Effects of the Protein Phosphatase Inhibitors, Okadaic Acid and Vanadate, on Localization of Occludin in Primary Cultures of Rat Hepatocytes

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ABSTRACT

To elucidate whether protein phosphorylation is associated with the localization of the tight junction protein occludin, we determined the changes of occludin protein expression in primary cultures of rat hepatocytes after treatment with the protein phosphatase inhibitors okadaic acid and vanadate. After 2 h of treatment with 1 μM okadaic acid or 5 mM vanadate, occludin immunoreactivity showing continuous lines in non-treated cells changed to a few spots on the plasma membrane. In western blots, broad bands above the occludin protein (65 kD) became conspicuous after treatment with okadaic acid and vanadate. We treated the same samples with alkaline phosphatase to examine whether the broad bands depended on the changes in the phosphorylation states of occludin protein. The broad bands disappeared and the occludin was observed as a narrow band corresponding to 65 kD. Neither a significant change in the mRNA of occludin nor a change in the immunoreac-

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Abbreviations used:

BrdU	: 5-bromo-2'-deoxyuridine	FITC	: fluorescein isothiocyanate
BSA	: bovine serum albumin	HBSS	: Hanks balanced salt solution
DAB	: 3,3'-diaminobenzidine	HRP	: horseradish peroxidase
DMSO	: dimethylsulfoxide	PBS	: phosphate-buffered saline
DTT	: dithiothreitol	RT	: room temperature
EGF	: epidermal growth factor	RT-PCR	: Reverse transcription polymerase chain reaction

tivity of the tight junction associated protein, ZO-1, was observed after treatment with okadaic acid or vanadate. These results suggested that the phosphorylation of occludin is closely associated with localization of the protein in cultured hepatocytes and that protein phosphatase inhibitors affect the localization of occludin but not ZO-1 on the plasma membrane.

Key words : Tight junctions, Occludin, ZO-1, Protein phosphatase inhibitor, Okadaic acid, Vanadate, Primary rat hepatocytes

INTRODUCTION

Tight junctions are the most apical component of the intercellular junctions and divide the apical from the basolateral cell surface domain to create and maintain cell polarity (1,2). A number of protein components of the tight junctions have been identified in recent years (3). Occludin, with a molecular weight of 65 kD is the only putative integral membrane and is a candidate for formatting the functional intercellular seal of the tight junctions. It has been shown to localize to tight junction strands by immunogold labeling of freeze-fracture replicas (4,5,6). However, the role of protein phosphorylation in the assembly and the sorting of occludin protein to the plasma membrane has been conflicting (7,8,9).

We previously showed that many tight junction strands in freeze-fracture replicas were observed and that they formed well-developed networks in primary cultures of adult rat hepatocytes using a medium containing epidermal growth factor (EGF) supplemented with 2% dimethylsulfoxide (DMSO) and 10^{-7} M glucagon (10). Recently, we found high expression of occludin protein in the cells. In the present study, to elucidate whether protein phosphorylation is associated with the localization of occludin, we determined the changes of occludin protein expression in cultured rat hepatocytes after treatment with the protein phosphatase inhibitors okadaic acid and vanadate.

MATERIALS AND METHODS

Isolation and culture of rat heoatocytes:

Male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) weighing about 300-400g were used to isolate hepatocytes by the two-step liver perfusion method of Seglen (11) with some modification. Briefly, the liver was perfused in situ through the portal vein with 150 ml of Ca^{2+}, Mg^{2+}-free Hanks balanced salt solution (HBSS) supplemented with 0.5 mM EGTA (Sigma Chemical Co., St. Louis, MO), 0.5 mg/L insulin (Sigma) and antibiotics. After the initial brief perfusion, the liver was per-

fused with 200 ml of HBSS containing 40 mg of collagenase (Yakult Co., Tokyo, Japan) for 10 min. The isolated cells were purified by Percoll iso-density centrifugation (12). Viability of the cells by the trypan blue exclusion test was more than 90% in these experiments. The cells were suspended in L-15 medium (GIBCO BRL, Gaithersburg, MD) with 0.2% bovine serum albumin (BSA; Seikagaku Kogyo Co., Tokyo, Japan), 20 mM HEPES (Dojindo, Kumamoto, Japan), 0.5 mg/L insulin (Sigma), 10^{-7} M dexamethasone (Sigma), 1 g/L galactose (Sigma), 30mg/L proline (Sigma), and antibiotics. The isolated hepatocytes were plated on 35 mm and 60 mm culture dishes (Corning Glass Works, Corning, NY), which were coated with rat tail collagen (500 μg of dried tendon/ml of 0.1% acetic acid) (13), and placed on a 100% air incubator at 37℃. Two to three hrs after plating, the medium was changed to L-15 medium supplemented with 0.2%BSA, 20 mM HEPES, 0.5 mg/L insulin, 10^{-7} M dexamethasone, 1 g/L galactose, 30 mg/L proline, 20 mM $NaHCO_3$, 5 mg/L transferrin (Wako Pure Chemical Inc., Osaka, Japan), 0.2 mg/L $CuSO_4 \cdot 5H_2O$, 0.5 mg/L $FeSO_4 \cdot 4H_2O$, 0.75 mg/L $ZnSO_4 \cdot 7H_2O$, 0.05 mg/L $MnSO_4$, 5 μg/L Na_2SeO_3, 10 ng/ml EGF (Becton Dikinson Labware, MA), and antibiotics. The cells were then placed in a humidified, 5% CO_2: 95% air incubator at 37℃. The medium was replaced with fresh medium every other day, and 2% DMSO (Aldrich Chemical Co., Inc., Milwaukee, WI) and 10^{-7} M glucagon (glucagon novo, Yamanouchi, Tokyo, Japan) were added to the medium after 96 h of culture (10). The cells were maintained until day 10.

Okadaic acid and vanadate treatment:

Cultured cells at day 10 were washed with the modified L-15 medium containing EGF with 2% DMSO and 10^{-7} M glucagon. Protein phosphatase inhibitors, 1 μM okadaic acid (GIBCO BRI) and 5 mM vanadate (sodium orthovanadate, Wako, Tokyo, Japan) were added to the medium for 30 min or 2 h.

Measurement of lactate dehydrogenase (LDH) activity:

To examine the cytotoxicities to primary cultured rat hepatocytes of okadaic acid and vanadate, LDH activity in the medium was measured using an LDH assay kit (Serotic Co., Sapporo, Japan). Three dishes were examined per experiment. The results are shown as a histogram.

Immunofluorescence Microscopy:

The cells grown on coated glass coverslips (BIOCOAT, Becton Dikinson Labware) were fixed with acetone for 30 min at -20℃. After rinsing with

PBS, the coverslips were incubated with a polyclonal anti-human occludin antibody (diluted 1:100, ZYMED, South San Francisco, CA) or a polyclonal anti-ZO-1 antibody (diluted 1:100, ZYMED) at room temperature (RT) for 1 h. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (diluted 1:50, DAKO, Copenhagen, Denmark) at RT for 1 h. All samples were examined with a Nikon Fx epifluorescence photomicroscope (Nikon, Tokyo, Japan).

Western blot analysis and densitometry analysis:

The dishes were washed with phosphate-buffered saline (PBS) twice and 1 ml of the buffer (1 mM $NaHCO_3$, 2 mM PMSF [Sigma] and 2 mg/L leupeptin [Sigma]) was added to 60-mm dishes. The cells were scraped and collected to eppendorf tubes and then sonicated for 30 sec. The sonicates were centrifuged at 4,500 × g for 10 min. The final pellets were resuspended in Laemmli sample buffer (14) without dithiothreitol (DTT). Some of the resuspended samples were incubated for 1 h at 37℃ in the presence of 5 units of calf alkaline phosphatase (Takara, Kyoto, Japan). For control reactions conducted in the presence of the phosphatase inhibitor, 10 mM sodium orthovanadate was added to the phosphatase reaction buffer. The protein concentration of the samples was determined using a protein assay kit (Bio-Rad, Richmond, CA). Twenty μg of protein of each sample was treated with DTT (final concentration of 100 mM), boiled for 3 min and then loaded on 4-20% SDS-polyacrylamide gel (Daiichi Pure Chemicals Co., Tokyo, Japan). After electrophoretic transfer to a nitrocellulose membrane (Bio-Rad) using semi-dry blotting for 6 h (0.65 mA/cm^2), the membrane was stained with Ponceau S (Sigma) and photographed. Thereafter, the membrane was saturated overnight at 4℃ with a blocking buffer (25 mM Tris, pH 8.0, 125 mM NaCl, 0.1% Tween 20, 4% skim milk) and was incubated with a polyclonal anti-human occludin antibody (diluted 1:1000, ZYMED) at RT for 2 h. The membrane was incubated with a horseradish peroxidase (HRP) - conjugated anti-rabbit IgG (diluted 1:1000, Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine (DAB) was used as a substrate. Scanning-densitometry was performed using a Macintosh LC-520 computer (Apple Computer, Cupertino, CA) and an EPSON GT-5000 scanner (Seiko Epson, Suwa, Japan). The signals were quantified by the NIH Image 1.52 Densimetric Analysis Program (Wayne Rasband, NIH, Bethesda, MD).

Reverse transcription polymerase chain reaction (RT-PCR) analysis:

RT-PCR was performed on total RNA extracted from the cultured rat

hepatocytes because the signals of Northern blot analysis for occludin mRNA of the cells were faint (data not shown). Total RNA was extracted from the cells using the single-step thiocyanate-phenol-chloroform extraction method (15) as modified by Xie and Rothblum (16). One μg of total RNA was reversed transcribed into cDNA using a mixture of oligo (dT) and MuLV reverse transcriptase following the recommended procedure (GeneAmp PCR kit, Perkin Elmer, Branchburg, NJ). Each cDNA synthesis was performed in a total volume of 20 μl for 30 min at 42℃ and terminated by incubation for 5 min at 99℃. PCR containing 100 pM of primer pairs and 0.5 μl of 20 μl total RT reaction was performed in 20 μl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, and 0.5 U of Taq DNA polymerase (Takara, Tokyo, Japan), applying 30 cycles with cycle times of 30 s at 94℃, 30 s at 50℃ and 30 s at 72℃ using a Perkin Elmer/Cetus Model 2400 Thermocycler. Final elongation time was 7 min at 72℃. Primers used to detect occludin by RT-PCR had the following sequence: upstream primer 5' TAAGGGAATATCCACCTATCACTT CAG 3', downstream primer 5' CATCAGCAGCAGCCATGTACTCTTCAC 3', corresponding to the published nucleotide sequence of the mouse occludin cDNA (5). In contrast, PCR reactions were performed with primers coding for the housekeeping gene, G3PDH (upstream primer 5' ACCACAGTCCATGCCATCAC 3', downstream primer 5' TCCACCACCCTGTTGCTGTA 3'), giving rise to a PCR product 452 bp in length to control for equal amounts of template cDNAs. Ten μl of the 20 μl total PCR reaction was analyzed in 1% agarose gel after being Stained with ethidium bromide.

RESULTS

Cytotoxicity of okadaic acid and vanadate

Figure 1 shows LDH activity in the medium of cultured rat hepatocytes

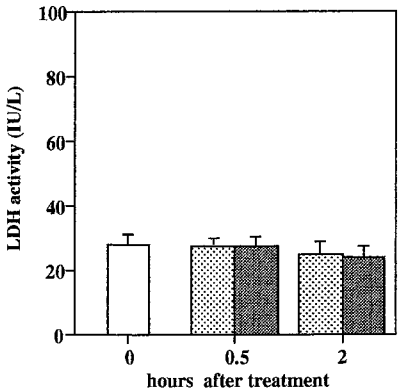

Fig. 1 LDH activity in the medium of primary cultured rat hepatocytes treated with 1 μM okadaic acid and with 5 mM vanadate.
□: non-treatment, ▨: okadaic acid, ▨: vanadate.

after treatment with 1 μM okadaic acid or 5 mM vanadate. No change of LDH activity in the medium was observed after treatment with okadaic acid or vanadate for 2 h, showing that the treatments were non-toxic to the cultured rat hepatocytes.

Immunofluorescence microscopy of occludin and ZO-1

Fluorescent immunocytochemistry on primary cultures of rat hepatocytes was carried out to examine changes of occludin and ZO-1 after treatments with okadic acid and vanadate (Fig. 2). In the cultures at day 10, both

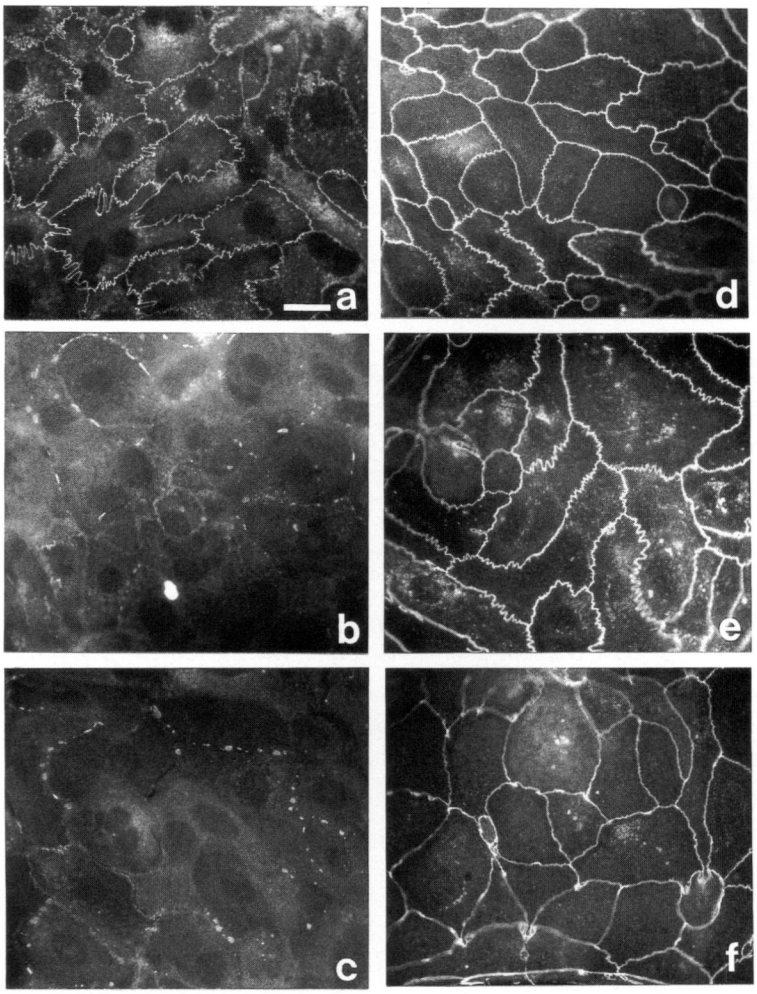

Fig. 2 Fluorescent immunocytochemistry of occludin (a, b, c) and ZO-1 (d, e, f) in primary cultured rat hepatocytes treated with 1 μM okadaic acid or with 5 mM vanadate. (a, d): non-treatment, (b, e): 2h after treatment with okadaic acid, (c, f): 2 h after treatment with vanadate. Figures are the same magnification. Bar, 20 μm.

occludin and ZO-1 immunoreactivities were strongly observed as lines between adjacent cells (Fig. 2 a and d). After 2 h of treatment with okadaic acid or vanadate, occludin immunoreactivity but not ZO-1 immunoreactivity, markedly decreased and it was observed as a few spots on the plasma membrane (Fig. 2 b, c, e and f). In the cells treated with okadaic acid or vanadate for 30 min, no change was observed (data not shown).

Western blot analysis of occludin protein

Figure 3 shows the changes of occludin by Western blot. In the

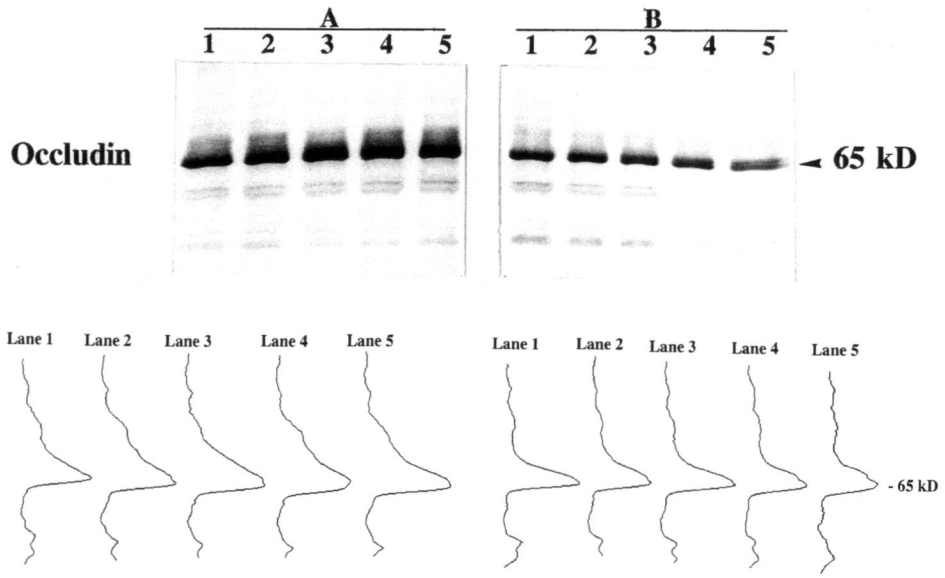

Fig. 3 Western blot analysis for occludin protein in primary cultured rat hepatocytes treated with 1 μM okadaic acid or with 5 mM vanadate. Whole cells were separated by electrophoresis in 4-20 % SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. After transfer, the blots were stained with an antibody against occludin. A: without alkaline phosphatase, B:with alkaline phosphatase. lane 1: non-treatment, lane 2: 30 min after treatment with okadaic acid, lane 3: 2 h after treatment with okadaic acid, lane 4: 30 min after treatment with vanadate, lane 5: 2 h after treatment with vanadate. Lower panel shows patterns of the signals quantified by the NIH Image analysis program.

non-treated hepatocytes, a broad band above the occludin protein (65 kD) was observed (Fig. 3A). In the cells treated with okadaic acid or vanadate for 30 min and 2 h, broad bands became conspicuous compared to those of non-treatment (Fig. 3A). To examine whether the broad bands depended on changes in the phosphorylation state of occludin, we performed alkaline phosphatase treatment of the samples. In all samples treated with alkaline phos-

phatase, the broad bands disappeared, forming a narrow single band (Fig. 3B). When the samples were incubated with alkaline phosphatase plus an excess amount of phosphatase inhibitor, broad bands were clearly observed (data not shown).

RT-PCR analysis of occludin mRNA

Figure 4 shows the changes in the amount of occludin mRNA. There

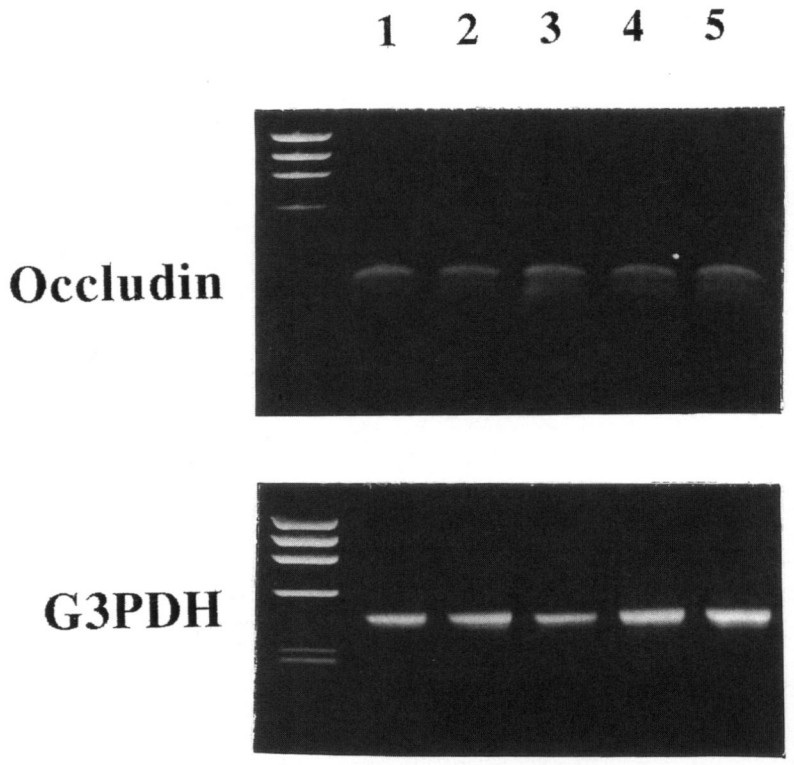

Fig. 4 RT-PCR analysis for occludin or G3PDH in primary cultured rat hepatocytes treated with 1 μM okadaic acid and with 5 mM vanadate. Total RNA was reverse transcribed and analyzed by PCR amplification using primers specific for occludin and G3PDH. Ten μl of the 20 μl total PCR reaction was analyzed in 1% agarose gel after being stained with ethidium bromide. The molecular weight marker (HaeIII) is showed in the left lanes. lane 1: non-treatment, lane 2: 30 min after treatment with okadaic acid, lane 3: 2 h after treatment with okadaic acid, lane 4: 30 min after treatment with vanadate, lane 5: 2 h after treatment with vanadate.

was no remarkable difference in the amount of occludin mRNA after treatment with 1 μM okadaic acid or 5 mM vanadate.

DISCUSSION

Tight junctions are regulated in response to various physiological and tissue-specific needs (1, 2, 3). However, very little is known about the regulatory mechanisms involved in assembly of tight junctions. Recently, Stuart and Nigam (17) reported that the assembly of tight junction-associated protein ZO-1 was regulated by protein kinase C (PKC). Although it is thought that one of regulations in the assembly of the tight junction proteins is protein phosphorylation, the mechanism of assembly of tight junction protein occludin is not yet clarified. For example, Sakakibara et al and Wong reported that phosphorylation of occludin is associated with the formation of tight junctions in MDCK cells (7,9), while Cordenonsi et al reported that occludin is dephosphorylated during development of Xenopus Laevis (8). In the present study, scanning densitometric analysis suggested that appropriate phosphorylation of occludin is required for the localization of occludin at tight junction of cultured hepatocytes, However, hyperphosphorylation of occludin affects the localization of occludin on the plasma membrane.

It was reported that sequence motifs of occludin have various phosphorylation domains (4, 5) and that occludin is a 65 kD phosphoprotein bound to ZO-1 by coimmunoprecipitation (17,18). In the present study, although metabolic labeling by [^{32}p] orthophosphate was not carried out, western blot analysis clearly showed that the occludin in the cutured rat hepatocyes was phosphorylated (Fig. 3). On the other hand, it is known that hyperphosphorylation by okadaic acid, an inhibitor of serine/threonine protein phosphatases, and by vanadate, an inhibitor of tyrosine protein phosphatases, affects the assembly of desmosome and adherens junctions in epithelial cells (19, 20). More recently, it was reported that okadaic acid increases intestinal epithelial paracellular permeability regulated by tight junctions (21). In the present study, treatments with okadaic acid and vanadate inhibited assembly of occludin protein but not ZO-1 protein on the plasma membrane in cultured hepatocytes by post-translational modifications. When the level of phosphorylation of occludin was completely restored to the control level by removal of okadaic acid and vanadate, occludin again forms continuous lines at cell-cell contacts(data not shown). These results suggest that the reversible phosphorylation of occludin in hepatocytes is closely associated with the localization of the protein on the plasma membrane.

It is also known that tight junctions may be regulated by various factors such as Ca^{++} and cyclic AMP, other tight junction-associated proteins and the organization of actin filaments (3,22). Furthermore, new membrane pro-

teins of tight junctions, claudin 1 and 2,were recently disclosed (23,24). Each of claudins is able to form tight junction strand without occludin (25), though the regulation of the functions of claudins, including protein phosphorylation, has not been unknown, so far. Thus, the possibilities still remain that the assembly of occludin in cultured hepatocytes might be influenced by those factors.

It is known that okadaic acid is a strong liver-tumor promoter and cell polarity is lost during the process of liver tumors. On the other hand, it is thought that cell polarity in epithelial cells may be detected by the fence function of tight junctions (1, 2, 3). As okadaic acid inhibited the assembly of the tight junction protein occludin in the hepatocytes, it may be very important to study the effects of changes of occludin on the loss of cell polarity during the process of liver tumors.

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