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Involvement of Akt/mTOR pathway on EGF-induced Cell Transformation

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Our previous study has demonstrated that phosphatidylinositol 3-kinase (PI3K) is necessary for epidermal growth factor (EGF)-induced cell transformation in mouse epidermal JB6 cells. Akt and the mammalian target of rapamycin (mTOR) are regarded as the PI3K downstream effectors. Therefore, in this study, we investigated the role of Akt and mTOR on EGF-induced cell transformation in JB6 cells using rapamycin, a specific mTOR inhibitor, and cells expressing dominant negative mutants of Akt1 (DNM-Akt1). We found that the treatment of cells with rapamycin inhibited EGF-induced cell transformation. Rapamycin only slightly inhibited JB6 cell proliferation at 72 hr. Although LY294002, a PI3K inhibitor, attenuated EGF-induced activator protein 1 (AP-1) activation, the treatment of rapamycin did not change AP-1 activity. The treatment with rapamycin inhibited EGF-induced phosphorylation and activation of ribosomal p70 S6 protein kinase (p70 S6K), a mTOR downstream target, but had no effect on phosphorylation and activation of Akt. Rapamycin also had no effect on EGF-induced phosphorylation of extracellular signal-regulated protein kinases. We indicated that an introduction of DNM-Akt1 into JB6 Cl 41 cells inhibits EGF-induced cell transformation without blocking the cell proliferation. The expression of DNM-Akt1 also suppressed EGF-induced p70 S6K activation as well as Akt activation. These results indicated an involvement of Akt/mTOR pathway on EGF-induced cell transformation in JB6 cells.

INTRODUCTION

Phosphatidylinositol 3-kinase (PI3K) plays a critical role in many biological effects including cell growth, apoptosis, insulin action, cell migration, and integrin function [1-3]. In addition, an involvement of the PI3K signaling in tumorigenesis has been suggested [4,5]. The expression in chicken cells of the oncogene v-p3k, a homolog to the gene encoding the catalytic subunit (p110) of PI3K, which was isolated from tumorigenic retroviruses, was shown to cause oncogenic transformation [6]. The oncogenic transformed phenotype was also observed in mammalian fibroblasts transfected with the constitutive active form of p110α [7]. In fact, numerous genetic or epigenetic changes lead to increased PI3K signaling in human cancer [8,9]. Mutations in the tumor suppressor phosphatase and Tensin homolog gene (PTEN), which antagonizes the PI3K, are frequently observed in a number of cancers [4,10]. Our previous studies also have demonstrated that the PI3K is necessary for 12-*O*-tetradecanoylphorbol-13-acetate (TPA)- or epidermal growth factor (EGF)-induced cell transformation in mouse epidermal JB6 cells [11,12]. In those studies, we found that LY294002 and wortmannin, PI3K inhibitors, inhibited tumor promoter-induced cell transformation as well as activator protein 1 (AP-1) activation. Because AP-1 was shown to be one of critical factors for the cell transformation in JB6 cell lines [13-15], the effects of PI3K inhibitors were suggested to be due to the attenuation of AP-1 activation. However, the investigation of the mechanism of PI3K activation recently has revealed several downstream effectors of its function. The serine/threonine proto-oncogene Akt [also known as protein kinase B] was first identified as a cellular homolog of the transforming oncogene product v-Akt [16] and it is activated by growth factors, oncogenes, and

integrins through the PI3K-dependent signaling. Akt also has been shown to be overexpressed in ovarian, prostate, breast and pancreatic cancers, which is associated with a poor prognosis and increased tumorigenicity [4,17-19]. Accumulating evidence shows that ribosomal p70 S6 protein kinase (p70 S6K) is also a crucial effector of PI3K in response to growth factors [20]. Elevated activation of p70 S6K has been indicated in PTEN-deficient tumor cells and the activation is attenuated to basal levels by reintroduction of PTEN [21]. PI3K and p70 S6K are constitutively activated in small cell lung cancer cells and anchorage-independent proliferation in their cells is mediated through Akt and p70 S6K-dependent pathway [22]. p70 S6K is also amplified or overexpressed in human breast cancer [23,24]. On the other hand, the activation of p70 S6K is known to require signaling through mammalian target of rapamycin (mTOR, also called FRAP) [25]. mTOR is reported to be essential for cell transformation induced by oncogenes *p3k* and *akt* [26] and to be directly phosphorylated by Akt [27,28]. Therefore, the inhibition of cell transformation by the PI3K inhibitors in JB6 cells may also be involved in the affects on PI3K downstream targets, including Akt, mTor or p70 S6K, in addition to the attenuation of AP-1 activation.

Rapamycin is a natural product macrolide that induces G1 growth arrest by interfering with the signaling functions of mTor [29,30]. In mammalian cells, rapamycin forms a complex with FK-506-binding protein 12 that binds specifically to mTor [30] and inhibits mTOR's ability to phosphorylate target proteins such as p70 S6K and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1), which play essential roles in ribosome biogenesis and cap-dependent translation, respectively [31-33]. Aoki *et al*. [26] showed that rapamycin inhibits formation of transformed cell foci induced by the expression of

Akt and P3k, the protein encoding oncogene *p3k*. Therefore, in this study, we investigated the role of Akt/mTOR pathway on EGF-induced cell transformation in JB6 cells by using rapamycin and cells expressing dominant negative mutants of Akt1.

MATERIALS AND METHODS

Materials

Eagle's minimal essential medium (MEM), L-glutamine and basal medium eagle (BME) were from Life Thechnologies (Rockville, MD); fetal bovine serum (FBS) and gentamicin were from BioWhittaker Biosciences (Walkersville, MD); PI3K inhibitors, wortmannin and LY29402, and the mTor inhibitor, rapamycin were from Calbiochem (La Jolla, CA); the Akt immunoprecipitation kinase assay kit, S6 kinase assay kit and dominant negative mutants of Akt1 (DNM-Akt1) were from Upstate Biotechnology Inc. (Lake Placid, NY); the Akt antibody and phospho-specific Akt (serine 473), p70 S6K antibody and phospho-specific p70 S6K (threonine 389), and PhosphoPlus p44/42 mitogen activator protein (MAP) kinase antibody kits were from Cell Signaling Technology Inc. (Beverly, MA); the anti-Akt1/2 antibody was from Santa Cruz (Santa Cruz, CA).

Cell Culture

The JB6 mouse epidermal cell line Cl 41 and its stable transfectants, P+1-1 (AP-1 reporter transfectant) and Cl 41 DNM-Akt1, were grown at 37°C 5% CO_2 in MEM supplemented with 5% heat-inactivated FBS, 2 mM L-glutamine, and 25 μg/ml gentamicin.

Generation of Stable Transfectant

The DNM-Akt1 plasmid was transfected in JB6 Cl41 cells by using LipofectAMINE (Life Technologies Inc.) following the manufacturer's instructors. The stable transfectants were obtained by selection for G418 resistance (300 μg/ml) and further confirmed by assay of activity.

Anchorage-independent Transformation Assay

JB6 Cl 41 cell line and its transfectant, Cl 41 DNM-Akt1, ($1x10^4$) were exposed to EGF (10 ng/ml) with or without the indicated concentration of rapamycin or LY294002 in 1 ml 0.33% BME agar containing 10% FBS over 3.5 ml 0.5% BME agar medium containing 10% FBS. The cultures were maintained in a 5% CO_2 incubator at 37°C and the cell colonies were scored at 14 days after cells were exposed to EGF.

Assay of AP-1 Activity

JB6 AP-1 reporter stable P+1-1 cells were seeded in a 96-well plate. After 24 h incubation, the cells were starved by replacing 0.1% FBS/MEM and incubating for 24 h. Then, the cells were pretreated with the indicated concentration of rapamycin or LY294002 for 1 h and cultured with 10 ng/ml EGF for 24 h. The cells were extracted with lysis buffer and luciferase activity was measured with a luminometer (Monolight 2010). Relative AP-1 activity was calculated as described previously [34].

Cell Proliferation Assay

Cells were suspended in 5% FBS/MEM at a density of $1x10^4$ cells/ml. The cell suspension (1 ml) were placed in each well of a 24-well plate and incubated for 24 h. Then, the indicated concentrations of rapamycin or LY294002 were added and further incubated for 48 h in a 5% CO_2 incubator at 37°C. After incubation for the indicated periods, cell number was measured under microscope.

Immunoblotting

Immunoblotting was carried out as described previously [34]. In brief, JB6 Cl 41 cells were cultured to 80% confluence. The cells were starved in 0.1 % FBS/MEM for 48 h at 37°C. Then media were changed to fresh 0.1% FBS/MEM and cells were incubated for another 2-4 h at 37°C. Before the cells were exposed to EGF, they were either treated or not treated with LY294002, wortmannin or rapamycin for 1 h. Then, EGF (10 ng/ml) was added and subsequently incubated for 30 min at 37°C in the presence or absence of inhibitors. The cells were then lysed and immunoblot analysis performed by using the antibodies against Akt, p70 S6K and extracellular signal-regulated protein kinases (Erks) or the phospho-specific antibodies against their phosphorylated proteins. Antibody-bound proteins were detected by fluorescence (ECF Western Blotting Kit, Amersham Pharmacia Biotech, Piscataway, NJ) and analyzed using the Storm 840 Scanner (Molecular Dynamics, Sunnyvale, CA).

Akt and p70 S6K Immunoprecipitation Kinase Assay

Cells were treated with the inhibitors before treatment with EGF (10 ng/ml) and lysates were prepared from the cells and the immunoprecipitation was carried out using 20 μl of

anti-Akt1/2 (Santa Cruz, Santa Cruz, CA) or 3 μg of anti-p70 S6K antibody (Cell Signaling). The enzyme immune complex was washed three times with 0.5 ml of lysis buffer, and once with 100 μl of assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, pH 7.0, 1 mM sodium orthovanadate, 1 mM DTT). For the Akt kinase assay, the enzyme immune complex was added to 10 μl of assay dilution buffer, 10 μM protein kinase A inhibitor peptide, 0.1 mM Akt substrate peptide, and 1 μCi [γ-^{32}P] ATP, and, for the p70 S6K assay, it was added to 20 μl of assay dilution buffer, 10 μl of inhibitor cocktail, 50 μM S6 kinase substrate peptide, and 1 μCi [γ-^{32}P] ATP. The reaction was incubated for 10 min at 30°C and centrifuged and then 30 μl of the supernatant fraction was transferred onto P81 phosphocellulose paper and allowed to bind for 30 s. The P81 papers were washed three times in 0.75% phosphoric acid then washed once in acetone and γ-^{32}P incorporation was measured by scintillation counting.

Statistical Analysis

Significant differences between samples were determined by both Student's *t* test and Welch's *t* test.

RESULTS AND DISCUSSION

We previously demonstrated that PI3K plays a pivotal role on TPA- or EGF-induced cell transformation in JB6 cells [11,12]. In those studies, we showed that the PI3K inhibitors, LY294002 and wortmannin, inhibited their tumor promoter-induced cell transformation as well as AP-1 activation, which is one of critical factors for the cell transformation in JB6 cells [13-15]. On the other hand, accumulating evidence has

suggested that the transforming effect of PI3K relates to the activation of its downstream effector kinase, including Akt, mTOR and p70 S6K [4,16-19]. In particular, several recent studies have suggested an essential role of mTOR in tumorigenesis mediated by PI3K signaling. Aoki *et al*. [26] showed that rapamycin, a mTOR inhibitor, effectively blocked oncogenic transformation induced by constitutively active PI3K or Akt. Treatment of PTEN heterozygous mice with CCI-779, an ester analog of rapamycin, has been reported to inhibit development of neoplastic lesions as well as decrease of p70 S6K activity [35]. Moreover, Neshat *et al*. [36] indicated that the increase of S6 kinase activity observed in PTEN-deficient human cancer cell lines was highly sensitive to CCI-779. Therefore, in this study, we investigated effect of rapamycin on EGF-induced cell transformation in JB6 cells. Consistent with our previous study [12], treatment of cells with LY294002, a PI3K inhibitor, inhibited EGF-induced cell transformation (Fig. 1A). Treatment with rapamycin also suppressed the cell transformation induced by EGF (Fig. 1A). Although LY294002 also attenuated EGF-induced AP-1 activation, treatment of rapamycin had no effect on AP-1 activation (Fig. 1B), indicating that the inhibition of cell transformation by rapamycin was independent of AP-1 activation. Several studies have demonstrated the impressive antiproliferative effects of rapamycin in different types of tumor cells [37]. The antiproliferative actions exhibited by treatment with rapamycin may result from inhibition of critical signal transduction pathways that control cell cycle progression from G1 to S phase [29]. However, the treatment of cells with rapamycin or LY294002 only slightly inhibited cell proliferation at 72 hr (Fig. 2), suggesting that the effect of rapamycin and LY294002 on blocking cell transformation is not due to cell growth inhibition.

In response to growth factors, mTOR is thought to control signal to translation initiation machinery via phosphorylation of p70 S6K and 4E-BP1 [31-33]. Activated p70 S6K phosphorylates the 40S ribosomal protein S6 and stimulates the translation of mRNAs with a 5' terminal oligopolypyrimidine including the protein they encode [38]. Phosphorylation of 4E-BP1 leads to dissociation of eukaryotic initiation factor (elf) 4E to 4E-BP1, and the free elf 4E forms the translation initiation complex at the cap of the mRNA [32,39]. To confirm whether the concentration of rapamycin that inhibited the cell transformation blocks mTOR activity induced by EGF, we examined the effect on EGF-induced activation of p70 S6K, a downstream target of mTOR. As showed in Fig. 3, rapamycin inhibited EGF-induced p70 S6K phosphorylation at threonine 389, a rapamycin-sensitive site [40], but had no effect on Akt phosphorylation. Similar results were observed on EGF-induced activation of p70 S6K and Akt by the treatment with rapamycin (Fig. 4). We also comfirmed that rapamycin had no effect on EGF-induced PI3K activity (data not shown). In contrast, treatment with the PI3K inhibitors, LY294002 and wortmannin, inhibited EGF-induced phosphorylation and activation of both p70 S6K and Akt (Figs. 3 and 4). EGF not only stimulates the PI3K signaling but activates mitogen-activated protein (MAP) kinases, such as Erks [41,42]. In addition, p70 S6K has been shown to be phosphorylated by MAP kinases [43,44]. However, treatment with rapamycin showed no change in EGF-induced Erks phosphorylation (Fig. 5). These results suggested that the effect of rapamycin on EGF-induced signaling appears to be specific to the mTOR. Based on the direct phosphorylation of mTOR by Akt [27,28] and p70 S6K activation by transfection with constitutively active Akt [45], Akt has been indicated to be a candidate as a downstream effector of PI3K in the mTOR-

dependent pathways. However, the regulation of mTOR by Akt is still controversial, because the phosphorylation site of mTOR by Akt has been shown to be not required for p70 S6K activation [28]. Thus, the inhibitory effect of rapamycin on EGF-induced cell transformation might be through PI3K/Akt-independent pathway. In contrast, recent studies have reported an important role of mTOR in PI3K/Akt function. Rapamysin or CCI-779 treatment of PTEN-deficient cells or activated PI3K- or Akt-expressing cells has been reported to block the phosphorylation of p70 S6K or 4E-BP1 [26,35,36]. Reintroduction of PTEN in PTEN-deficient tumor cells has been shown to correct elevated activation of p70 S6K [21]. We indicated that an introduction of DNM-Akt1 into JB6 Cl 41 cells inhibits EGF-induced cell transformation without blocking the cell proliferation (Fig. 6). The expression of DNM-Akt1 also suppressed EGF-induced p70 S6K activation as well as Akt activation (Fig. 7). Therefore, it suggested that Akt/mTOR pathway is closely involved in EGF-induced cell transformation in JB6 Cl 41 cells, and the inhibitory effect of rapamycin may be due to blocking mTOR. In this study, we demonstrated that rapamycin, a mTOR inhibitor, suppressed EGF-induced cell transformation without blocking of AP-1 activation or cell proliferation in mouse JB6 cells. The expression of DNM-Akt1 also inhibited cell transformation and p70 S6K activation induced by EGF. These indicated that Akt/mTOR pathway is involved in EGF-induced cell transformation in addition to AP-1 activation. mTOR is also reported to regulate translation of c-Myc, a transcription factor deregulated in a wide variety of human cancers, and activates Stat 3, which is activated in many human cancers and causes cell transformation [46-49]. The inhibitory effect by rapamycin might result in blocking activation of these targets. Aberrant activation of the PI3K signaling occurs in

numerous and diverse human cancers, and mTOR and its downstream target, p70 S6K, are the critical factors in the PI3K-dependent tumorigenesis. Therefore, the results from this study may provide suggestions in prevention or therapy for tumorigenesis with deranged PI3K signaling.

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Abbreviations: 4E-BP1, elf4E binding protein; AP-1, activator protein 1; BME, basal medium eagle; DNM-Akt1, dominant negative mutants of Akt1; EGF, epidermal growth factor; Erk, extracellular signal-regulated protein kinase; FBS, fetal bovine serum; JB6 Cl 41, JB6 mouse epidermal cell line Cl 41; MAP, mitogen activated protein; MEM, minimal essential medium; mTOR, mammalian target of rapamycin; MTT, methylthiazoletetrazolium; p70 S6K; ribosomal p70 S6 protein kinase, PI3K, phosphatidylinositol-3 kinase; PTEN, phosphatase and Tensin homolog, TPA; 12-*O*-tetradecanoylphorbol-13-acetate

FIGURE LEGENDS

Figure 1. **Effects of rapamycin on EGF-induced cell transformation and AP-1 activation.** *A,* Aliquots of 10^4 JB6 Cl 41 cells were treated with EGF (10 ng/ml) with or without the indicated concentration of rapamycin or LY294002 in 0.33% BME agar containing 10% FBS over 0.5% BME agar medium containing 10% FBS. Cell colonies were scored after 14 days incubation at 37˚C in 5% CO2. Data are the means ± SD (n=8). *B,* JB6 cell AP-1 reporter stable P+1-1 cells were seeded in a 96-well plate. After 24 h incubation, the cells were starved by replacing 0.1% FBS/MEM for 24 h. Then, the cells were pretreated with the indicated concentration of rapamycin or LY294002 for 1 h and cultured with 10 ng/ml EGF for 24 h. The AP-1 activity was measured with a luciferase activity assay as described under ‘‘Material and Methods’’. The results showed as relative AP-1 activity are the means ± SD (n=5-6). * Significantly different from the untreated control at $P < 0.01$.

Figure 2. **Effect of rapamycin on cell proliferation.** JB6 Cl 41 cells were suspended in 5% FBS/MEM at a density of $1x10^4$ cells/ml. The cell suspension (1 ml) were placed in each well of a 24-well plate and incubated for 24 h. Then, the indicated concentrations of rapamycin or LY294002 were added and further incubated for 48 h in a 5% CO_2 incubator at 37°C. After incubation for the indicated periods, cell number was measured under microscope. Data are the means ± SD (n=4).

Figure 3. **Effect of rapamycin on EGF-induced phosphorylation of Akt and p70 S6K.** JB6 Cl 41 cells (80% confluence) were starved by replacing the medium with 0.1% FBS/MEM and culturing for 48 h. The cells were then pretreated with rapamycin, LY294002 or wortmannin for 1 h at the indicated concentration. The cells were treated with EGF (10 ng/ml) and subsequently cultured for 30 min. Then, the cells were lysed

and the phosphorylation levels were estimated by immunoblotting with phospho-specific antibodies for Akt or p70 S6K.

Figure 4. **Effect of rapamycin on EGF-induced activation of Akt and p70 S6K.** JB6 Cl 41 cells (80% confluence) were starved by replacing the medium with 0.1% FBS/MEM and culturing for 48 h. The cells were then pretreated with rapamycin, LY294002 or wortmannin for 1 h at the indicated concentration. The cells were treated with EGF (10 ng/ml) and subsequently cultured for 30 min. Then, the cells were lysed and Akt or p70 S6K was immunoprecipitated using anti-Akt1/2 antibody or anti-p70 S6K antibody. Their activities are assessed using their substrate peptide and γ-^{32}P ATP. Each bar indicates the mean ± S.E (n=3-4). * Significantly different from the untreated control at $P < 0.05$.

Figure 5. **Effect of rapamycin on EGF-induced Erks phosphorylation.** JB6 Cl 41 cells (80% confluence) were starved by replacing the medium with 0.1% FBS/MEM and culturing for 48 h. The cells were then pretreated with rapamycin, LY294002 or wortmannin for 1 h at the indicated concentration. The cells were treated with EGF (10 ng/ml) and subsequently cultured for 30 min. The cells were lysed and the phosphorylation levels were estimated by immunoblotting with phospho-specific p44/42 MAP kinase (Erks) antibody.

Figure 6. **Inhibition of EGF-induced cell transformation by introduction of DNM-Akt1.** *A*, Aliquots of 10^4 JB6 Cl 41 cells or Cl 41 DNM-Akt1 cells (DNM-Akt1) were treated with EGF (10 ng/ml) in 0.33% BME agar containing 10% FBS over 0.5% BME agar medium containing 10% FBS. Cell colonies were scored after 14 days incubation at 37˚C in 5% CO2. Data are the means ± SD (n=8). *B*, Cells were suspended in 5% FBS/MEM at a density of $1x10^4$ cells/ml. The cell suspension (1 ml) were placed in each

well of a 24-well plate and incubated for 24 h. Then, the indicated concentrations of rapamycin or LY294002 were added and further incubated for 48 h in a 5% CO_2 incubator at 37°C. After incubation for the indicated periods, cell number was measured under microscope. Data are the means ± SD (n=4).

Figure 7. **Inhibition of EGF-induced p70 S6Kactivation by introduction of DNM-Akt1.** JB6 Cl 41 cells and Cl 41 DNM-Akt1 cells (DNM-Akt1) (80% confluence) were starved by replacing the medium with 0.1% FBS/MEM and culturing for 48 h. Their cells were treated with EGF (10 ng/ml) and subsequently cultured for 30 min. Then, the cells were lysed and Akt or p70 S6K was immunoprecipitated using anti-Akt1/2 antibody or anti-p70 S6K antibody. Their activities are assessed using their substrate peptide and γ-^{32}P ATP. Each bar indicates the mean ± S.E (n=3-4). * Significantly different from the untreated control at $P < 0.05$.

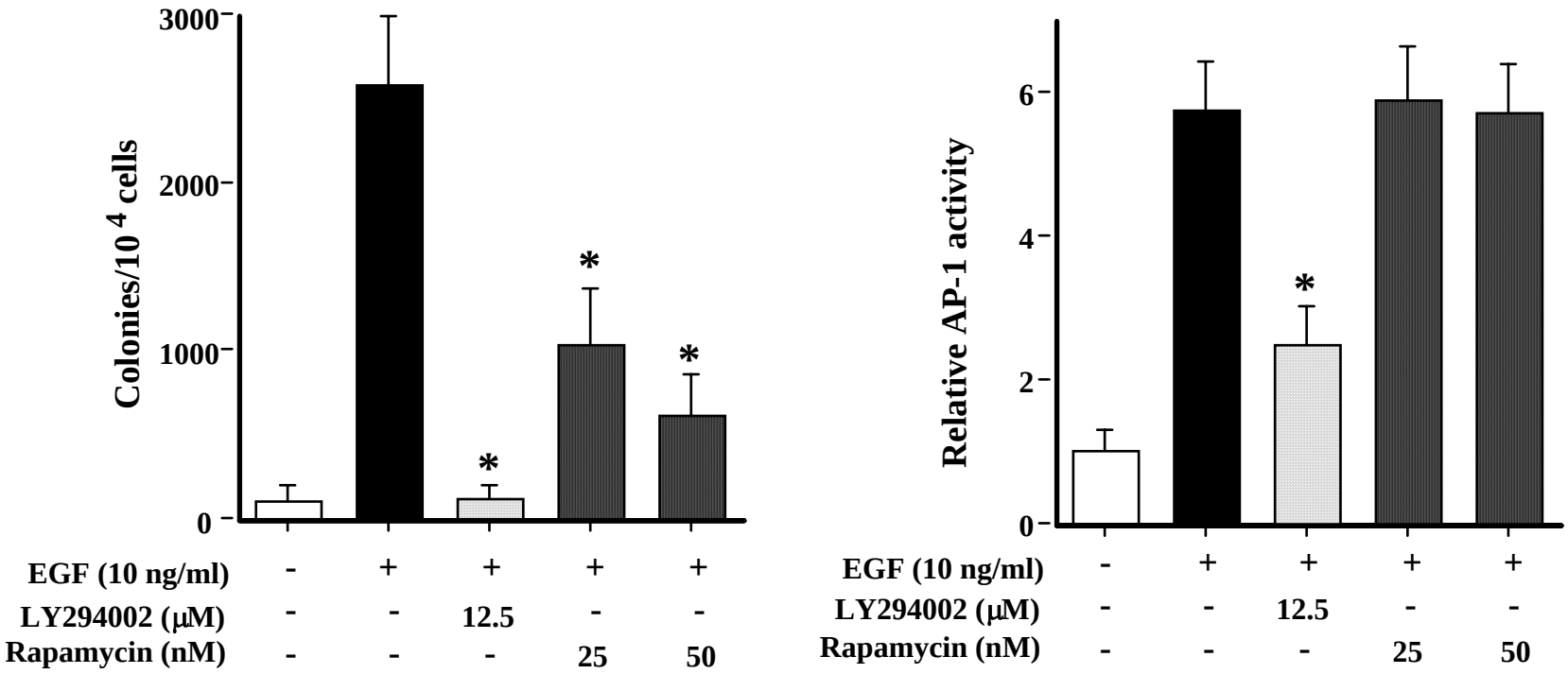
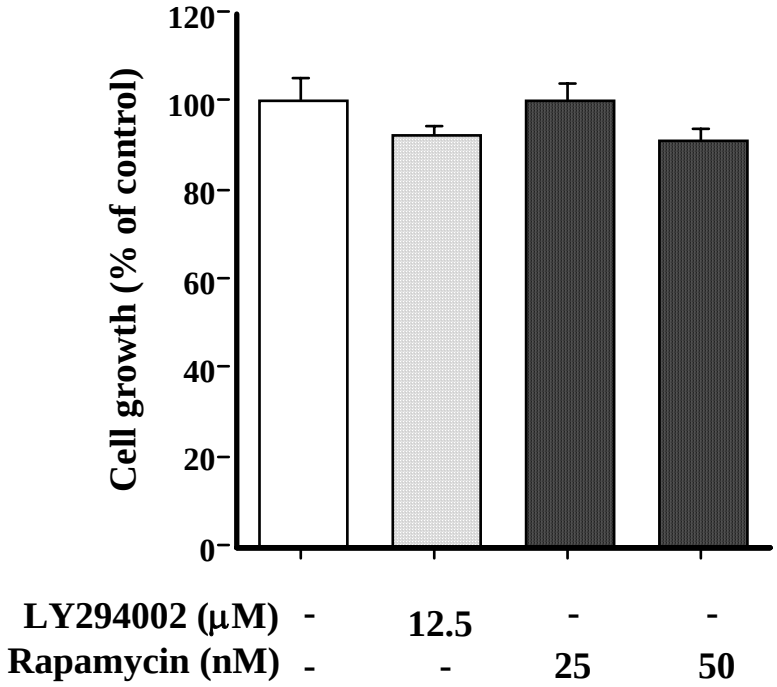
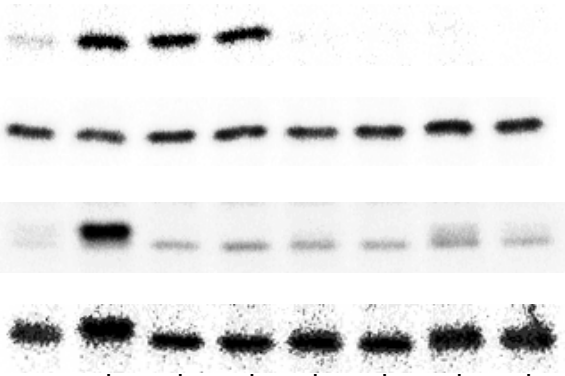


Figure 1. Nomura et al.


Cell growth (% of control)
120
100
80
60
40
20
0
LY294002 (μM) - 12.5 - -
Rapamycin (nM) - - 25 50



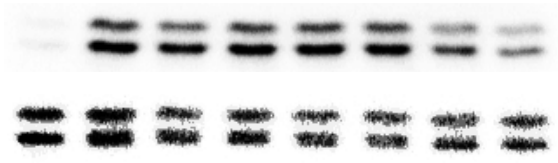
Phospho-Akt (Ser 473)

Akt

Phospho-p70 S6K (Thr 389)

p70 S6K

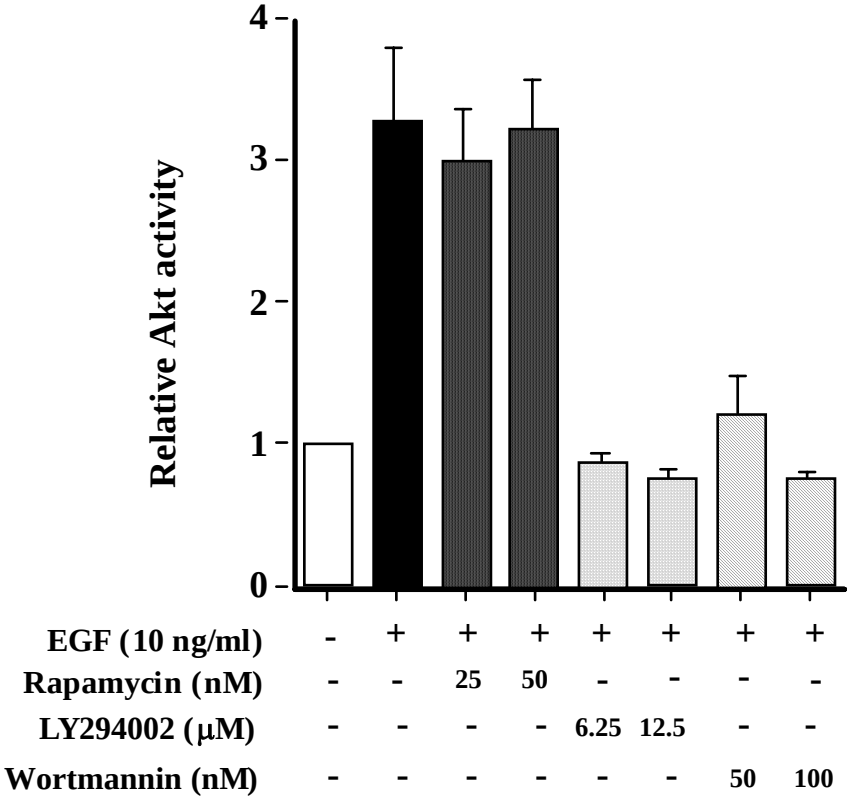
EGF (10 ng/ml)	-	+	+	+	+	+	+	+
Rapamycin (nM)	-	-	25	50	-	-	-	-
LY294002 (μM)	-	-	-	-	6.25	12.5	-	-
Wortmannin (nM)	-	-	-	-	-	-	50	100

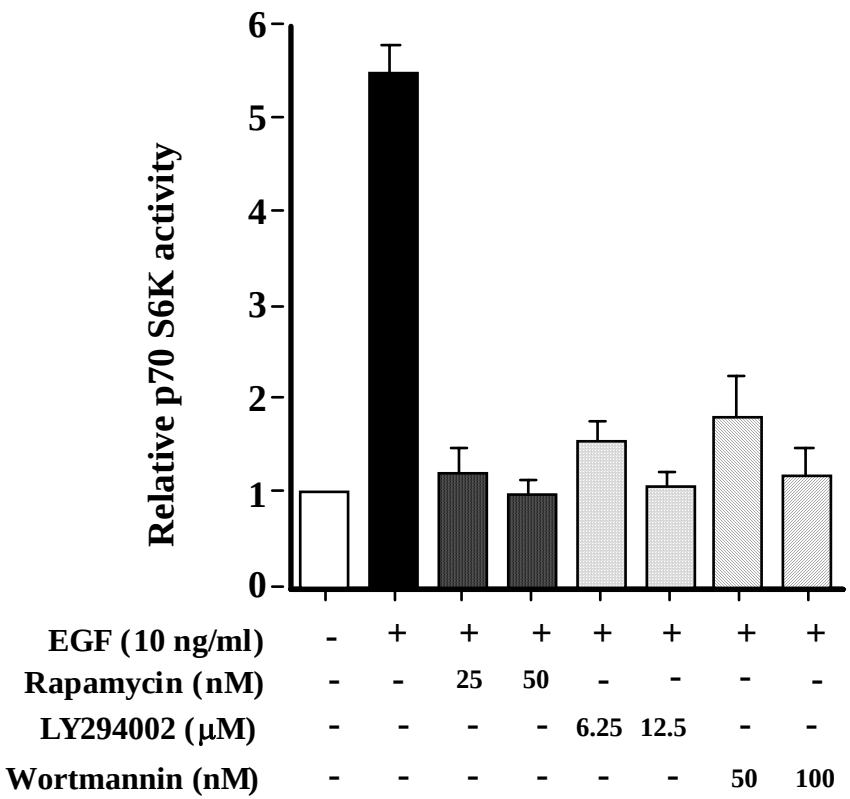


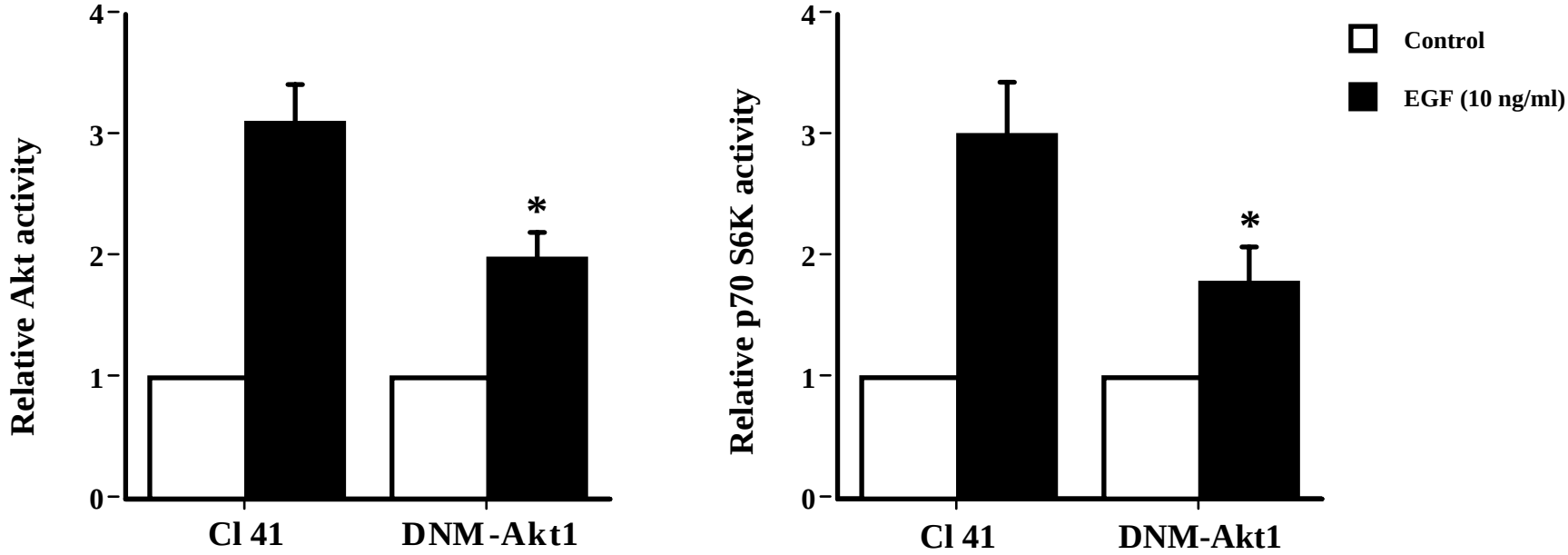
Phospho-Erks

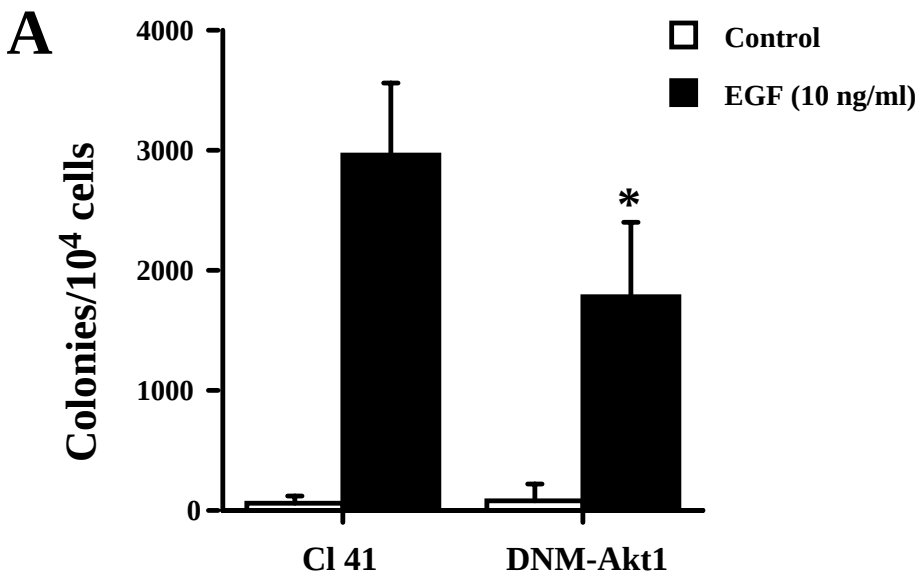
Erks

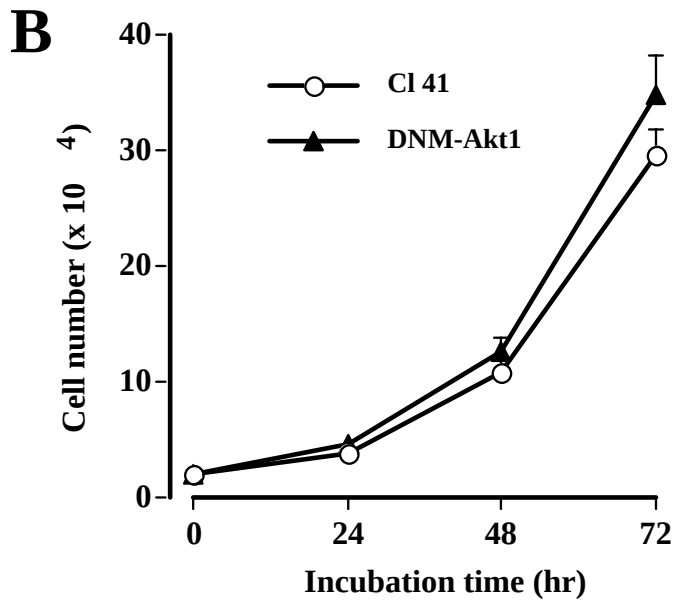
EGF (10 ng/ml)	-	+	+	+	+	+	+	+
Rapamycin (nM)	-	-	25	50	-	-	-	-
LY294002 (μM)	-	-	-	-	6.25	12.5	-	-
Wortmannin (nM)	-	-	-	-	-	-	50	100


Relative Akt activity
4
3
2
1
0
EGF (10 ng/ml) - + + + + + + +
Rapamycin (nM) - - 25 50 - - - -
LY294002 (μM) - - - - 6.25 12.5 - -
Wortmannin (nM) - - - - - - 50 100


Relative p70 S6K activity
6
5
4
3
2
1
0
EGF (10 ng/ml) - + + + + + + +
Rapamycin (nM) - - 25 50 - - - -
LY294002 (μM) - - - - 6.25 12.5 - -
Wortmannin (nM) - - - - - - 50 100


Relative Akt activity
4
3
2
1
0
*
Cl 41
DNM-Akt1
Relative p70 S6K activity
4
3
2
1
0
*
Cl 41
DNM-Akt1
Control
EGF (10 ng/ml)


A
4000
3000
2000
1000
0
Colonies/10^4 cells
Control
EGF (10 ng/ml)
*
Cl 41
DNM-Akt1


B
40
30
20
10
0
Cell number (x 10 4)
Cl 41
DNM-Akt1
0
24
48
72
Incubation time (hr)