Cytoskeletal changes during epithelial-to-fibroblastoid conversion as a crucial mechanism of des-γ-carboxy prothrombin production in hepatocellular carcinoma

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Abstract. Des-gamma-carboxy prothrombin (DCP) is a well-established tumor marker for hepatocellular carcinoma (HCC), but the precise mechanism by which HCC cells produce DCP remains unknown. Our preliminary experiments demonstrated that HepG2 cells with chemical induction of epithelial-to-fibroblastoid conversion (EFC) produced DCP through impairment of vitamin K uptake via cytoskeletal rearrangement. Therefore, in this study we further examined this mechanism *in vitro* and using human HCC samples. Hepatoma cell lines (HepG2 and PLC/PRF/5) were induced EFC or epithelial-mesenchymal transition (EMT) by phorbol 12-myristate 13 acetate (TPA) or transforming growth factor (TGF)-ß1. We analyzed these cells by ELISA, Western blotting and immunofluorescent studies. We also examined DCP production and E-cadherin expression in human surgically resected HCC samples by immunohistochemical studies. Labeled low-density lipoprotein (LDL) uptake (as a surrogate for vitamin K) was significantly impaired in DCP-producing hepatoma cells following induction of EFC or EMT. Further, filamentous actin, which plays a critical role in clathrin-mediated endocytosis, was dissociated in DCP-producing cells. Latrunculin A, an actin depolymerizer, induced naïve hepatoma cells to produce DCP with impairment of labeled-LDL uptake. In addition, an E-cadherin neutralizing antibody did not induce DCP production. Finally, immunohistochemical studies demonstrated that DCP production was inversely correlated with the intensity of E-cadherin expression in human HCC cells. In conclusion, cytoskeletal changes during EFC or EMT plays a critical mechanistic role in DCP production via impairments in vitamin K uptake.

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Introduction

Prothrombin is a vitamin K-dependent blood coagulation protein that is synthesized only in the liver, and the biological active form of prothrombin is dependent on the use of vitamin K as a cofactor in the posttranslational carboxylation of specific glutamyl residues of prothrombin precursor (1). In the absence of vitamin K, or when its action is antagonized by vitamin K antagonists, abnormal prothrombin (des-γ-carboxy prothrombin, DCP), also known as 'protein induced by vitamin K absence or antagonist II' (PIVKA-II), is released into the blood (1,2). DCP is a well-established tumor marker for hepatocellular carcinoma (HCC) (3). Several authors have suggested that DCP production in HCC cells requires a reduction of γ-carboxylase gene expression (4), excessive synthesis of prothrombin precursors (5) or reduction of vitamin K content (6). However, the precise mechanism by which HCCs produce DCP remains unclear.

Cell migration is an essential function in various physiologic processes, including wound healing, as well as in pathophysiologic processes, such as tumor cell invasion and metastasis (7). Cancer cells can gain migratory and/or invasive properties when they were exposed to noxious stimuli, such as chemicals (8) and hypoxia (9), in an attempt to escape from an unfavorable microenvironment. In these situations, cancer cells lose their epithelial properties and acquire mesenchymal properties. This phenomenon is known as epithelial-mesenchymal transition (EMT) (10), which is a process initially observed in embryonic development. Recently, we showed preliminary experiments that HepG2 cells with chemical induction of epithelial-to-fibroblastoid conversion (EFC) produced DCP through impairment of vitamin K uptake via cytoskeletal rearrangement (11), suggesting that HCC cells may change their phenotype to produce DCP when they gain the migratory or invasive properties. Here, we attempt to further examine the mechanism of DCP production *in vitro* and investigate using human surgically resected HCC samples.

Materials and methods

Cell culture. Hepatoma cell lines, HepG2 and PLC/PRF/5 cells, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). For immunofluorescence studies, cells were cultured in chamber slides (Nagle Nunc International, Rochester, NY).

Enzyme-linked immunosorbent assay (ELISA) for DCP. Cells were seeded at a density of $2x10^5$ cells into 24-well plates. The next day, the medium was replaced with medium containing various concentrations of phorbol 12-myristate 13 acetate (TPA) (Sigma, St. Louis, MO), transforming growth factor (TGF)-ß1 (R&D Systems, Minneapolis, MN) or 1 μM latrunculin A (Lat A) (Sigma) with or without vitamin K2 (Sigma), and the cells were cultured for a 24 h (48 h for TGF-ß1). In other experiments, cells were cultured with 500 μg/ml of suramin (Sigma) with or without 100 nM vitamin K2. Cells were also treated with 3 μg/ml SHE78-7 (Takara, Tokyo, Japan) or control IgG (BD Biosciences, San Jose, CA) for 48 h. The supernatant of the culture medium was subjected to ELISA for DCP. Control cells were incubated with 10% DMEM containing 0.1% DMSO (control for TPA), or 10% DMEM containing 4 nM HCl (control for TGF-ß1). The DCP concentration was determined using a PIVKA-II ELISA kit (Eisai, Tokyo, Japan) according to the manufacturer's instructions.

Immunofluorescent microscopy. After cells were fixed with 4% paraformaldehyde, primary antibodies were applied overnight at room temperature. Anti-rabbit E-cadherin (1:50, IBL Co., Fujioka, Japan) or anti-mouse PIVKA-II (anti-DCP) (MU-3, 1:100, a kind gift from Eisai, Co., Japan) were used as primary antibodies. After washing, the cells were incubated with FITC-conjugated rabbit IgG (Sigma) or AlexaFluor 568-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA). The cell nuclei were labeled with mounting medium containing DAPI (Vector, Burlingame, CA). FITC-conjugated phalloidin (Sigma) was applied for 40 min at room temperature to reveal filamentous actin. Images were obtained using the 'All-In-One Immunofluorescence Microscopy' system (Keyence Inc., Tokyo, Japan).

Western blotting. After incubation with or without the indicated drugs for 48 h, cellular proteins were resolved using SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were incubated with anti-mouse vimentin (1:1000, Chemicon, Billerica, MA), or anti-mouse E-cadherin (1:2500, BD Transduction Laboratories, San Jose, CA). Antibodies against ß-actin (1:5000, abcam, Tokyo, Japan) were used as an internal control. The proteins were detected using electro-chemiluminescence techniques (Pierce Chemicals, IL). Densitometric quantification was performed using Image Quant 5.2 (GE Healthcare, Piscataway, NJ).

Uptake of low-density lipoprotein (LDL). Cells were cultured in serum-free DMEM containing 1% bovine serum albumin with 0.1% DMSO (control) or 50 nM TPA for 24 h, or with 4 nM HCl (control) or 1 ng/ml TGF-ß1 for 48 h. Cells were incubated with 1 μg/ml 3,3'-dioctadecylindocarbocyanin (Dil)-LDL (Biomedical Technologies, Stoughton, MA) and 10 μg/ml of human LDL (Biomedical Technologies) for 20 min at 37˚C, then fixed with 4% paraformaldehyde. After anti-mouse PIVKA-II was applied overnight, monolayer was incubated with FITC anti-mouse IgG (Sigma).

Fluorescence-activated cell-sorting (FACS) analysis. Cells were cultured in serum-free DMEM containing 1% bovine serum albumin with 50 nM TPA, 1 ng/ml TGF-ß1 or each control. Cells were incubated with human LDL receptor antibody (Acris-antibodies, Hiddenhausen, Germany) on ice for 15 min. After FITC-conjugated anti-rabbit IgG (Sigma) was applied, cells were subjected to FACS analysis. The stained cells were analyzed using FACSCalibur and CellQuest software (Becton-Dickinson, San Jose, CA).

Filamentous (F) actin/globular (G) actin in vivo assay. The ratio of F actin versus G actin was analyzed using an F actin/G actin *in vivo* assay kit (Cytoskeleton Inc., Denver, CO) according to the manufacturer's instructions. Briefly, cells were lysed using a cell lysis and F actin stabilization buffer, and homogenized using 25G syringes. The cell lysates were centrifuged at 100000 g for 60 min at 37˚C. The supernatants (G actin) were separated from the pellets (F actin). The pellets were resuspended to the same volume as the supernatants using ice-cold distilled water containing 10 μM cyto-chalasin D and were incubated on ice for 60 min. The samples were analyzed by Western blotting. The ratio of F actin versus G actin was quantified using Image Quant 5.2 (GE Healthcare).

Immunohistochemical studies of human HCC samples. Surgically resected specimens were collected between 2005-2007 at Jichi Medical University Hospital and Koseiren Suzuka Chuo Hospital, Japan. The Ethics Committees of both hospitals approved the protocol. Both serum DCP and AFP were measured in all patients. Paraffin-embedded samples were consecutively prepared as 4-μm sections and routinely stained with hematoxylin & eosin (H&E). The specimens were examined immunohistochemically by the avidin-biotin complex (ABC) method. Anti-mouse E-cadherin (1:400, BD Transduction Laboratories) and anti-mouse PIVKA-II (1:100) were used as primary antibodies. An ABC kit was obtained from Vector Laboratories. The sections were retrieved by autoclaving. After blocking endogenous peroxidase activity by 0.3% H_2O_2, each primary antibody was applied and reacted overnight at room temperature. For color development, 0.05% wt/vol 3,3'-diamino-benzidine tetrahydrochloride (DAB) was used. The sections were counterstained with hematoxylin. The immunohistochemical staining intensity, as well as the histological grade of HCC, was evaluated independently by three authors (K. Murata, H. Suzuki and A. Sakamoto) without providing them with any clinical information. Staining intensity of DCP or E-cadherin was graded as follows: 0, negative; 1, weakly positive; 2, moderately positive; and 3, strongly positive. The staining intensity of E-cadherin was evaluated into 'moderately positive' when the staining intensity was as strong as that for normal hepatocytes. The staining intensity of E-cadherin and DCP were evaluated in the same HCC lesions that showed the strongest DCP staining in the section.

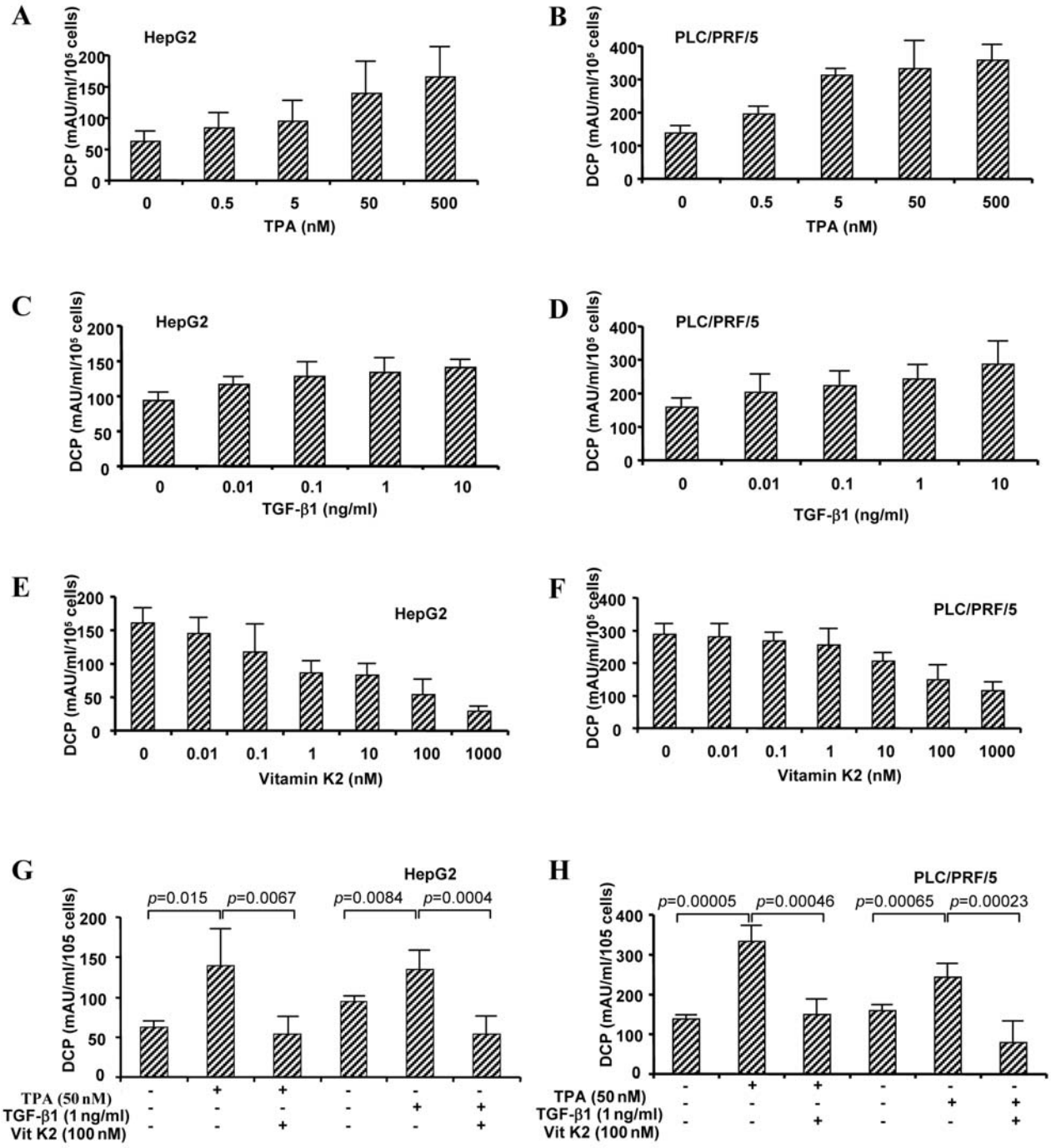

Figure 1. Enzyme-linked immuno-sorbent assay (ELISA). TPA induced DCP production in a dose-dependent manner in HepG2 (A) and PLC/PRF/5 (B) cells (p=0.00095, p<0.00001, respectively, ANOVA). TGF-ß1 also induced DCP production in a dose-dependent manner in HepG2 (C) and PLC/PRF/5 (D) cells (p=0.0098, p=0.00019, respectively, ANOVA). Supplementary vitamin K2 inhibited TPA (50 nM)-induced DCP production in a dose-dependent manner in HepG2 (E) and PLC/PRF/5 (F) cells (p<0.00001, p<0.00001, respectively, ANOVA). (G and H) A summary of inhibitory effects of vitamin K2 on DCP production in both cells is shown (paired t-test). Values are means ± SEM.

Statistical analysis. All data are expressed as means ± SEM. Multiple comparisons were performed using One-way ANOVA. Intergroup comparisons were performed using paired t-tests and Bonferroni's correction for multiple comparisons. Values of p<0.05 were considered statistically significant.

Results

Dose-dependent DCP production by TPA or TGF-ß1 treatment and effects of vitamin K2 on DCP production. TPA triggers the morphological transition from epithelial to fibroblastic appearance accompanied by scattering and migration of HepG2 cells (12), and TGF-ß1 promotes conversion to EMT in an HCC cell line (13). Thus, hepatoma cells were treated with TPA or TGF-ß1 to induce EMT. Western blotting with MU-3 antibody failed to detect DCP, but ELISA was successful. In fact ELISA demonstrated that TPA treatment significantly induced both HepG2 (Fig. 1A) and PLC/PRF/5 (Fig. 1B) cells to produce DCP in a dose-dependent manner. TGF-ß1 also significantly induced both cells to produce

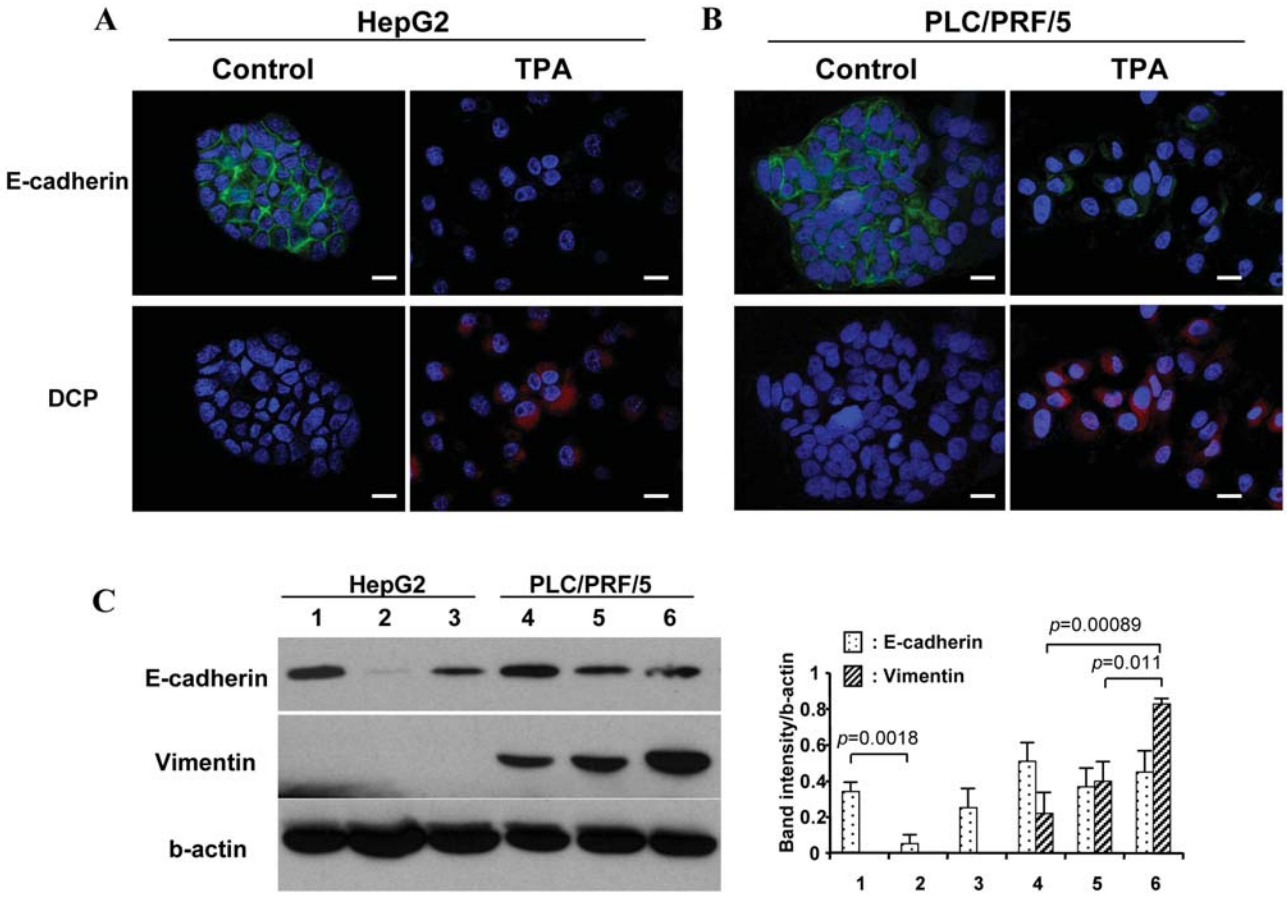

Figure 2. DCP production in cells with reduced E-cadherin expression. (A and B) In an immunofluorescent study, DCP production (red) was observed clearly in TPA-treated cells along with reduced E-cadherin expression. In both control cells, linear E-cadherin expression (green) was observed around the cell boundary. Cell nuclei were stained with DAPI (blue). The bars represent 20 μm. (C) Western blotting showed that TPA down-regulated E-cadherin expression in both cells in comparison with control cells, but this effect was less prominent in PLC/PRF/5 cells. TGF-ß1 up-regulated vimentin expression only in PLC/PRF/5 cells. Lanes 1 and 4, control; lanes 2 and 5, TPA; lanes 3 and 6, TGF-ß1.

DCP in a similar fashion (Fig. 1C and D). DCP production in TPA (50 nM)-treated HepG2 (Fig. 1E) and PLC/PRF/5 (Fig. 1F) cells was significantly inhibited by vitamin K2 in a dose-dependent manner. As shown in the figures (Fig. 1G and H), DCP production was inhibited by vitamin K2 (100 nM) in TGF-ß1 (1 ng/ml)-treated cells as well.

DCP production in hepatoma cells following chemical induction of EMT. To confirm whether TPA or TGF-ß1 induced hepatoma cells to undergo EMT and whether EMT-induced cells produced DCP, immunofluorescent studies and Western blotting were performed. Immunofluorescent studies demonstrated that TPA reduced E-cadherin expression in both HepG2 (Fig. 2A) and PLC/PRF/5 (Fig. 2B) cells, whereas linear E-cadherin expression around the cell boundary was observed clearly in both control cells. Treated cells became scattered and adopted a fibroblast-like appearance, whereas control cells were round-shaped and clustered. In addition, inhibition of E-cadherin expression in TPA-treated cells resulted in DCP production. Western blotting (Fig. 2C) demonstrated that TPA down-regulated E-cadherin expression in both cell types, but its effect on E-cadherin expression in PLC/PRF/5 cells was less prominent. Treatment with TGF-ß1 reduced E-cadherin expression as well, but this effect was weaker than that of TPA in both cell types. By contrast, TPA and TGF-ß1 up-regulated vimentin expression only in PLC/PRF/5 cells.

Impairment of LDL uptake in DCP-producing cells. Next, the effect of EFC or EMT on vitamin K uptake in hepatoma cells was examined. As previously reported (11), we used labeled-LDL (3,3'-dioctadecylindocarbocyanin LDL: Dil-LDL) as a surrogate for vitamin K because it is difficult to measure vitamin K uptake directly. Fluorescent studies demonstrated prominent accumulation of Dil-LDL in the cytoplasm of control HepG2 (Fig. 3A) and PLC/PRF/5 (Fig. 3B) cells but not in TPA-treated hepatoma cells. Interestingly, cells with an impairment in Dil-LDL uptake produced DCP, while cells with marked accumulation of Dil-LDL did not produce DCP (Fig. 3A and B). TGF-ß1 reduced Dil-LDL uptake and increased DCP production in both cell types, although TGF-ß1 did not show a prominent effect on E-cadherin expression (Fig. 2C). The numbers of LDL-receptor were higher after TPA treatment when compared with control cells (Fig. 3C and D) and were similar after TGF-ß1 treatment, suggesting that impairment of Dil-LDL uptake in TPA- or TGF-ß1-treated cells was not due to a decrease in the numbers of LDL-receptors. Suramin inhibits hepatic uptake of chylomicron remnants through interference with the function of heparin sulfate proteoglycan (HSPG) (15) and significantly

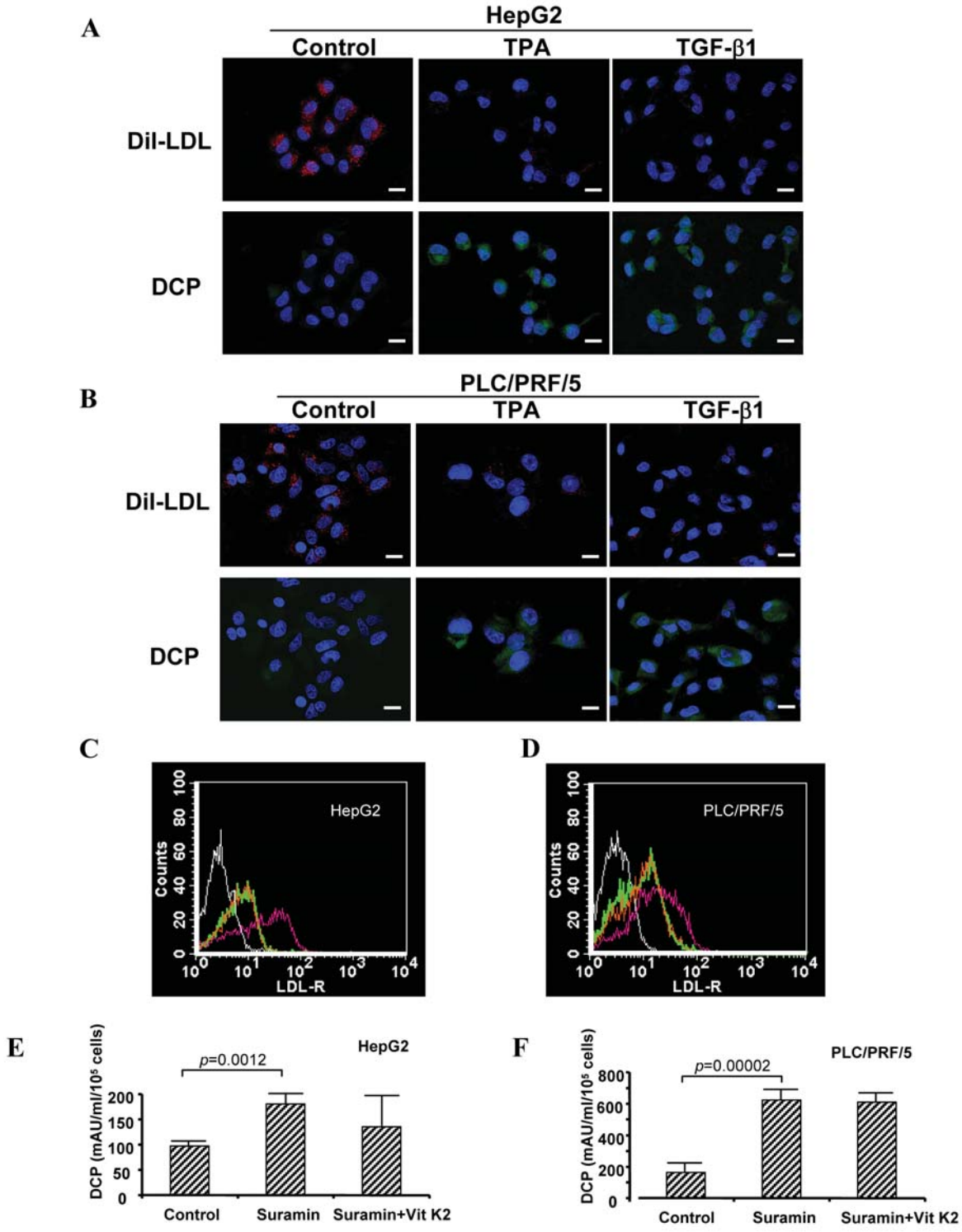

Figure 3. Immunofluorescent studies of Dil-LDL uptake and DCP production. (A and B) Dil-LDL uptake (red) was significantly impaired in both treated-cells in comparison with control cells. Cells with impairment of Dil-LDL uptake produced DCP (green), whereas control cells with well uptake of Dil-LDL did not. Cell nuclei were stained with DAPI (blue). The bars represent 20 μm. (C and D) FACS demonstrated that the intensity curve was shifted to the right in TPA-treated cells (pink) relative to control cells (green) and was similar in TGF-ß1-treated cells (red). The white curve represents control cells treated with FITC-anti-rabbit IgG, but without anti-LDL receptor. (E and F) Suramin (500 μg/ml) significantly induced DCP production in both cells (paired t-test) without any inhibitory effects of vitamin K2 (100 nM). Values are means ± SEM.

induced DCP production in both cell types without reduced effects of vitamin K2 (Fig. 3E and F).

Filamentous actin rearrangement in DCP-producing cells. Dynamic polymerization of endogenous filamentous (F) actin plays an important role in clathrin-mediated endocytosis, including endocytosis of fat-soluble vitamins (i.e., vitamin K). Therefore, the contribution of cytoskeletal rearrangement during EFC or EMT to DCP production in hepatoma cells was investigated. An immunofluorescent study using FITC-conjugated phalloidin revealed a subcortical typical network of F actin in control HepG2 (Fig. 4A and C) and PLC/PRF/5

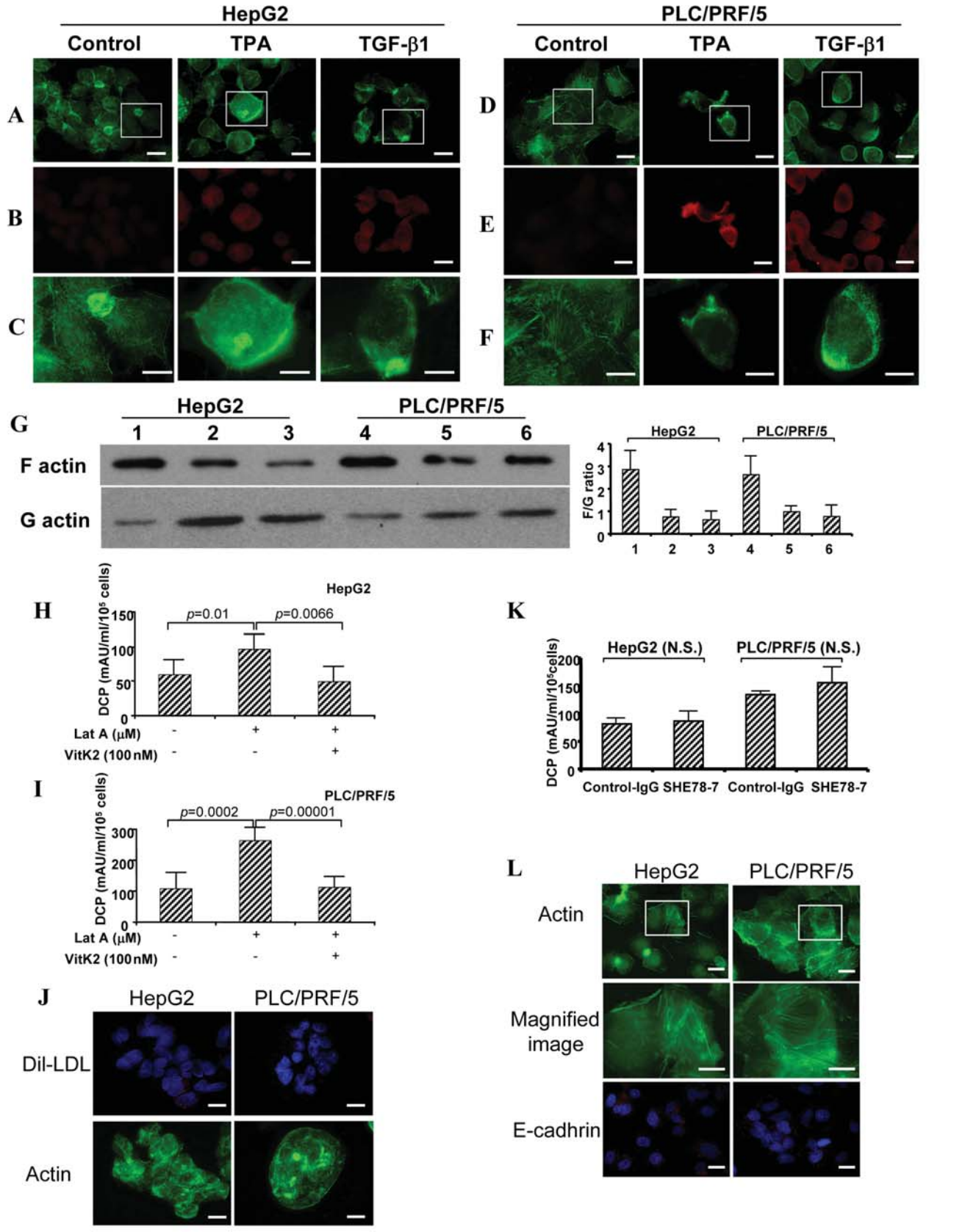

Figure 4. Disruption of F actin and DCP production. (A and D) FITC-conjugated phalloidin staining showed fine linear F actin in control cells, whereas F actin was disrupted in both TPA- and TGF-ß1-treated cells. (B and E) Double staining of phalloidin and DCP showed that cells with disrupted F actin produced DCP (red), whereas control cells with fine F actin network did not. (C and F) Magnified images of the inlet of A and D. (G) The amount of F actin was significantly reduced in both TPA- and TGF-ß1-treated cells relative to control cells (lanes 1 and 4, control; lanes 2 and 5, TPA; lanes 3 and 6, TGF-ß1). (H and I) Lat A (1 μM) induced naïve cells to produce DCP, which was inhibited by 100 nM vitamin K2 (paired t-test). (J) Lat A (1 μM) impaired Dil-LDL uptake (red) in both cell types. Fine network of F actin (green) was disappeared in Lat A-treated cells. (K) SHE78-7 (3 μg/ml) did not induce DCP production (NS, paired t-test). (L) SHE78-7 did not affect fine filamentous actin network (green) with loss of linear structures of E-cadherin (red). Cell nuclei were stained with DAPI (blue). The bars represent 20 μm except for E and F (10 μm). Values are means ± SEM.

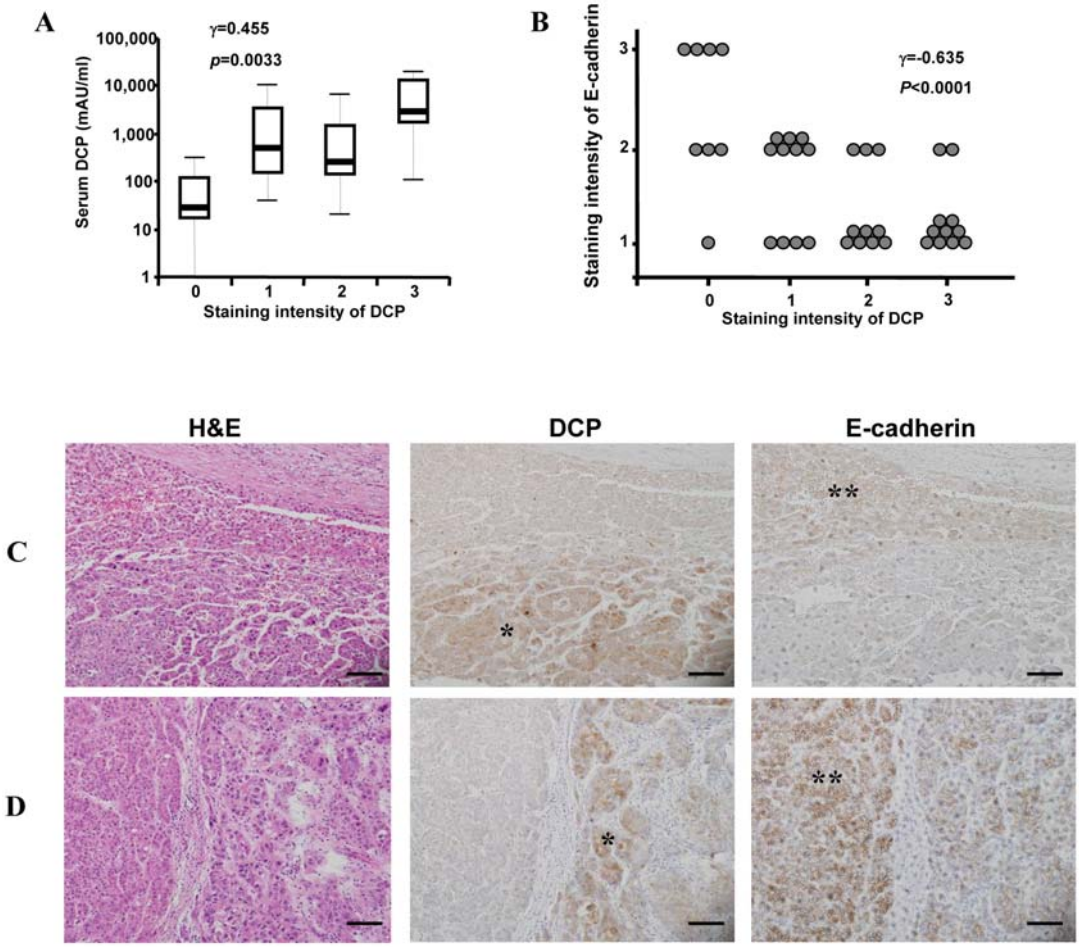

Figure 5. Immunohistochemical studies of E-cadherin and DCP in human HCC. (A) Serum DCP levels correlated with staining intensity of DCP (γ=0.455, p=0.0033). Staining intensity was classified as negative; 0, weekly positive; 1, moderately positive; 2, and 3, strongly positive. (B) Staining intensity of DCP was inversely correlated with that of E-cadherin (γ=-0.635, p<0.0001). Typical reciprocal staining patterns of DCP and E-cadherin in case 2 (C) and case 38 (D) are shown. *Area with weak staining for E-cadherin in cells that produce DCP. **Area with strong staining for E-cadherin in cells without DCP production. The bars represent 100 μm.

(Fig. 4D and F) cells, whereas these structures were disrupted and phalloidin-positive dots were observed after TPA or TGF-ß1 treatment. In addition, DCP production was clearly observed in TPA- or TGF-ß1-treated HepG2 (Fig. 4B) and PLC/PRF/5 (Fig. 4E) cells, in which linear F actin structures were absent, whereas DCP production was not observed in the control cells that showed a fine network of F actin filaments. Further, the amount of F actin was significantly reduced, and the amount of G actin was significantly increased in TPA- and TGF-ß1-treated cells relative to control cells (Fig. 4G). To confirm the contribution of F actin disruption to DCP production, naïve HepG2 and PLC/PRF/5 cells were treated with latrunculin A (Lat A), an actin depolymerizer. Lat A treatment significantly induced DCP production in naïve HepG2 (Fig. 4H) and PLC/PRF/5 (Fig. 4I) cells. In addition, Lat A-induced DCP production was inhibited by administration of vitamin K2 (100 nM) in both cell types (Fig. 4H and I). Further, Lat A treatment resulted in impairments in Dil-LDL uptake in both cell types (Fig. 4J). To determine whether E-cadherin expression directly contributes to DCP production, hepatoma cells were treated with SHE78-7, a neutralizing antibody against human E-cadherin. Linear E-cadherin expression around the cell boundary disappeared after SHE78-7 treatment. SHE78-7 treatment alone did not induce DCP production (Fig. 4K). In addition, liner F actin network was clearly observed after SHE78-7 treatment in both cell types (Fig. 4L), suggesting that inhibition of E-cadherin expression alone did not affect F actin structure and is therefore not an essential mechanism for DCP production.

Immunohistochemical study of E-cadherin and DCP in human HCC samples. A total of 40 patients with surgically resected HCC were included in this study. Characteristic features of enrolled patients are summarized in Table I. The median age was 66±11 years (male:female = 29:11) at the time of operation. The cause of chronic liver disease was hepatitis B infection (n=8), hepatitis C infection (n=23) and cryptogenic cirrhosis (n=9). Serum DCP levels were higher in patients with moderately- or poorly-differentiated HCC than those in patients with well-differentiated HCC (5329±6344 mAU/ml, 272±653 mAU/ml, respectively, p=0.0031). Serum DCP was significantly higher in patients with vascular invasion than those without (5992±7026 mAU/ ml, 1100±2318 mAU/ml, respectively, p=0.0033), which is consistent with prior reports (16,17). Immunohistochemistry demonstrated that the staining intensity of DCP correlated with the level of serum DCP in HCC patients (γ=0.455, p=0.0033) (Fig. 5A). DCP and E-cadherin were not homogenously stained, even in

Table I. Patient characteristics.

No.	Age	Cause	DCP	AFP	Histological grade	Vascular invasion
1.	70	C	24	12	W	-
2.	72	C	9,595	55,980	M	-
3.	60	C	3,564	20	P	+
4.	40	B	508	2,131	M	+
5.	55	B	2,692	45	W	-
6.	64	NBNC	42	15	W	-
7.	75	NBNC	217	4	W	-
8.	65	C	20	5	W	-
9.	78	B	11,266	2	M	+
10.	77	C	139	396	P-M	+
11.	71	C	18,192	15	M	++
12.	76	C	113	24	W	-
13.	74	NBNC	5,900	3	M	+
14.	63	NBNC	696	8	M	-
15.	65	C	1,735	210	M-P	-
16.	78	C	18	2,272	W	-
17.	71	C	15	70	W	-
18.	70	C	985	18,090	M-P	+
19.	69	C	205	52	W	-
20.	65	C	469	192	M	-
21.	70	C	321	24	M	-
22.	71	C	52	13	W	-
23.	44	B	274	50,000	W	-
24.	62	NBNC	1,880	1,060	M	+
25.	40	B	16,500	50,000	M-P	+
26.	74	C	2,340	31	M	-
27.	79	NBNC	6,520	4	M	-
28.	51	B	339	67,600	W	-
29.	78	C	1,872	60	M	-
30.	74	C	83	5	W	-
31.	71	B	71	2	W	+
32.	56	C	10,734	14	M-P	+
33.	59	B	8,343	59,633	M	+
34.	69	NBNC	1	2,772	M	+
35.	70	C	250	52	M	+
36	75	C	20,629	43,088	P-M	+
37.	68	C	21	514	W	-
38.	55	NBNC	108	64	M-P	-
39.	47	NBNC	360	6	M	-
40.	73	C	159	20,109	W	-

W: well-, M: moderate-, P: poorly-differentiated.

the same lesion. Interestingly, the staining intensity of E-cadherin negatively correlated with the staining intensity of DCP in the same lesions (γ=-0.635, $p<0.0001$) (Fig. 5B). Typical staining patterns are shown in Fig. 5C (case 2) and Fig. 5D (case 38). In these cases, DCP production was observed in HCC cells with weak staining intensity of E-cadherin, whereas DCP production was not observed in HCC cells with strong staining intensity of E-cadherin. These findings are consistent with observations from our *in vitro* experiments.

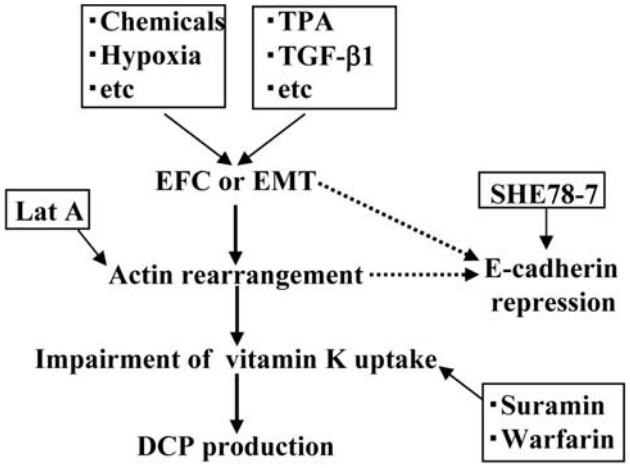

Figure 6. Schematic summary of proposed mechanism for DCP production in HCC cells.

Discussion

DCP is a well-known tumor marker for HCC, but the precise mechanism underlying DCP production in HCC cells remains unclear. By contrast to AFP and other tumor markers, DCP levels can be reduced (via administration of vitamin K2 analogues) without decreasing tumor burden (14), which was confirmed *in vitro* (18). Importantly, DCP is not specific for HCC, as all hepatocytes can produce DCP in the absence of vitamin K action (2) such as with vitamin K nutritional deficiencies or following warfarin administration. It is also well known that vitamin K-rich foods reduce the effect of warfarin (19). The above evidence led us to the hypothesis that vitamin K uptake is impaired in DCP producing HCC cells. Indeed, the present study demonstrated that TPA- or TGF-ß1-induced morphological changes into EFC or EMT resulted in impairments in Dil-LDL uptake and enhanced DCP production. Further, functional inhibition of HSPG by suramin induced DCP production in hepatoma cells.

Actin-based cytoskeleton reorganization is required to reshape the plasma membrane, including different forms of endocytic internalization and the protrusion of lamellipodia or filopodia during cell migration (20-22). Particularly with endocytic processes, the polymerization of actin provides the force for the deformation and movement of a membrane at different steps (21). Fat-soluble vitamins (including vitamin K) are taken up into cells through clathrin-mediated endocytic internalization (23), in which the dynamic polymerization of endogenous F actin plays a crucial role (24,25). In the present study, TPA- or TGF-ß1-treatment resulted in the loss of the fine network of phalloidin-positive F actin (Fig. 5). Actin rearrangement during TPA or TGF-ß1 treatment was confirmed by F actin/G actin in an *in vivo* assay (Fig. 4G). Further, the present study found that cells with actin rearrangement produced DCP, whereas DCP production was not observed in cells with liner F actin structure. In addition, Lat A, an actin depolymerizer, induced naïve hepatoma cells to produce DCP and induced an impairment in Dil-LDL uptake, suggesting that polymerization of F actin is required for vitamin K uptake and that impairment of F actin polymerization results in DCP production.

Down-regulation of E-cadherin,which is a central component of cell-cell adhesion junctions, is involved in the early recurrence of HCC after surgical treatment (26) or metastasis (27). Vimentin is also expressed in migrating epithelial cells, such as in the context of wound healing (28) or tumor invasion (29,30). Vimentin expression in cancer cells is associated with tumor metastases (29,31). Thus, both E-cadherin down-regulation and vimentin up-regulation are associated with tumor spread. Since high serum DCP in HCC patients is well associated with tumor spread (16,32,33), DCP-producing HCC cells might be a migratory or invasive phenotype. Indeed, the present study demonstrated that hepatoma cells produced DCP when they morphologically changed their phenotype to EFC or EMT. In Western blotting, different effects of TPA or TGF-ß1 on E-cadherin or vimentin expression were observed between the two cell lines. Since vimentin expression levels in each naïve cell is different, naïve HepG2 cells might have strong epithelial characteristics whereas naïve PLC/PRF/5 cells might have characteristics intermediate between those epithelial and mesenchymal phenotypes, which may explain those different effects. However, both treatments induced hepatoma cells to produce DCP, regardless of their effects on E-cadherin or vimentin expression. In addition, treatment with SHE78-7, a neutralizing antibody against human E-cadherin, did not induce DCP production and did not affect F actin structures. Therefore, E-cadherin down-regulation or vimentin up-regulation does not appear to be essential for DCP production.

Immunohistochemical studies demonstrated inverse correlation between DCP production and E-cadherin expression in surgically resected specimens. However, DCP-producing HCC cells did not always show reduced E-cadherin expression. Since the present *in vitro* study demonstrated that cytoskeletal changes, not E-cadherin repression, might have crucial role for DCP production, actin rearrangement may affect the juxtamembrane region of E-cadherin, thereby altering E-cadherin expression in DCP-producing HCC cells rather than direct effects of reduced E-cadherin expression on DCP production. Other investigators have reported that E-cadherin attaches to the cytoskeleton via connections to several cytoplasmic proteins, including ß-catenins (34), although the mechanistic link between E-cadherin expression and cytoskeletal rearrangement remains unclear. The present study was unable to directly investigate the association between DCP production and actin rearrangement in human HCC samples. This is because it is difficult to stain F actin in formalin-fixed tissues and because F actin is unstable and is easily depolymerized. In addition, extracellular matrix within the HCC samples also contains F actin, making it difficult to assess HCC F actin content. However, further investigation is necessary to address this issue.

The reduction of DCP production by vitamin K2 in TPA-, TGF-ß1- or Lat A-treated cells is due to supplementary effects, not due to cytotoxic effects, as the dose of vitamin K2 required to reduce DCP levels (<100 nM) is less than that required to inhibit HCC cell growth (50-100 μM) (13). F actin is critical for endocytosis in yeasts (35). However, in mammalian cells, F actin has a facilitatory rather than an essential role in endocytosis (36). In addition, inhibition of endocytosis in mammalian cells by Lat A tends to reduce the rate of internalization rather than blocking it completely

(37). This evidence suggests that mammalian cells have actin-independent pathway for endocytosis. Therefore, it is possible that supplementary vitamin K2 is taken up through a functionally impaired-F actin dependent pathway or through an F actin-independent pathway in DCP-producing HCC and that additional vitamin K2 may recover total uptake into the cells.

Taken together, the present study demonstrated that phenotypic changes of hepatoma cells during EFC or EMT resulted in enhanced DCP production via impairments of vitamin K uptake, as reflected by decreased labeled-LDL uptake and cytoskeletal rearrangement (Fig. 6). Importantly, this mechanistic notion is consistent with substantial clinical evidence (14,16,17,26,32,33).

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