研究报告

# Driving Functional E-Cadherin onto Cell Surface by Elevation of PKB Activity in SMMC 7721 Hepato-carcinoma Cells*

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**Abstract** In order to study the regulation of E-cadherin by protein kinase B (PKB), wild type SMMC 7721 hepato-carcinoma cells and a Gag-PKB/SMMC 7721 cell line where PKB activity is markedly increased compared with control cells were used. Interestingly, increasing PKB activity via insulin stimulation or Gag-PKB transfection does not enhance the E-cadherin in the level of mRNA and that of protein by using Northern blot and Western blot analysis, but markedly drives E-cadherin protein to cell surface by using flow cytometry analysis and immunofluorescence analysis localization of E-cadherin, which resulted in the increase of cell aggregation and the inhibition of cell apoptosis mostly via E-cadherin. Therefore, new evidences that elevation of PKB activity could drive functional E-cadherin molecule to cell surface are provided.

**Key words** E-cadherin, protein kinase B, cell aggregation, anoikis

E-cadherin is a transmembrane glycoprotein which localizes at the sites of adherent junctions and is involved in a number of biological processes, including cell adhesiveness, cell growth and differentiation, cell recognition, and sorting during developmental morphogenesis, as well as aggregation-dependent cell survival[1,2]. Previous studies showed that E-cadherin could participate in transducing outside-in signals. Engagement of E-cadherins in homophilic calcium-dependent cell-cell interactions resulted in a rapid phosphatidylinositol 3-kinase (PI3K)-depenent activation of protein kinase B (PKB) and the subsequent translocation of PKB to the nucleus in MDCK cells[3]. Increasing E-cadherin expression can promote cell aggregation and survival of MPT cells in suspension via activation of PI3K[4]. This suggests that surface E-cadherin can initiate signal to regulate PKB activity. However, little is known about the action of PKB on E-cadherin. In this study, the increasing PKB activity via insulin stimulation or Gag-PKB transfection was used to investigate the effect of PKB on E-cadherin in SMMC 7721 hepato-carcinoma cells.

## 1 Materials and methods

### 1.1 Cell lines and reagents

The SMMC 7721 cells were obtained from Institute of Biochemistry and Cell Biology, The Chinese Academy of Sciences. The stable Gag-PKB/SMMC 7721 cell line was established as described previously[5]. The goat polyclonal anti-E-cadherin (N-20) antibody and the donkey anti-goat-HRP secondary antibody were purchased from Santa Cruz Biotechnology. The PKB kinase assay kit was purchased from New England Biology. RPMI 1 640 medium, Trizol were purchased from Gibco/BRL. DNA restriction endonucleases and random primer labeling kit were purchased from Promega. Hybone-N$^+$ nylon membrane, [$\alpha$-$^{32}$P] ATP (>11.1×10$^{10}$ Bq/(mol·L$^{-1}$)), Hybond-polyvinylidene difluoride (PVDF) membrane, enhanced chemiluminescence (ECL) assay kit and $^{32}$P-dATP were purchased from Amersham Corp. Poly-HEMA, Leupeptin, aprotinin, insulin, wortmannin, and PMSF were purchased from Sigma Chemical Co. Other reagents were commercially available in China.

### 1.2 Cell culture

SMMC 7721 and derived cells were cultured as described previously[5]. For inducing cells anoikis[5], the cells ($10^6$ cells per 60mm dish) were trypsinized, and plated in cell suspension dishes previously coated with polyHEMA to be cultured as indicated time.

### 1.3 Preparation of E-cadherin cDNA probe and Northern blot analysis

E-cadherin cDNA probe was obtained by RT-PCR of polyA$^+$ mRNA from SMMC 7721 cells according to

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the manufaturer's instructions. The primers 5′-GGTTCAAGCTGCTGACCTTC-3′ and 5′-CTCAAA-ATCCTCCCTGTCCA-3′ used for this amplification resulted in a cDNA fragment of 627 bp, and was verified by sequencing. Northern blot analysis was performed as described previously[5].

### 1.4 PKB kinase assay and Western blot analysis

PKB kinase assay was performed as described previously[5]. To detect the E-cadherin protein level, Western blot experiment was performed using anti-E-cadherin antibody as primary antibody[5].

### 1.5 Flow cytometry analysis

To assess E-cadherin expression on cell surface[6], cells were trypsinized with 0.01% trypsin containing 1 mmol/L $CaCl_2$, then divided into two groups and adjusted to $10^6$ cells per 100 μl PBS. One group was immunostained with anti-E-cadherin antibody (1∶100) at 4℃ for 45 min, the other control group was incubated with nonimmune serum instead of the primary antibody (1∶200), and then both followed by a FITC-conjugated donkey anti-goat secondary antibody at 4℃ for 45 min in darkness. The cells were washed three times with PBS, then were suspended in PBS and detected by flow cytometry. To assess apoptotic cells, flow cytometry analysis was performed as described previously[5].

### 1.6 Immunofluorescence analysis

Localization of E-cadherin was detected by indirect immunofluorescence[7]. Briefly, the cells grown on glass coverslips were washed three times with PBS, followed by fixation in 3.7% paraformaldehyde for 10 min. After extensive washing, the coverslips were blocked with PBS containing 1% BSA and 0.1% Triton X-100 for 30 min, and then followed by anti-E-cadherin antibody and the FITC-conjugated donkey anti-goat secondary antibody. After washing with PBS, coverslips were visualized under a fluorescence microscope.

### 1.7 Aggregation assay

Cell aggregation assays were performed according to the methods described by Nagafuchi *et al.*[8]. Briefly, cells grown to confluence were detached by incubating with 0.01% trypsin in PBS in the presence of 1 mmol/L $CaCl_2$. $10^5$ cells suspended in 1 ml PBS in the presence or absence of 10 mmol/L $CaCl_2$ with or without anti-E-cadherin (1∶100) antibody were seeded into wells pre-coated with poly-HEMA, and incubated at 37℃ on a gyratory shaker at 80 r/min for 30 min. Cell aggregation was measured by calculating the ratio of the total particle number at the end of the assay to the initial particle number, with the latter being identical to the total number of cells added.

## 2 Results

### 2.1 Increasing PKB activity markedly drives E-cadherin to cell surface

The functions of E-cadherin could be influenced by the amount of cell surface E-cadherin. A lot of evidence showed that PI3K/PKB signal pathway could be induced by insulin . To investigate whether elevation of PKB activity can alter cell surface E-cadherin, we studied the PKB activity and cell surface E-cadherin when SMMC 7721 cells were treated with insulin. After cells were treated with insulin for 10 min, PKB activity was markedly increased. At the same time, surface E-cadherin was also increased by flow cytometry analysis. However, these increasing of PKB activity and surface E-cadherin were both inhibited when SMMC

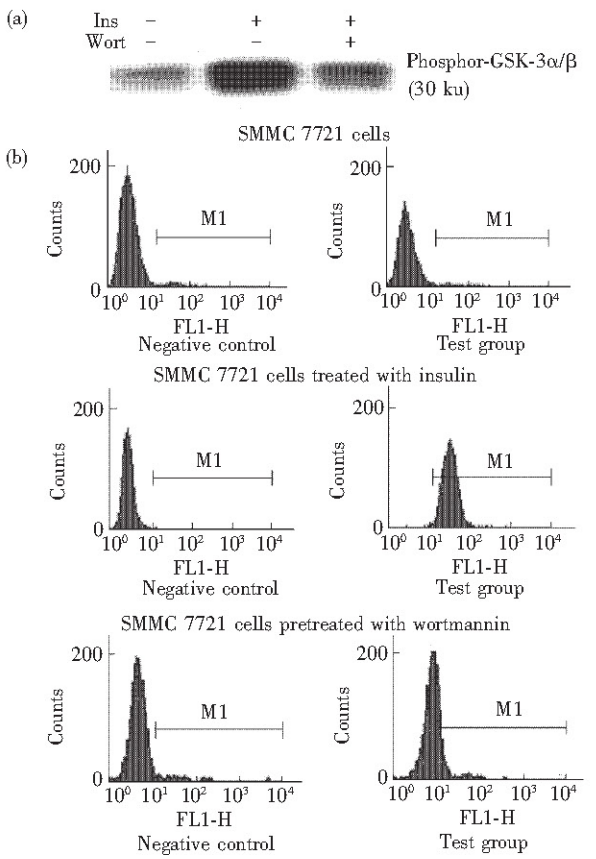


**Fig. 1 Surface E-cadherin in SMMC 7721 cells treated with insulin and/or wortmannin**

(a) PKB activity was measured in SMMC 7721 cells treated with insulin and/or wortaminnin. SMMC 7721 cells were starved in serum-free RPMI 1640, pretreated with or without wortmannin (100 nmol/L) for 30 min, followed with insulin (100 nmol/L) for 10 min, then the cells were used to assay PKB activity (see materials and methods). (b) FCM analysis of surface E-cadherin in SMMC 7721 cells treated with insulin and/or wortmannin. Cells were immunostained with anti-E-cadherin antibody followed by FITC-conjugated secondary antibody as described in **Materials and methods.**

7721 cells were pretreated with wortmannin, an inhibitor of PI3K (Figure 1). To further confirm the effect of PKB on surface E-cadherin, a stable Gag-PKB/SMMC 7721 cell lines where PKB activity is markedly increased compared with control cells was used to identify cell surface E-cadherin by using flow cytometry analysis and immunofluorescence. The results of flow cytometry analysis revealed that increasing PKB activity could markedly increase cell surface E-cadherin, whereas almost no E-cadherin on the cell surface of SMMC 7721 cells or Gag/SMMC 7721 cells was detected (Figure 2). Immunofluorescence microscopy also showed that E-cadherin was clear on cell surface, especially at cell-cell borders of Gag-PKB/SMMC 7721 cells. However, no E-cadherin could be found on control cells (Figure 3).

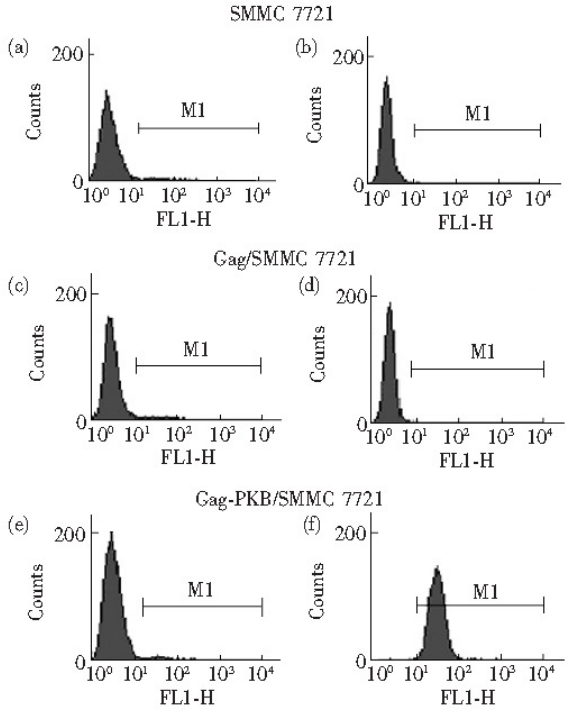


**Fig. 2 Flow cytometry analysis of surface E-cadherin in Gag-PKB/SMMC 7721 and control cells (including SMMC 7721 and Gag/SMMC 7721 cells)**

The group of analysis (a, c, e) is negative control of the test group (b, d, f) accordingly

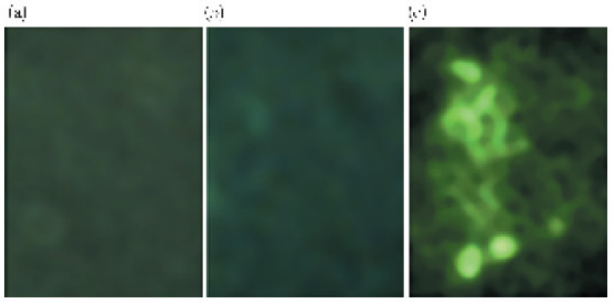

**Fig. 3 Immunofluorescence localization of E-cadherin in Gag-PKB/SMMC 7721 and control cells**

The cells were immunostained with anti-E-cadherin antibody followed by FITC-conjugated secondary antibody as described in **Materials and methods**. Immunofluorescence microscopy analysis showed that E-cadherin was clear on cell surface, especially at cell-cell borders of Gag-PKB/SMMC 7721cells (c). However, nothing can be found in control cells such as SMMC 7721 cells (a), and Gag/SMMC 7721 cells (b). Magnification: 10×40.

## 2.2 Over-expressing PKB does not change E-cadherin mRNA or total protein expression

To further investigate whether the increasing effect of PKB on cell surface E-cadherin results from alteration of E-cadherin expression, Northern blot and Western blot analysis were used to measure the E-cadherin mRNA level and protein level simultaneously in control cells and Gag-PKB/SMMC 7721 cells. It was revealed that increasing PKB activity could not alter E-cadherin mRNA level or total protein level when compared to that of the SMMC 7721 cells or Gag/SMMC 7721 cells (Figure 4).

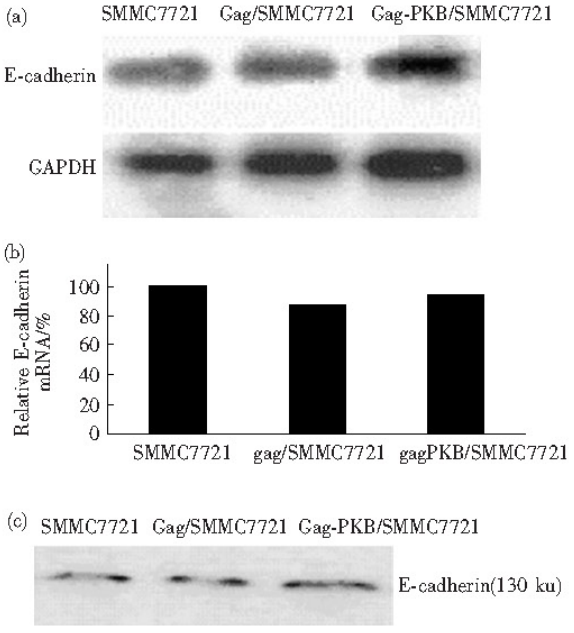


**Fig. 4 Determination of E-cadherin expression in Gag-PKB/SMMC 7721 and control cells**

(a) Northern blot analysis of E-cadherin mRNA expression in Gag-PKB/SMMC 7721 and control cells. Total RNA (40 μg) samples isolated from each sample were electrophoresed, and the blot was sequentially probed with E-cadherin and GAPDH fragment (1~759). (b) Determination of relative E-cadherin mRNA expression in Gag-PKB/SMMC 7721 and control cells. Each lane in (a) was scanned by laser densitometry and the relative E-cadherin mRNA levels was determined from the ratio of their absorbances relative to GAPDH in each lane and was expressed as arbitrary units. (c) Western blot analysis of E-cadherin expression in Gag-PKB/SMMC 7721 and control cells. Equal lysate derived from each sample were electrophoresed on 10% SDS-PAGE, transferred eletrophoretically onto a PVDF membrane and blotted with E - cadherin antibody. Similar results were obtained in three independent experiments.

### 2.3 Over-expressing PKB increases $Ca^{2+}$- and E-cadherin-dependent cell aggregation as well as cell survival in an E-cadherin dependent fashion

Cell aggregation assays were performed under conditions favoring cadherin-mediated adhesion to address the functionality of the E-cadherin. As illustrated in Figure 5, we detected a significant enhancement of $Ca^{2+}$- and E-cadherin-dependent cell-cell adhesion in the Gag-PKB/SMMC 7721 cells compared to control cells because the enhancement of cell aggregation was blocked either in the absence of calcium, or applying antibody recognizing E-cadherin. Also, it was found that Gag-PKB/SMMC 7721 cells revealed less apoptosis by using flow cytometry analysis compared to SMMC 7721 cells or Gag/SMMC 7721 cells when cells were deprived of cell matrix adhesion, but Gag-PKB/SMMC 7721 cells recovered the most sensitivity to anti-E-cadherin antibody when they were induced apoptosis in suspension (Figure 6). Therefore, these results suggested that elevation of PKB activity could increase $Ca^{2+}$- and E-cadherin-dependent cell aggregation as well as cell survival mostly in an E-cadherin dependent fashion.

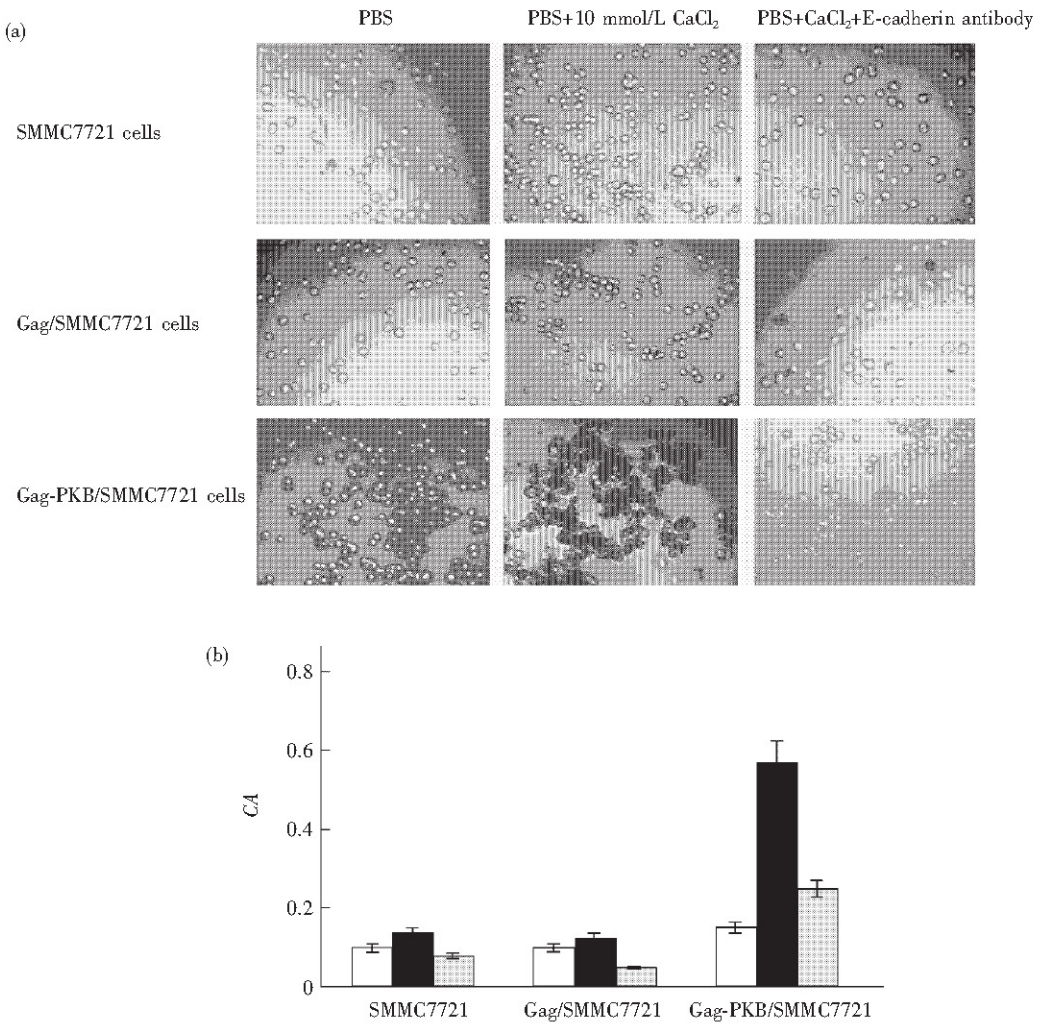


**Fig.5 Cell aggregation of Gag-PKB/SMMC 7721 and control cells**

Cell aggregates in a calcium- and cadherin-dependent manner (see **Materials and methods**). (a)Cells showed the process of aggregations. When suspended in poly-HEMA coated wells for 30 min in different culture. Magnification: 10×10. (b)Cell aggregation was expressed as the formula: $CA=1-N_{30}/N_0$. $CA$, cell aggregation; $N_{30}$, the number of particles in suspension at 30 min; $N_0$, the number at time zero. □: PBS; ■: PBS+Ca; ▨: PBS+Ca+anti-E-cadherin Ab.

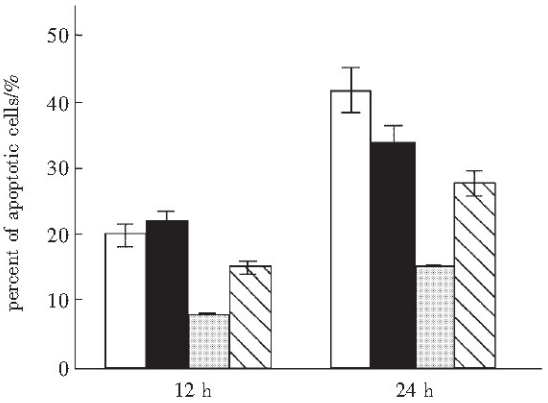


**Fig.6 Flow cytometry analysis of the apoptotic cells induced by deprivation of cell matrix adhesion**

Cells were induced by deprivation of cell matrix adhesion and were analyzed the percent of apoptosis by flow cytometry analysis. Data represent the $(\bar{x} \pm s)$ from three separate experiments. $P < 0.05$ compared to 7721 cells or Gag/SMMC7721 cells. □: SMMC7721; ■: Gag/SMMC7721; ▨: Gag-PKB/SMMC7721; ▧ : Gag-PKB/SMMC7721+anti-E-cadherin Ab.

## 3 Discussion

In this study we demonstrated that cell surface E-cadherin was increased due to elevation of PKB activity in SMMC 7721 cells. Redistribution of E-cadherin at the cell surface were verified by stimulating PKB activity with insulin treatment in SMMC 7721 cells or increasing PKB activity via Gag-PKB transfection, and the surface E-cadherin was decreased by blocked PI3K/PKB with wortmannin. The enhancement of cell surface E-cadherin was not dependent on the increasing of E-cadherin transcription or translation detected by Northern blot and Western blot analysis. Gag-PKB/SMMC 7721 cells were potentiated to cell aggregation and survival. These data suggested that PKB could drive functional E-cadherin molecule to cell surface.

A pool of surface E-cadherin can be constantly trafficked through an endocytic, recycling pathway. The surface E-cadherin results from the balance between internalizing and recycling a portion of E-cadherin on the cell surface[9]. Our results showed that increasing PKB activity doesn't produce newly synthesized E-cadherin protein, but increases surface E-cadherin. This discovery indicated that PKB might break the balance of recycling regulation, and resulted in the accumulation of surface E-cadherin. These increasing of surface E-cadherin consequently promoted calcium- and E-cadherin-dependent cell-cell adhesion and cell survival in suspension. The PI3K/PKB pathway is a major regulator of cell survival[10]. Expression of activated PKB can block cell apoptosis induced by detachment of epithelial cells from extracellular matrix, which is also known as anoikis[11]. In addition, previous work revealed that enhancement of E-cadherin-mediated cell-cell adhesion could promote cell survival in suspension. Therefore, our studies suggested that PKB might protect SMMC 7721 cells from anoikis via increasing of surface E-cadherin. However, we found that the cell survival of Gag-PKB/SMMC 7721 cells was not completely inhibited by anti-E-cadherin antibody. Thus, other pathways may also contribute to Gag-PKB/SMMC 7721 cells survival in suspension.

Previous work indicated that over expression of E-cadherin resulted in a rapid PI3K-dependent PKB activation[4]. In this report we showed that the elevation of PKB activity could promote E-cadherin to cell surface, which increased cell aggregation and cell survival. Therefore, it was suggested that a positive feedback loop might occur. PKB signaling which is initiated by E-cadherin drives the functionally E-cadherin to cell surface to prolong E-cadherin-mediated cell survival signal. Although positive feedback loop is less obvious than negative feedback control of signaling kinetics, the use of positive feedback loop involves in some processes of animal development signal transduction. The frequently quoted example is the positive feedback loop that maintains the expression of the *Ubx* gene in parasegment 7 of the *Drosophila* gut, and another example came from the positive feedback cycle between Ras/MAPK pathway and EGF receptor signal[12]. However, as in engineering positive feedback in biology always carries with it the risk of loss of control of signaling, and a subsequent breakdown of homeostasis that can lead to disease. Nevertheless, cells often receive restricted signals to integrate the information, and then respond appropriately. In the developing *Drosophila* gut as described above, high levels of Wingless signaling can repress *Ubx* transcription, thereby closing the positive feedback loop to ensure that *Ubx* expression is restricted to the correct region of the gut[13]. So it was speculated that in normal cells certain unknown pathway also might attenuate the positive feedback loop round E-cadherin and PKB. It is presumed that at least there are two pathways involved in the negative regulation of E-cadherin-PKB loop: one is the contact inhibition of cell growth such as the effect of N-cadherin in CHO cells[14], the other possibility is that PI3K activated by E-cadherin leads to the activation of protein kinase Cζ, an inhibitor of PKB

[15~17]. Through the integration of the positive and negative feedback loops in normal cells, surface E-cadherin can reach balance and epithelial tissues maintain the normal structure and homeostasis.

In summary, this study demonstrates that PKB could drive E-cadherin to cell surface, and consequently promote E-cadherin-mediated cell-cell adhesion and increase cell survival partly via enhancement of surface E-cadherin, thereby shedding new insights into the regulation of E-cadherin and the role of E-cadherin in PKB-mediated anti-apoptosis pathway. Currently we are working further to elucidate the mechanism involved in E-cadherin recycling that regulated by PKB.

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# SMMC 7721 肝癌细胞蛋白激酶 B 活性增高可驱动有功能的上皮钙粘着蛋白到细胞表面*

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摘要 为了研究蛋白激酶 B (PKB) 对上皮钙粘着蛋白 (E-cadherin) 的调节, 使用了用胰岛素处理的野生型 SMMC 7721 细胞及稳定表达持续激活 PKB 的 SMMC 7721 细胞株 (Gag-PKB/SMMC 7721). 用 RNA 印迹法和蛋白质印迹法检测细胞 E-cadherin 表达, 发现通过胰岛素刺激或在细胞中表达持续激活 PKB 从而增加 PKB 活性, 不影响 E-cadherin 的转录和蛋白质合成, 但用流式细胞术和免疫荧光定位 E-cadherin, 则发现 PKB 活性增加能明显驱动 E-cadherin 到细胞表面, 从而导致部分通过 E-cadherin 途径的细胞粘聚增加和细胞凋亡的抑制. 因此, 我们提供新的证据表明, 增加 PKB 活性可驱动有功能的 E-cadherin 分子到细胞表面.

关键词 上皮钙粘着蛋白, 蛋白激酶 B, 细胞粘聚, 失巢凋亡

学科分类号 Q5

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