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THE ALTERATION OF EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK) ACTIVITY OF COS7 CELLS FROM INTERPHASE TO MITOSIS

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Abstract – Although many reports have suggested that ERK1/2 activity is increased in interphase and inhibited in mitosis, the change of ERK1/2 activity from interphase to mitosis is largely undefined. In this study, we examined the alteration of ERK1/2 activity in COS7 cells in response to nocodazole. Nocodazole-treated or nocodazole-untreated COS7 cells were stimulated by EGF. We found that ERK1/2 activity of nocodazole-treated cells was decreased with prolonged time of cells treated with nocodazole while being increased in nocodazole-untreated cells. COS7 cells became round after being treated with nocodazole for 4 hr. And from the time point, ERK1/2 activity was gradually and significantly inhibited. Furthermore, we showed that MEK activity was greatly decreased compared with nocodazole-untreated cells after the cells were treated by nocodazole for 8 hr and then EGF. In addition, the migration of cells was inhibited in response to nocodazole. Taken together, we concluded that ERK1/2 activity was gradually inhibited from interphase to mitosis and the cell migration was affected by nocodazole.

Key words: ERK1/2 activity, COS7 cell, nocodazole, EGF.

INTRODUCTION

The cell cycle, or cell-division cycle, is a series of events that takes place in a cell leading to its division and replication. In eukaryotes, the standard cell cycle can be divided into two brief periods: the interphase and the mitotic phase. At cellular interphase, distinct signaling mechanisms in ERK pathway facilitate progression through G1/S and survival in G1 and through processes involved in nuclear transcription factor phosphorylation, immediate-early gene induction, expression of cell cycle genes that direct DNA synthesis, and regulation of translational initiation (16, 27). To this extent, the strategies that have been used to disrupt ERK function have reduced growth-factor-stimulated DNA replication (4, 7, 20). Nevertheless, in mitotic phase, one of the marked changes is general inhibition of membrane traffic. Many studies have showed that membrane traffic is inhibited during mitosis and mitotic cells likely fail to respond to transmembrane signaling (2, 3, 18, 19, 21-26, 28). Some signal pathways are affected, such as MAPK pathway. The reports showed that ERK activity appears to inhibit the transition from G2 to M phase of the cell cycle (1, 5, 9). At M phase, EGF-induced intracellular signaling is obliterated by hyperphosphorylation of its receptor, which decreases ligand binding affinity (13). Dangi and Shapiro (6) further showed that Cdc2 inhibits EGF-mediated ERK activity during mitosis. These M phase-specific attenuation of growth factor signaling might be required for proper cell cycle progression and division.

Nocodazole is usually used to synchronize the cell division cycle. Cells treated with nocodazole arrest with a G2- or M-phase DNA

Abbreviations: **EGF**, epidermal growth factor; **ERK**, extracellular signal-regulated kinase; **MEK**, mitogen-activated proteinor extracellular signal-regulated kinases

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content when analysed by flow cytometry. Microscopy of nocodazole-treated cells shows that they do enter mitosis but cannot form metaphase spindles because microtubules (of which the spindles are made) cannot polymerise. Prolonged arrest of cells in mitosis due to nocodazole treatment typically results in the apoptotic-like cell death (8).

Although it is known that ERK1/2 activity is increased in interphase and inhibited in mitosis, many events remain unknown. The report presented here addresses the change of ERK1/2 activity from interphase to mitosis in COS7 cells response to nocodazole. We found that ERK1/2 activity was gradually decreased with prolonged time of cells treated by nocodazole. COS7 cells became round after cells treated by nocodazole for 4 hr. Moreover, the migration of cells was affected.

MATERIALS AND METHODS

Cells lines and culture

COS7 cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS, penicillin, and streptomycin and were maintained in a 5% CO_2 atmosphere.

Antibodies and Chemicals

Rabbit anti-ERK1/2 and anti-MEK1, mouse anti-pp-ERK1/2 and anti-tubulin, and goat anti-p-MEK were from Santa Cruz Biotech (Santa Cruz, CA). HRP conjugated secondary antibodies were purchased from Bio-Rad (Hercules, CA). EGF was obtained from Upstate Biotechnology, Inc. (Lake Placid, N.Y.). Nocodazole was from Calbiochem. DMEM was from GIBCO. Unless otherwise specified, all the chemicals were purchased from Sigma (St. Louis, MO).

Cell treatment

To detect ERK activity of cells at interphase and mitosis, COS7 cells were serum starved with DMEM for 24 hr, and then stimulated with EGF with a final concentration of 50 ng/ml for indicated time (0, 5, 15, 30, 60 and 120 min) and after that cells were scraped and lysated to prepare the protein samples of the interphase; on the other hand, cells were treated with nocodazole (200 ng/ml) for 24 hr, and then stimulated with EGF with a final concentration of 50 ng/ml for indicated time (0, 5, 15, 30, 60 and 120 min) and after that mitotic cells were collected and lysated to prepare the protein samples of the mitosis.

To detect the alteration of ERK activity from interphase to mitosis, COS7 cells were firstly treated with or without nocodazole (250 ng/ml) for the indicated time (0, 0.5, 1, 2, 4, 8, 12, 24 hr) and then stimulated with EGF (50 ng/ml) for 15 min. Finally, all of cells were collected and lysated.

Gel Electrophoresis and Immunoblotting

The protein samples were separated by electrophoresis through 10% polyacrylamide SDS-containing gels depending on the size of protein interested (37.5:1 Acrylamide /bis, Tris-Cl; pH 8.8, TEMED, Ammonium persulfate). Prestained protein markers (Sigma) were used for molecular weight standards. Proteins were electrophoretically transferred onto nitrocellulose membranes (BioRad, Hercules, CA). The transfer was done using a semi-dry blotting apparatus (Model SD transfer cell, BioRad) at 15 V per minigel for 45 min in transfer buffer. Blots were blocked with 3% skim milk in 0.05% Tween-PBS (blocking buffer) for 30 min.

Membranes were then probed with polyclonal rabbit anti-ERK1/2 and anti-MEK1, monoclonal mouse anti-pp-ERK1/2 and anti-tubulin, or polyclonal goat anti-p-MEK in blocking buffer overnight. And the primary antibodies were detected with a polyclonal goat anti-rabbit Ig coupled to HRP, a polyclonal rabbit anti-goat Ig coupled to HRP or a polyclonal goat anti-mouse Ig coupled to HRP followed by enhanced chemiluminescence, with SuperSignal ECL western Blotting Detection Reagents (Pierce Chemical) and light detection with Fuji Photo Film (Tokyo, Japan).

Observation of Cell morphology

COS7 cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS, and were maintained in a 5% CO_2 atmosphere. When being 80% confluence, COS7 cells were treated with nocodazole (0.25 μg/ml) for 0, 0.5, 1, 2, 4, 8, 12, 24 hr followed by watching cells under microscope. Images for COS7 cells were acquired at magnifications of ×10, respectively.

Wound-healing assay

In response to nocodazole and EGF, Cell migration was checked by the wound-healing assay (29). COS7 cells were seeded into 6-well plates and grown to the confluence. Confluent cell monolayer was scratched by a sterile micropipette tip, and wounded monolayer was then washed with PBS to remove cell debris. Cells were further incubated with or without nocodazole (250 ng/ml). After 4 hr, cells were treated with EGF (100 ng/ml) and continued to culture. Wounds were photographed at 0, 24 and 48 hr after wounding. The cell migration was evaluated by measuring the width of the wounds.

Statistics

All experiments were repeated three times. Results were expressed as mean±SE. The data were analyzed with the software package SPSS 16.0. P-value$<$0.05 were considered significant.

RESULTS

ERK activity in interphase and mitosis

Although ERK activity is essential for mediating cell cycle progression at interphase such as DNA synthesis, EGF receptor-mediated activity of the ERK pathway is inhibited in mitotic cells (10). To identify ERK activity in interphase and mitosis, COS7 cells were stimulated by EGF after treated with or without nocodazole. Nocodazole is an anti-neoplastic agent which exerts its effect on cells by interfering with the polymerization of microtubules or by perturbation of the anaphase-promoting complex/cyclosome (8). According to Fig.1, we found that pp-ERK1/2 was

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significantly decreased in mitosis compared with that in the interphase. The discrepancy in ERK1/2 activity observed in mitotic and interphase cells prompted us to examine the factors responsible for ERK1/2 activity alteration.

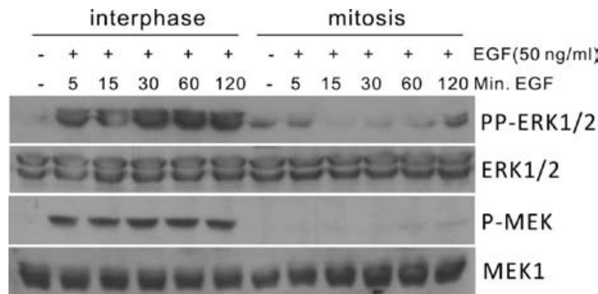

Figure 1. Status of ERK1/2 and MEK activity in the interphase and the mitosis. Asynchronous or nocodazole-arrested mitotic cells were treated without or with EGF (50 ng/ml) for 5, 15, 30, 60 and 120 min. Active ERK1/2 and MEK (pp-ERK1/2 and p-MEK) were analyzed by immunoblotting. Serum starved cells without EGF stimulation were used as control. Data are representative of three independent experiments.

Changes of ERK activity in nocodazole-treated cells

The previous study found that ERK activity was affected in response to nocodazole (12). To further investigate the event, COS7 cells were treated with or without nocodazole for 0, 0.5, 1, 2, 4, 8, 12, 24 hr, respectively. It was shown from Fig.2A and 2C that ERK1/2 phosphorylation in nocodazole-untreated cells was increased by 0.5 hr and kept to 2 hr followed by being gradually decreased with prolonged serum starvation. Nevertheless, after COS7 cells were treated with nocodazole, we found that ERK1/2 phosphorylation was increased by 0.5 hr followed by being decreased compared with ERK activity at 0 hr (fig. 2D).

Surabhi Dangi identified that treatment with EGF failed to activate the ERK pathway in mitotic cells (6). To investigate the changes of ERK1/2 activity, COS7 cells were treated by EGF after cells were treated with or without nocodazole for indicated periods. Fig.3C witnessed that ERK1/2 activity in response to EGF was obviously increased in nocodazole-untreated cells. However, after COS7 cells were treated with nocodazole and then EGF, ERK1/2 activity was gradually decreased with the time extending (Fig. 3D).

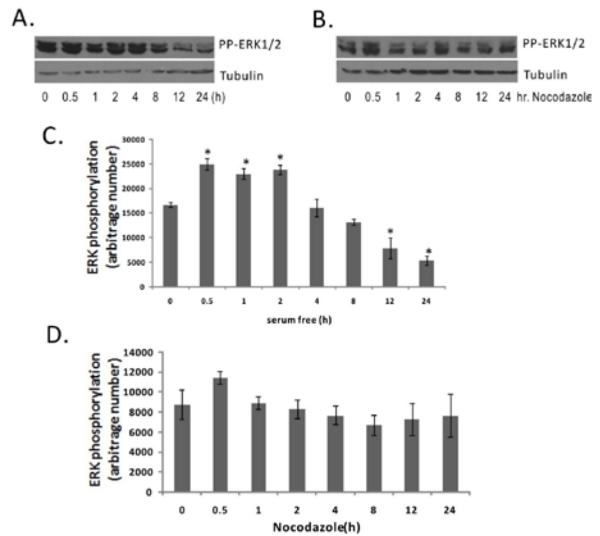

Figure 2. COS7 cells were treated with or without nocodazole (0.25 μg/ml) for 0, 0.5, 1, 2, 4, 8, 12 and 24 hr. Then cells were lysated and the activities of ERK1/2 were checked by immunoblotting. Tubulin expression was used for a protein loading control. Representative results were shown (2A, 2B). The band intensity of pp-ERK was shown by arbitrage numbers which were measured using Image J software. Data are means ± standard errors of three separate experiments (2C, 2D). ERK activity at 0 hr was acted as control. **P*<0.05 vs. control group.

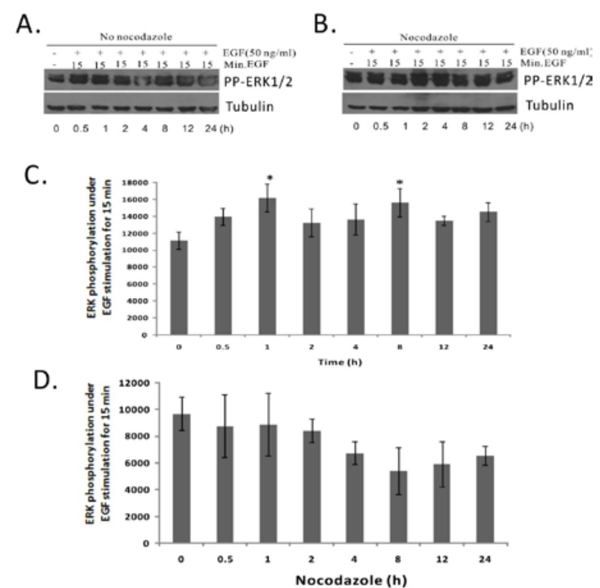

Figure 3. COS7 cells were untreated or treated with nocodazole (0.25 μg/ml) for 0, 0.5, 1, 2, 4, 8, 12 and 24 hr. Then Cells were treated with or without EGF (50 ng/ml) for 15 min. Active ERK1/2 was detected by immunoblotting. Tubulin expression was used for a protein loading control. Representative results were shown (3A, 3B). The band intensity of pp-ERK was shown by arbitrage numbers which were measured using Image J software. Data are means ± standard errors of three independent experiments (3C, 3D). ERK activity at 0 hr was acted as control. **P*<0.05 vs. control group.

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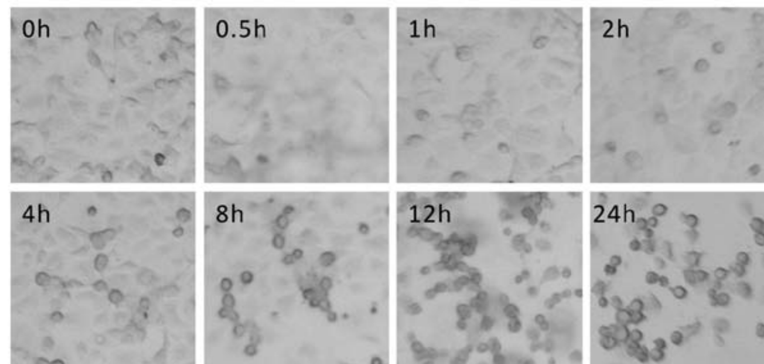
Cell morphology and ERK1/2 activity

After treated with nocodazole, COS7 cells were morphologically changed. Before cells were treated with nocodazole for 4 hr, almost no change was observed under microscope, which showed that cells were most in interphase. At 4 hr, a few cells started to become round followed by more cells becoming round afterwards (Fig.4A). Finally, cells were arrested in mitosis. It was predicted from the change of cell morphology that cellular innate signal pathways have altered gradually. Therefore, we investigated the ERK activity. From Fig.4B, we found that ERK1/2 activity was affected in the process. Before 4 hr, ERK activities in cells incubated with EGF were almost the same between the presence and absence of nocodazole. After 4 hr, ERK activity in EGF stimulation with nocodazole-untreated cells was higher than that of nocodazole-treated cells, especially significant at 24 hr (Fig.4B). It was shown from the above that ERK activity was gradually and significantly inhibited in cells transition from interphase to mitosis.

MEK activity in response to nocodazole

According to the MAPK pathway, we know that ERK activity is related to MEK activity. To explore the mechanism of ERK inhibition, we examined MEK activity. Fig.1 witnessed that MEK was greatly phosphorylated under EGF stimulation in the interphase while the stimulation by EGF did not increase MEK phosphorylation in mitotic cells. After that, MEK activity was measured after cells treated with nocodazole for certain time. From 0.5 hr to 4 hr, the results showed that MEK was greatly phosphorylated after EGF stimulation. After 4 hr, MEK activity has changed. MEK activity of nocodazole-induced cells stimulated with EGF was lower than that of nocodazole-untreated cells. And the discrepancy is more significant at 24 hr (Fig.4B).

A.



B.

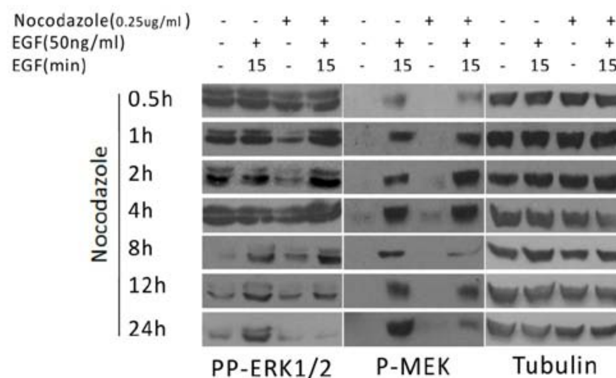


Figure 4. After cultured to 80% confluent COS7 cells were treated with nocodazole (0.25 μg/ml) for 0, 0.5, 1, 2, 4, 8, 12 and 24 hr. Then the cells were watched under the microscope. Representative images were shown (4A). Next cells were untreated or treated with EGF (50 ng/ml) for 15 min after COS7 cells were treated with or without nocodazole (250 ng/ml) for the indicated time (0, 0.5, 1, 2, 4, 8, 12, 24 hr). Active ERK1/2 and MEK (pp-ERK1/2 and p-MEK) were checked by immunoblotting. Tubulin expression was used for a protein loading control. Data are representative of three independent experiments (4B).

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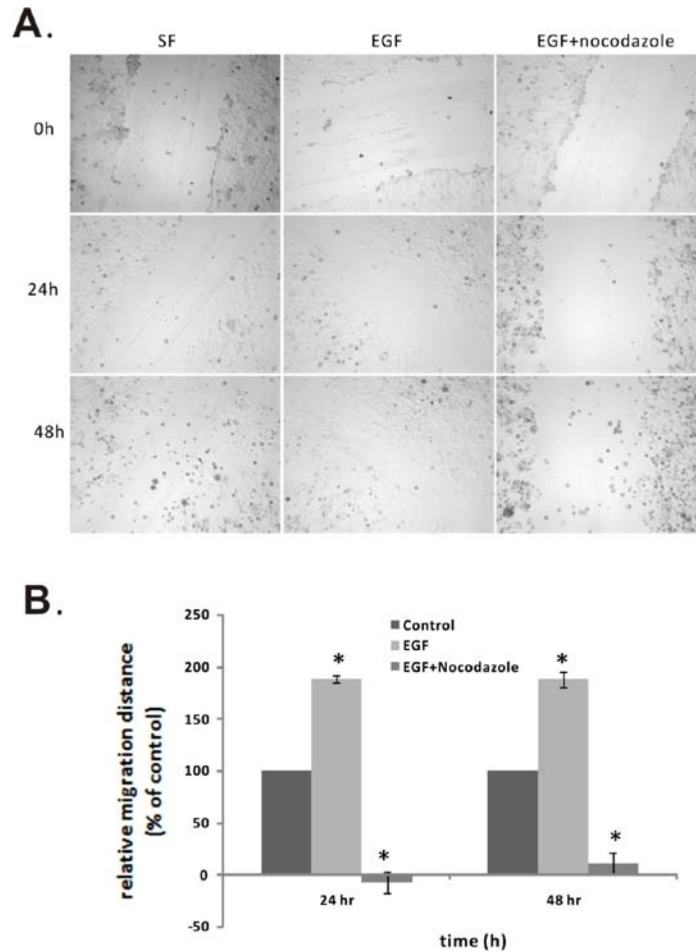

Figure 5. Confluent COS7 cell monolayer was scratched by a sterile micropipette tip. Then they were further cultured with or without nocodazole (250 ng/ml); after 4 hr, they were treated by EGF (100 ng/ml). Wounds were photographed at 0, 24 and 48 hr after wounding. Images were acquired at magnifications of ×10, respectively. Representative images were shown (Fig.5A). Wounding data are expressed as a percentage of migrating distance of 24 and 48 hr without EGF and nocodazole in control, respectively (Fig. 5B). $^*P < 0.05$ vs. control group.

Cell migration

At the cellular level, one of the most prominent functions of the ERK pathway is to regulate cell cycle progression. When entering into the mitosis from the interphase, cells keep the energy needed for dynamic structural changes and the ERK pathway is almost closed. To further investigate the effect of ERK activity on cells, wound healing assay was used. It is seen from Fig.5A, nocodazole-untreated cells showed powerful ability to migrate during EGF stimulation and the wound became thin at 24 and 48 hr, especially very thin in the presence of EGF. Nevertheless, the wound in nocodazole-treated cells was almost not reduced at 24 and 48 hr though cells were treated with EGF. Fig. 5B showed that the discrepancy of cell migration in the presence of EGF or EGF and nocodazole was significant compared with the control ($P < 0.05$). Therefore, we considered that the migration of cells was greatly inhibited under ERK activity affected by nocodazole.

DISCUSSION

In the present study, we demonstrated that the cells were arrested in mitosis and ERK activity was gradually inhibited with prolonged time of cells treated by nocodazole. In mitotic cells, ERK phosphorylation was almost completely inhibited compared with interphase cells (Fig.1). These changes of cells seem important to ensure that general cessation of transcription and translation events occurs to preserve the energy requirements needed for dynamic structural changes occurring in mitotic cells. In addition, we also knew that MEK activity in nocodazole-treated cells was

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decreased and the migration of cells was affected.

Previous findings indicated that ERK activity is inhibited in nocodazole-arrested mitotic cells (11). After nocodazole-induced mitotic COS7 cells were released for 2 hr, ERK activity started to increase and peaked at 8 hr. After this peak, ERK phosphorylation is precipitously diminished until 16 hr post-release (12). Here we collected all nocodazole-treated cells to investigate ERK activity response to nocodazole. ERK activity in nocodazole-treated cells was increased by 0.5 hr and decreased afterwards compared with that at 0 hr (Fig.2D). Due to ERK activity in nocodazole-untreated cells being increased at 0.5 to 2 hr ($p<0.05$) and then gradually decreased (Fig.2C), We considered that ERK activity was greatly related to serum starvation before 2 hr and was not increased by nocodazole, and the reason should be further investigated. From cellular interphase to mitotic phase, the activity of EGF receptor is inhibited (10, 13, 14, 17). Similarly, EGF receptor-mediated activity of the ERK pathway is inhibited in mitosis (10). For example, Hela cells were arrested in mitosis by treating with nocodazole (100 ng/ml) and then the mitotic cells were collected and treated with EGF from 0 to 90 min. The results showed that ERK phosphorylation was greatly decreased compared with asynchronous cells (6). After EGF stimulation to COS7 cells, we found that ERK1/2 activity was increased in nocodazole-untreated cells while gradually decreased in nocodazole-induced cells with the time extending (Fig.3C and 3D). ERK1/2 activity of mitotic cells was almost completely inhibited compared with interphase cells (Fig.1). It is concluded from the above that ERK1/2 activity was inhibited with cells transition from interphase to mitosis.

In mitosis, the cell morphology was round. It was known from Fig.4A that some cells were round after they were treated with nocodazole for 4 hr and more cells being round with the time extending. Therefore, we considered that the point of 4 hr is possibly a vital point for the changes of cell signal pathways. So we checked ERK activity. After COS7 cells were treated with nocodazole for 4 hr, and consequently stimulated with EGF, ERK1/2 activity was gradually and significantly decreased compared with nocodazole-untreated cells. We concluded that cellular innate signal pathways, such as MAPK pathway, possibly affect the cellular morphology and control the cell cycle.

As reported before, the change of ERK phosphorylation is related to MEK activity, which is greatly suppressed in mitosis (12, 15, 30). MEK activity was examined to explore the mechanism of ERK inhibition in cells transition from interphase to mitosis. We identified that MEK activity was indeed inhibited in mitotic cells (Fig.1). By measuring MEK activity for certain time, we found that it was decreased after cells were treated with nocodazole for 8 hr and then EGF for 15 min, which greatly affected downstream protein-ERK activity.

On the whole, our results showed that ERK activity is gradually inhibited in cells transition from interphase to mitosis. EGF receptor-mediated activity of ERK is significantly decreased in mitosis compared with that in interphase. In order to find out the reason for alteration of ERK activity, MAPK cascade should be further checked.

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