2 (Fig. 2M) with the myofibroblast marker α-SMA (Fig. 2N) was not observed (Fig. 2O). These results indicate that Thy1+ HOCs expressed COX-2 and c-Met but not EpCAM or α-SMA.

The next experiment assessed COX-2 expression in total RNA extracted from normal liver, 2AAF/PHx-treated liver (on day 9), and PHx-treated liver (after 12 hours), and sorted Thy1+ HOCs by RT-PCR (Fig. 3A). COX-2 expression was not detected in normal liver, but was detected in the activated HOC model (2AAF/PHx), the liver regeneration model (PHx), and the sorted Thy1+ HOCs, suggesting that COX-2 participates in HOC activation during liver regeneration. Furthermore, COX-2 and c-Met (HGF receptor) expression was examined in the activated HOC model by Western blotting. Normal liver did not induce COX-2 expression, but HOC induction resulted in a progressive increase in COX-2 expression on days 3-13 after 2-AAF/PHx (Fig. 3B). The detection of COX-2 on day 3 after 2-AAF/PHx suggests that the COX-2-induced PG pathway plays a role in HOC activation. Our immunofluorescence staining results revealed that the HOCs expressed COX-2 and the HOC markers (albumin and AFP), but that they did not express CD45. Interestingly, FN was also evident in the HOCs, indicating that it might play a role in HOC activation or proliferation in the 2AAF/PHx liver model (Fig. 3C).

2. Detection of PG receptors in HOCs

The finding that COX-2 was expressed in HOCs indicated that a COX-2-mediated PG pathway might be involved in the HOC proliferation mediated by PG receptors. Indeed, PGE2 binds its receptors, designated EP1, EP2, EP3, and EP4, which have been implicated in carcinoma cell proliferation.[39,40] RT-PCR was performed for each EP receptor subtype to determine their expression profiles in HOCs (Fig. 4). Tissues from normal rat liver and rat liver after PHx, as well as sorted Thy1+ HOCs expressed all four PG receptors (though faint *EP3* expression was noted in the Thy1+ HOCs), indicating the existence of these receptors in Thy1+ HOCs and their possible role in proliferation.

3. Role of COX-2 and ECM in activated HOCs

To explore the downstream events in the PG pathway, HOCs were cultured with 100 μM NS398, which suppressed HOC proliferation. CTGF is a downstream mediator of the fibrogenic properties of transforming growth factor (TGF)-β1, which
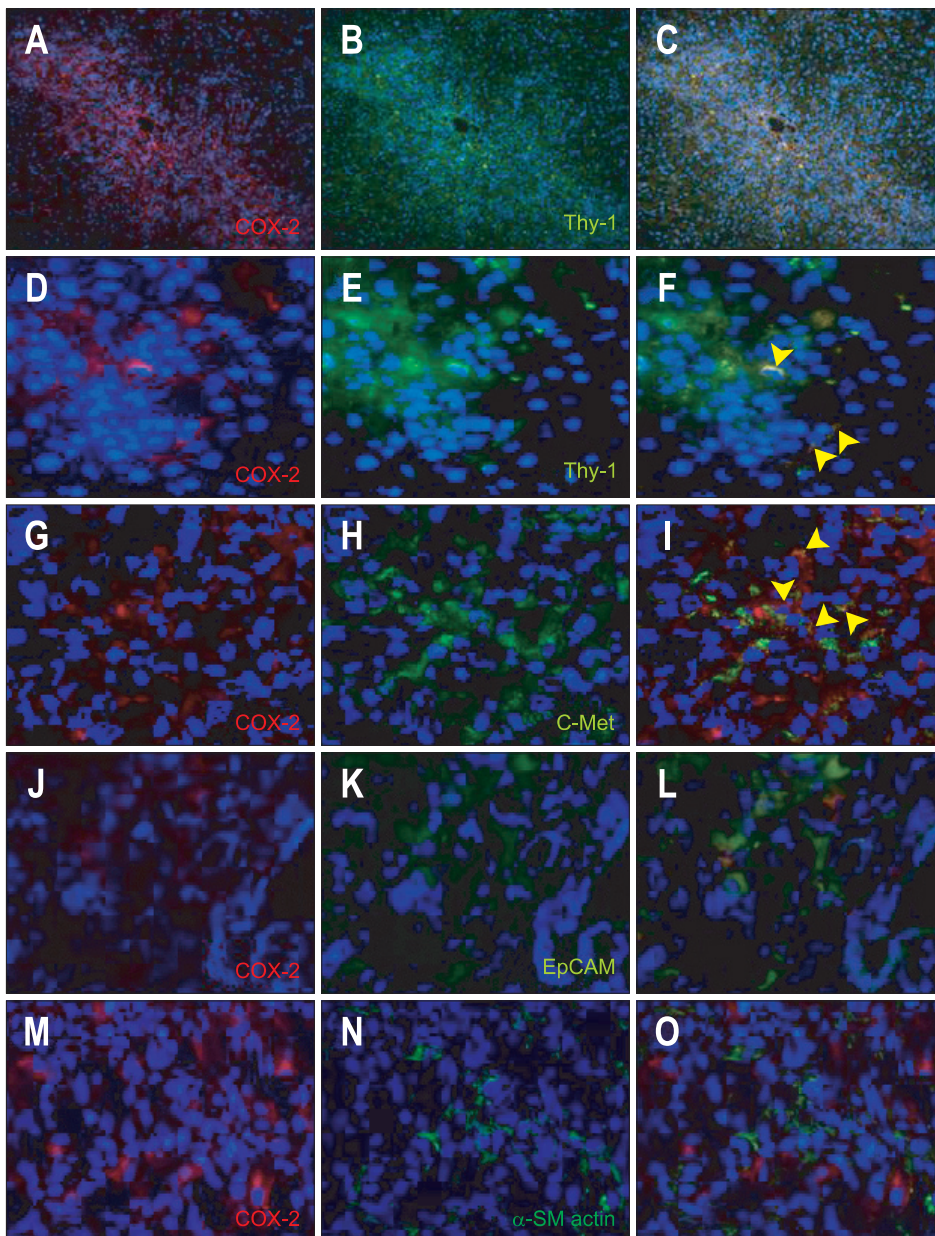
Fig. 2. Co-localization of cyclooxygenase (COX)-2 with thymus cell antigen1 (Thy1), c-Met, epithelial cell adhesion molecule (EpCAM), and α-smooth muscle actin markers. Double-immunofluorescence labeling with COX-2 (red; left panels in A, D, G, J, and M) and Thy1, c-Met, EpCAM, and α-smooth muscle actin, respectively (middle panels in B, E, H, K, and N). Yellow staining on the merged pictures indicates co-localization of the two proteins in hepatic oval cells (HOCs). Co-localization of COX-2 (A, D, G, J, and M) and Thy1 (B and E; merged images, C and F), c-Met (H; merged image, I), EpCAM (K; merged image, L), and α-smooth muscle actin (N; merged image, O) showing colocalization. 4',6-Diamidino-2-phenylindole counterstaining, blue (Original magnification: A-C, ×100; D-O, ×400).

stimulates the expression of ECM genes in many biological processes. Western blotting of HOCs cultured with NS398 showed increased levels of the ECM proteins CTGF, FN, and COX-2 (Fig. 5). Moreover, AKT phosphorylation was inhibited by COX-2 signaling, whereas ERK 1/2 phosphorylation was not. These results are consistent with the expression of CTGF, FN, and phospho-serine-specific AKT in activated HOCs via the PG pathway, and they indicate that ERK functions independently of PG signaling. FN is an ECM molecule that induces cell proliferation by inducing COX-2 signaling and subsequent PGE2 production.[33] Furthermore, we examined the HOC proliferation rate in FN-coated cultured dishes using BrdU staining. When HOCs were cultured on non-FN-coated dishes as a control and FN-coated dishes in the absence of NS398, dramatic proliferation was evident in the FN-coated dishes (Fig. 6A). However, the proliferation on non-FN-coated dishes was suppressed significantly when HOCs were treated with NS398. Interestingly, HOC proliferation was not inhibited significantly on FN-coated dishes, despite NS398 treatment (Fig. 6B). These observations are evidence of a positive interaction between HOCs and FN, which is critical for HOC proliferation.

COX-2 expression increased in a time-dependent manner following a change to HGF-containing medium after NS398 exposure for 24 hours (Fig. 7), suggesting a role for HGF in increased COX-2 expression during HOC proliferation.

DISCUSSION

We demonstrated that COX-2 is expressed and that ECM proteins were produced in activated HOCs through COX-2-induced

A

B

C
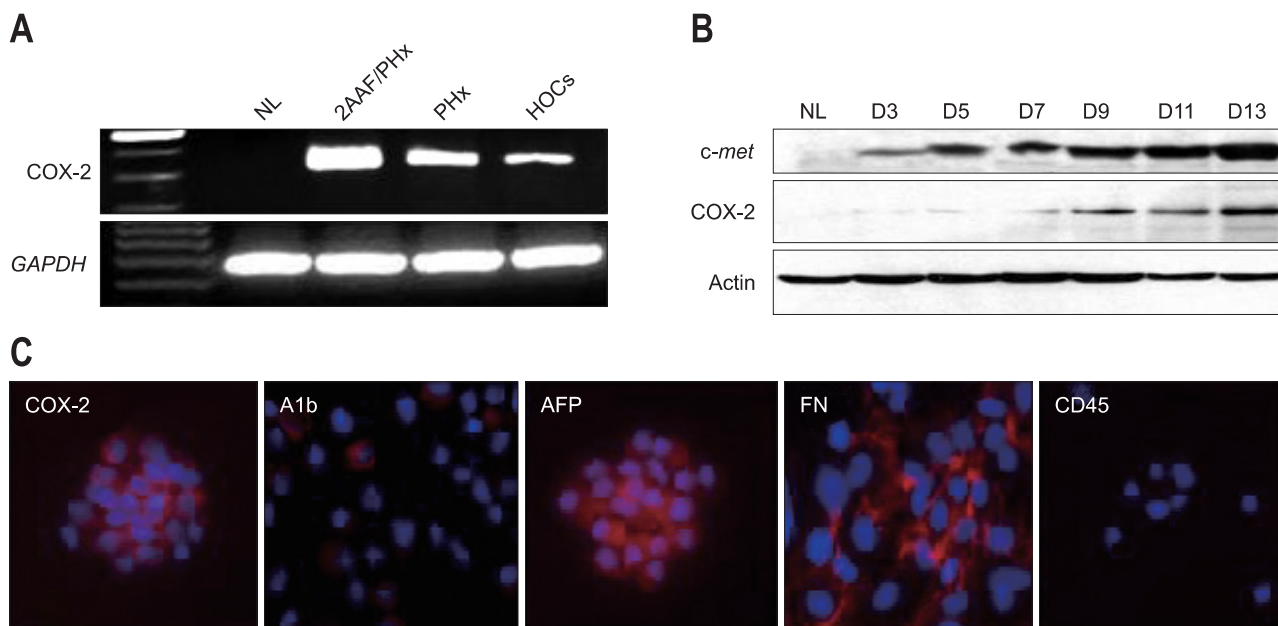
Fig. 3. Analysis of cyclooxygenase (COX)-2 expression in the 2-acetylaminofluorene (2-AAF)/70% partial hepatectomy (PHx) model and in sorted hepatic oval cells (HOCs). (A) RT-PCR analysis of COX-2 in normal liver (NL), 2AAF/PHx liver (day 9), PHx liver (after 12 hours), and Thy1+-sorted HOCs. (B) Western blot of a whole-liver homogenate demonstrating the accumulation of c-Met and COX-2. β-actin is used as a loading control. (C) Immunofluorescence staining (Texas Red) to examine the expression of COX-2, albumin, alpha-fetoprotein (AFP), fibronectin (FN), and CD45 in Thy1+ HOCs sorted from the livers of 2AAF/PHx-treated rats on day 9 (original magnification, ×600)
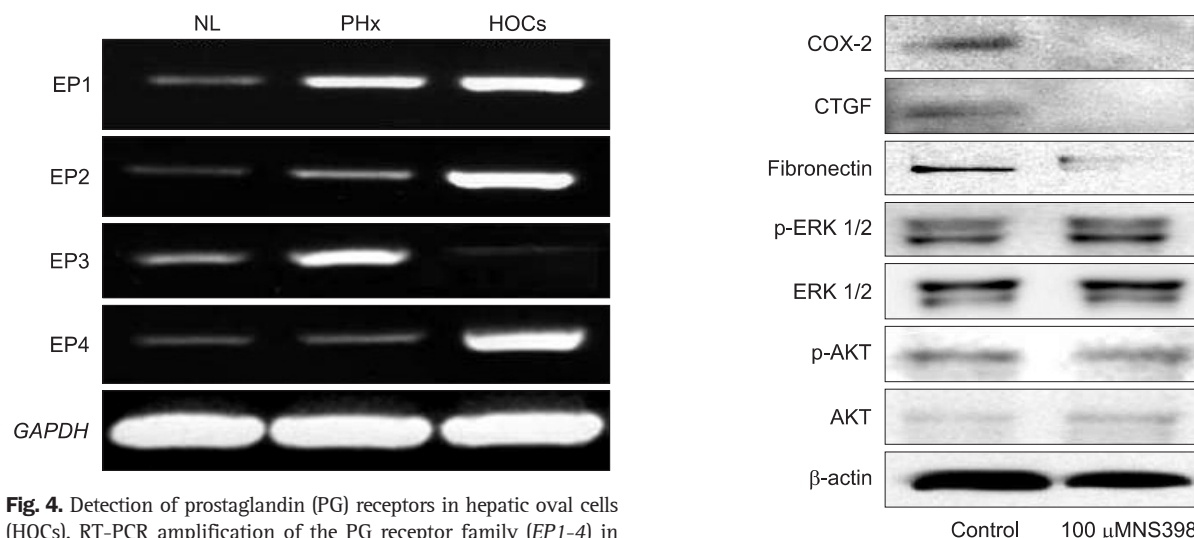

Fig. 4. Detection of prostaglandin (PG) receptors in hepatic oval cells (HOCs). RT-PCR amplification of the PG receptor family (*EP1-4*) in normal liver (NL), the liver regeneration model (PHx), and Thy1+-sorted HOCs. *GAPDH* is used as an internal standard.

Fig. 5. Western blot analysis of NS398-treated hepatic oval cells (HOCs). HOCs cultured with 100 μM NS398 for 24 hours are examined. Control cells are treated with DMSO without NS398. NS398 suppress cyclooxygenase (COX)-2, connective tissue growth factor (CTGF), fibronectin (FN), and AKT expression, but not ERK expression. β-Actin is used as a loading control.

PG signaling during liver regeneration. The relationship between COX-2 and HOC proliferation-related signals involved two ECM proteins (CTGF and FN) and AKT activity. The COX-2 inhibitor NS398 decreased HOC proliferation and significantly suppressed CTGF and FN expression and AKT activation, but not ERK 1/2 activation. Moreover, the observation that NS398 did not affect HOC proliferation on FN-coated dishes indicates that the FN-induced proliferation of HOCs occurs through COX-2-induced PG signaling. The observation that HOCs treated with NS398 were shifted to an HGF-containing medium for HOC regulation

is entirely consistent with a role for HGF in HOC proliferation.

In the liver regeneration model, HOCs participate in liver regeneration by activating a complex mixture of compounds, including cytokines and growth factors.[2-6] Several growth factors (e.g., HGF and epidermal growth factor) and intracellular signaling pathways (e.g., ERK and Akt) are important regulators of HOC-mediated liver regeneration.[41-43] Furthermore, the upreg-

A

B
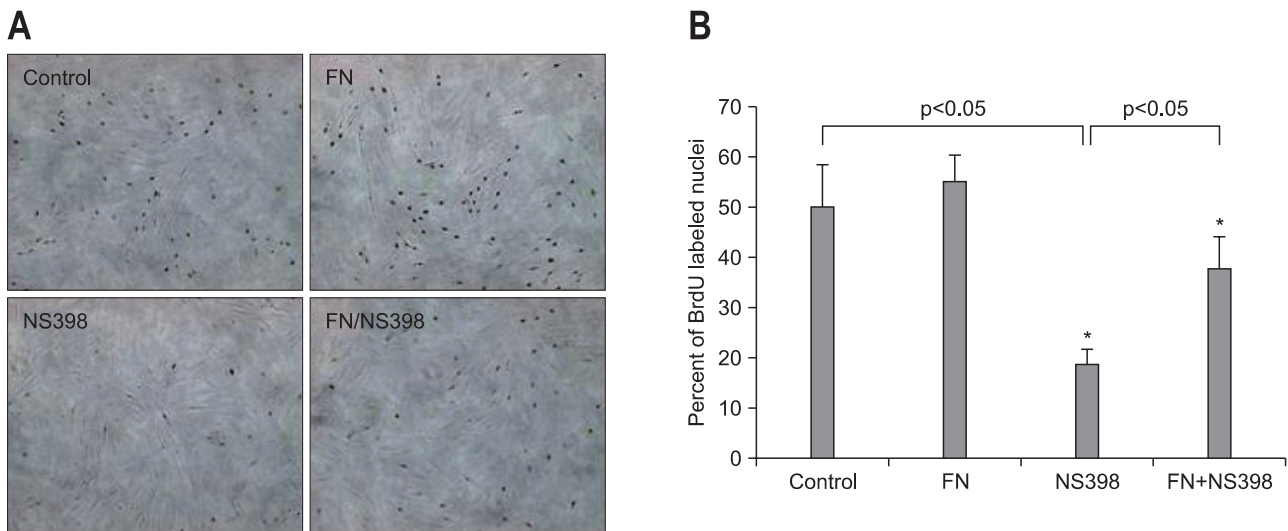
Fig. 6. Analysis of the proliferation of hepatic oval cell (HOC) grown on fibronectin (FN)-coated culture dishes by BrdU staining. (A) BrdU-labeled nuclei in HOCs. Control, non-FN-coated dish in the absence of NS398; FN, FN-coated dish in the absence of NS398; NS398, non-FN coated dish with NS398; FN/NS398, FN-coated dish with NS398 (*p<0.05) (original magnification, ×200). (B) Quantification of proliferating HOCs by BrdU staining.
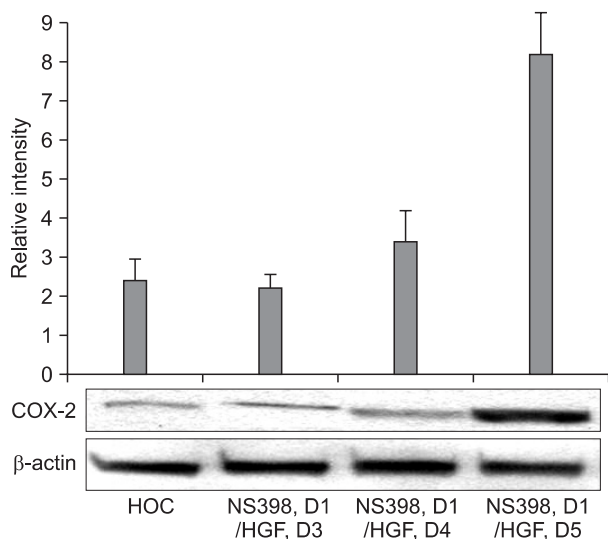
Fig. 7. Hepatic growth factor (HGF) induces cyclooxygenase (COX)-2 expression in hepatic oval cells (HOCs) treated with NS398. HOCs were cultured in 100 μM NS398 for 24 hours and transferred to medium containing 5 ng/mL HGF. A representative Western blot is shown. The time-dependent increase in COX-2 expression following HGF exposure is apparent.

ulation of ECM proteins is important for HOC proliferation during liver regeneration induced by 2-AAF/PHx.[12,31] However, the underlying mechanism linking the COX-2-induced PG signaling pathway with HOC-mediated liver regeneration is unclear.

We determined that COX-2 expression occurred in the HOC activation model, in which 2AAF pellets were implanted into animals to inhibit hepatocyte proliferation. Normal liver tissue did not express COX-2, and *COX-2* and *EP1-4* were expressed and upregulated in liver tissue obtained from the 2AAF/PHx model and from isolated HOCs.

EpCAM is a cell surface marker for HOCs.[44] Immunofluorescence microscopy showed that COX-2-expressing cells were stained with the HOC markers albumin (albeit weakly), AFP, FN, and c-Met, but not with EpCAM or α-SMA. Therefore, the sorted Thy1^{+} cells were HOCs, contrary to the opinion that EpCAM^{+} cells are representative of the HOC population.

An increase in c-Met and COX-2 expression was observed over the course of regeneration, suggesting that the PG signaling pathway plays a role in HOC activation and proliferation. Previous studies reported that selective COX-2 inhibitors reduced cancer growth in carcinogen-treated rodents, inhibited various cancer cell lines,[45,46] and delayed wound healing by reducing inflammation-induced epithelial cell proliferation.[47] We found that NS398 suppressed HOC proliferation and decreased CTGF, FN, and AKT expression during liver regeneration. In contrast, the proliferation of HOCs cultured on FN-coated dishes was not inhibited by COX-2 inhibitor treatment. These results indicate that COX-2 and ECM proteins might be involved in HOC activation through PG-dependent and -independent signaling pathways.

The physical condition of the normal subendothelial ECM is essential for maintaining different functions in all liver cells. We found that NS398 decreased COX-2, CTGF, FN, and AKT expression, but not ERK expression, and that it suppressed HOC proliferation. However, NS398-mediated inhibition was obviated by the presence of FN on the culture dishes. These results suggest that COX-2 is critical for HOC proliferation during liver regeneration, and that the interaction between ECM proteins and COX-2 is essential for liver regeneration after liver injury.

HGF is a multifunctional cytokine with mitogenic, morphogenic, and tumor-suppressing activities, which are present on the majority of epithelial cells, including HOCs.[48,49] Despite this

broad spectrum of biological activities, HGF is most likely the physiological hepatotrophic factor that triggers or modulates liver regeneration. HGF induces HOC proliferation in a 2-AAF/PHx rat model,[50] and PGs induce HGF expression in skin fibroblasts. In contrast, the inhibition of PG synthesis by dexamethasone reduces HGF production in these cells.[51] In the present study, c-Met and COX-2 protein levels increased during liver regeneration in the rat 2-AAF/PHx model, and COX-2 expression decreased gradually following NS398 treatment in a time-dependent manner, suggesting that the effects of NS398 on HOC proliferation are mediated by a COX-2-dependent signaling pathway (data not shown). Our findings are consistent with those of a previous report, although a reduction in the number of oval cells is associated with a reduced incidence of liver cancer in mice.[22] In the context of human chronic liver disease, these results are of considerable relevance, as liver regeneration is paralleled by changes in the number of hepatic progenitor cells, which can differentiate into hepatocytes and bile ductular cells.

COX-2 expression in HOCs was upregulated following exposure to an HGF-containing medium after NS398 treatment. These results suggest that HGF has a proliferative effect on HOCs, and that COX-2 mediates the effects of HGF on HOC proliferation due to binding of the growth factor to the c-Met receptor on the HOC surface. Thus, COX-2 may be used with ECM and HGF to investigate the regulation of HOC proliferation during liver regeneration.

There are some limitations to this study. First, the identification of the PGE2 receptors was preliminary; additional functional assays are needed. Second, a clear organized analysis of the signal transduction mechanisms involved in HOC proliferation, including total and phosphorylated ERK and AKT, is required.

In conclusion, we showed that COX-2 is expressed in activated HOCs, which proliferate through COX-2, extracellular FN, and AKT signaling, during liver regeneration after acute liver injury. HGF also stimulated HOC proliferation by increasing COX-2 expression. Therefore, COX-2 plays an important role in HOC proliferation after liver injury.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGEMENTS

REFERENCES

1. Michalopoulos GK, DeFrances MC. Liver regeneration. Science 1997;276:60-66.

2. Fausto N, Campbell JS. The role of hepatocytes and oval cells in liver regeneration and repopulation. Mech Dev 2003;120:117-130.

3. Shafritz DA, Dabeva MD. Liver stem cells and model systems for liver repopulation. J Hepatol 2002;36:552-564.

4. Evarts RP, Nagy P, Nakatsukasa H, Marsden E, Thorgeirsson SS. In vivo differentiation of rat liver oval cells into hepatocytes. Cancer Res 1989;49:1541-1547.

5. Petersen BE, Zajac VF, Michalopoulos GK. Hepatic oval cell activation in response to injury following chemically induced periportal or pericentral damage in rats. Hepatology 1998;27:1030-1038.

6. Thorgeirsson SS. Hepatic stem cells in liver regeneration. FASEB J 1996;10:1249-1256.

7. Petersen BE, Goff JP, Greenberger JS, Michalopoulos GK. Hepatic oval cellsexpress the hematopoietic stem cell marker Thy-1 in the rat. Hepatology 1998;27:433-445.

8. Sahin MB, Schwartz RE, Buckley SM, et al. Isolation and characterization of a novel population of progenitor cells from unmanipulated rat liver. Liver Transpl 2008;14:333-345.

9. Goldschneider I, Gordon LK, Morris RJ. Demonstration of Thy-1 antigen on pluripotent hemopoietic stem cells in the rat. J Exp Med 1978;148:1351-1366.

10. Craig W, Kay R, Cutler RL, Lansdorp PM. Expression of Thy-1 on human hematopoietic progenitor cells. J Exp Med 1993;177:1331-1342.

11. Bae SH, Choi JY, Yoon SK, et al. Thy1-positive bone marrow stem cells express liver-specific genes in vitro and can mature into hepatocytes in vivo. Hepatol Int 2008;2:63-71.

12. Pi L, Oh SH, Shupe T, Petersen BE. Role of connective tissue growth factor in oval cell response during liver regeneration after 2-AAF/PHx in rats. Gastroenterology 2005;128:2077-2088.

13. Yovchev MI, Grozdanov PN, Zhou H, Racherla H, Guha C, Dabeva MD. Identification of adult hepatic progenitor cells capable of repopulating injured rat liver. Hepatology 2008;47:636-647.

14. Hla T, Neilson K. Human cyclooxygenase-2 cDNA. Proc Natl Acad Sci U S A 1992;89:7384-7388.

15. Wen PZ, Warden C, Fletcher BS, Kujubu DA, Herschman HR, Lusis AJ. Chromosomal organization of the inducible and constitutive prostaglandin synthase/cyclooxygenase genes in mouse. Genomics 1993;15:458-460.

16. Funk CD, Funk LB, Kennedy ME, Pong AS, Fitzgerald GA. Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. FASEB J 1991;5:2304-2312.

17. Lee SH, Soyoola E, Chanmugam P, et al. Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. J Biol Chem 1992;267:25934-25938.

18. Jones DA, Carlton DP, McIntyre TM, Zimmerman GA, Prescott SM. Molecular cloning of human prostaglandin endoperoxide

synthase type II and demonstration of expression in response to cytokines. J Biol Chem 1993;268:9049-9054.

19. Fletcher BS, Kujubu DA, Perrin DM, Herschman HR. Structure of the mitogen-inducible TIS10 gene and demonstration that the TIS10-encoded protein is a functional prostaglandin G/H synthase. J Biol Chem 1992;267:4338-4344.

20. Hamasaki Y, Kitzler J, Hardman R, Nettesheim P, Eling TE. Phorbol ester and epidermal growth factor enhance the expression of two inducible prostaglandin H synthase genes in rat tracheal epithelial cells. Arch Biochem Biophys 1993;304:226-234.

21. Fosslien E. Molecular pathology of cyclooxygenase-2 in neoplasia. Ann Clin Lab Sci 2000;30:3-21.

22. Davies RA, Knight B, Tian YW, Yeoh GC, Olynyk JK. Hepatic oval cell response to the choline-deficient, ethionine supplemented model of murine liver injury is attenuated by the administration of a cyclo-oxygenase 2 inhibitor. Carcinogenesis 2006;27:1607-1616.

23. Marsden ER, Hu Z, Fujio K, Nakatsukasa H, Thorgeirsson SS, Evarts RP. Expression of acidic fibroblast growth factor in regenerating liver and during hepatic differentiation. Lab Invest 1992;67:427-433.

24. Nakamura T, Nawa K, Ichihara A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem Biophys Res Commun 1984;122:1450-1459.

25. Schmidt C, Bladt F, Goedecke S, et al. Scatter factor/hepatocyte growth factor is essential for liver development. Nature 1995;373:699-702.

26. Bottaro DP, Rubin JS, Faletto DL, et al. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science 1991;251:802-804.

27. Tsukada Y, Miyazawa K, Kitamura N. High intensity ERK signal mediates hepatocyte growth factor-induced proliferation inhibition of the human hepatocellular carcinoma cell line HepG2. J Biol Chem 2001;276:40968-40976.

28. Li G, Schaider H, Satyamoorthy K, Hanakawa Y, Hashimoto K, Herlyn M. Downregulation of E-cadherin and Desmoglein 1 by autocrine hepatocyte growth factor during melanoma development. Oncogene 2001;20:8125-8135.

29. Webster CR, Anwer MS. Phosphoinositide 3-kinase, but not mitogen-activated protein kinase, pathway is involved in hepatocyte growth factor-mediated protection against bile acid-induced apoptosis in cultured rat hepatocytes. Hepatology 2001;33:608-615.

30. Yin L, Lynch D, Sell S. Participation of different cell types in the restitutive response of the rat liver to periportal injury induced by allyl alcohol. J Hepatol 1999;31:497-507.

31. Pi L, Ding X, Jorgensen M, et al. Connective tissue growth factor with a novel fibronectin binding site promotes cell adhesion and migration during rat oval cell activation. Hepatology 2008;47:996-1004.

32. Han S, Ritzenthaler JD, Wingerd B, Rivera HN, Roman J. Extracellular matrix fibronectin increases prostaglandin E2 receptor

subtype EP4 in lung carcinoma cells through multiple signaling pathways: the role of AP-2. J Biol Chem 2007;282:7961-7972.

33. Han S, Sidell N, Roser-Page S, Roman J. Fibronectin stimulates human lung carcinoma cell growth by inducing cyclooxygenase-2 (COX-2) expression. Int J Cancer 2004;111:322-331.

34. Parker J, Kaplon MK, Alvarez CJ, Krishnaswamy G. Prostaglandin H synthase expression is variable in human colorectal adenocarcinoma cell lines. Exp Cell Res 1997;236:321-329.

35. Oh SH, Miyazaki M, Kouchi H, et al. Hepatocyte growth factor induces differentiation of adult rat bone marrow cells into a hepatocyte lineage in vitro. Biochem Biophys Res Commun 2000;279:500-504.

36. Sum EY, Shackleton M, Hahm K, et al. Loss of the LIM domain protein Lmo4 in the mammary gland during pregnancy impedes lobuloalveolar development. Oncogene 2005;24:4820-4828.

37. Bae SH, Jung ES, Park YM, et al. Expression of cyclooxygenase-2 (COX-2) in hepatocellular carcinoma and growth inhibition of hepatoma cell lines by a COX-2 inhibitor, NS-398. Clin Cancer Res 2001;7:1410-1418.

38. Oh SH, Witek RP, Bae SH, et al. Bone marrow-derived hepatic oval cells differentiate into hepatocytes in 2-acetylaminofluorene/partial hepatectomy-induced liver regeneration. Gastroenterology 2007;132:1077-1087.

39. Regan JW. EP2 and EP4 prostanoid receptor signaling. Life Sci 2003;74:143-153.

40. Mutoh M, Watanabe K, Kitamura T, et al. Involvement of prostaglandin E receptor subtype EP(4) in colon carcinogenesis. Cancer Res 2002;62:28-32.

41. Jobin C, Morteau O, Han DS, Balfour Sartor R. Specific NF-kappaB blockade selectively inhibits tumour necrosis factor-alpha-induced COX-2 but not constitutive COX-1 gene expression in HT-29 cells. Immunology 1998;95:537-543.

42. Lowes KN, Croager EJ, Abraham LJ, Olynyk JK, Yeoh GC. Upregulation of lymphotoxin beta expression in liver progenitor (oval) cells in chronic hepatitis C. Gut 2003;52:1327-1332.

43. Okano J, Shiota G, Matsumoto K, et al. Hepatocyte growth factor exerts a proliferative effect on oval cells through the PI3K/AKT signaling pathway. Biochem Biophys Res Commun 2003;309:298-304.

44. Okabe M, Tsukahara Y, Tanaka M, et al. Potential hepatic stem cells reside in EpCAM+ cells of normal and injured mouse liver. Development 2009;136:1951-1960.

45. Reddy BS, Rao CV, Seibert K. Evaluation of cyclooxygenase-2 inhibitor for potential chemopreventive properties in colon carcinogenesis. Cancer Res 1996;56:4566-4569.

46. Baek JY, Hur W, Wang JS, Bae SH, Yoon SK. Selective COX-2 inhibitor, NS-398, suppresses cellular proliferation in human hepatocellular carcinoma cell lines via cell cycle arrest. World J Gastroenterol 2007;13:1175-1181.

47. Baatar D, Jones MK, Pai R, et al. Selective cyclooxygenase-2 blocker delays healing of esophageal ulcers in rats and inhibits ulceration-triggered c-Met/hepatocyte growth factor receptor in-

duction and extracellular signal-regulated kinase 2 activation. Am J Pathol 2002;160:963-972.

48. Oh SH, Miyazaki M, Kouchi H, et al. Hepatocyte growth factor induces differentiation of adult rat bone marrow cells into a hepatocyte lineage in vitro. Biochem Biophys Res Commun 2000;279:500-504.

49. Okano J, Shiota G, Matsumoto K, et al. Hepatocyte growth factor exerts a proliferative effect on oval cells through the PI3K/AKT signaling pathway. Biochem Biophys Res Commun 2003;309:298-

304.

50. Shiota G, Kunisada T, Oyama K, et al. In vivo transfer of hepatocyte growth factor gene accelerates proliferation of hepatic oval cells in a 2-acetylaminofluorene/partial hepatectomy model in rats. FEBS Lett 2000;470:325-330.

51. Matsunaga T, Gohda E, Takebe T, et al. Expression of hepatocyte growth factor is up-regulated through activation of a cAMP-mediated pathway. Exp Cell Res 1994;210:326-335.