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## Role of epidermal growth factor in pathogenesis of uterine leiomyomas

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### ABSTRACT

**Objective:** To investigate the role of epidermal growth factor (EGF) in the pathogenesis of uterine leiomyomas. **Methods:** Human myometrial smooth muscle cells (HM-SMCs) and smooth muscle cells of human uterine leiomyomas (HL-SMCs) were separated from patients' specimens and cultured. After processed by EGF or PD98059 (inhibitor of MKK/MEK) +EGF, the proliferation rate of both SMCs was detected by BrdU method and the phosphorylation level of p44/42 mitogen-activated protein kinase (MAPK) was determined by Western-blot. After different processing time by EGF, the phosphorylation levels of p44/42 MAPK and AKT and p27 expression level in both SMCs were detected by Western-blot. **Results:** EGF could significantly promote HL-SMCs proliferation and PD98059 could inhibit this effect ( $P < 0.05$ ); besides, PD98059 could inhibit the increase of the phosphorylation level of p44/42 MAPK in both SMCs induced by EGF. When the processing time by EGF was over 15min, the phosphorylation levels of p44/42 MAPK and AKT in both SMCs decreased sharply and were close to zero; p27 expression in HM-SMCs raised significantly while the upregulation in HL-SMCs was little. **Conclusions:** EGF could not cause activation of EGFR because of the dephosphorylation of p44/42 MAPK and AKT in HL-SMCs, which caused p27 expression insufficiently and cell cycle dysregulation.

## 1. Introduction

Uterine leiomyomas were characterized by an increase in smooth muscle cells (SMCs) proliferation and excessive deposition of extracellular matrix proteins, primarily collagens type I and III[1,2]. Formation of leiomyomas was viewed as a multistep process, with involvement of ovarian steroid hormones, cytokines and growth factors. Steroid hormones induced mitogenic effects directly through their receptors or by regulating expression of growth factors

such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF) and their respective receptors[3]. Mitogen-activated protein kinase (MAPK) signal pathway was the main transduction path which can regulate cell growth, proliferation and differentiation and so on[4–6]. Many tumors, through autocrine or paracrine modes, could produce EGF which could combine with EGFR to form a dimer, activating their inherent tyrosine kinase and consequently activating the signal pathways in cells (such as MAPK pathway)[7,8].

In the study, human myometrial smooth muscle cells (HM-SMCs) and smooth muscle cells of human uterine leiomyomas (HL-SMCs) were processed by EGF, and then we investigated the trends of the phosphorylation levels of p44/42 MAPK in order to explore the role of EGF in the pathogenesis of uterine leiomyomas.

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## 2. Materials and methods

### 2.1. Cultivation of SMCs

10 human uterine leiomyomas specimens and 10 normal myometrium specimens from cervical intraepithelial neoplasia patients undergone the total hysterectomy were collected after informed consent. Then the uterine leiomyomas and normal myometrium were separated and cut into pieces. Then the pieces were placed into 25 cm<sup>3</sup> culture flask with 0.1% collagenase I in 5% CO<sub>2</sub> incubator at 37 °C for 5-6 h. After the pieces were digested into single cells, they were centrifuged at 1 000 rpm for 8min and beat repeatedly with dulbecco's modified eagle medium (DMEM) for adjusting concentration at 1×10<sup>5</sup> cells/mL. After that, the cells were incubated in 5% CO<sub>2</sub> incubator at 37 °C [9].

### 2.2. Bromodeoxyuridine (BrdU) method

HM-SMCs and HL-SMCs in logarithmic phase were plated in 96-well plate at 5×10<sup>4</sup> cells/mL and grew to confluence. Then the cells were processed, the processing design was as followed: a: control cells in DMEM; b: cells in DMEM with EGF (100 ng/mL, for 24 h); c: cells pretreated with 20 μM PD98059 (inhibitor of MKK/MEK, for 1h) in DMEM + EGF (100 ng/mL, for 24 h).

When the processing time by EGF was left 1 hour, the medium was switched to culture medium containing 10 μM BrdU and cells were incubated for 1 hour. BruU incorporation rate was measured by using a microplate reader. Three independent experiments were performed, and each assay was carried out in triplicate.

### 2.3. Western-blot

After being starved for 24h, the cells were processed by 100 ng/mL EGF for 15 min; besides, PD98059 was added before EGF in another group and the experimental design in detail was according to 2.2. After the cells were lysed and went through the incubation of primary antibody and secondary antibody, the grey value of target protein was measured by Quantity One software.

In order to observe the trends of the phosphorylation levels of p44/42 MAPK and AKT and expression level of p27, both SMCs were processed by 100 ng/mL EGF for 15min, 30min, 60min, 120min and 240min, respectively. And then, the cells were collected at different time points. The target proteins expression level were detected and measured.

**Table 1**

Relative expression level of p44/42MAPK and p-p44/42MAPK protein before and after the treatment of EGF.

	HM-SMCs		HL-SMCs	
	EGF(-)	EGF(+)	EGF(-)	EGF(+)
p44/42MAPK	1.38±0.19	1.43±0.21	1.41±0.25	1.47±0.24
p-p44/42MAPK	0.28±0.06	0.90±0.14 <sup>#</sup>	0.22±0.05	0.42±0.09 <sup>*#</sup>

Note: \* After the same treatment, in HM-SMCs vs in HL-SMCs,  $P < 0.05$ ; # in the same SMCs, before treatment vs. after treatment,  $P < 0.05$ .

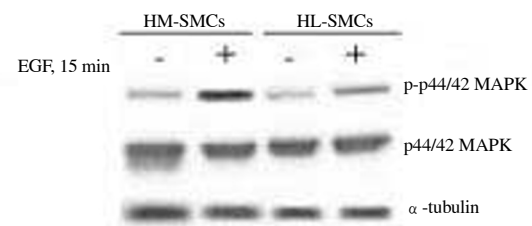
### 2.4. Statistical analysis

SPSS18.0 was used for statistical analysis, all the data were showed by mean±SD. Statistical analysis was performed using One-Way ANOVA with Dunnett's multiple comparisons test.  $P < 0.05$  was considered to be statistical significance.

## 3. Results

### 3.1. Effect of EGF on phosphorylation of p44/42 MAPK

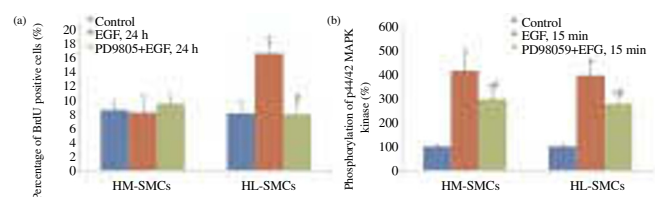
After the processing by EGF, the phosphorylation level of p44/42 MAPK in both SMCs increased significantly ( $P < 0.05$ ), and it was remarkably higher in HM-SMCs than in HL-SMCs ( $P < 0.05$ ) (Figure 1, Table 1).



**Figure 1.** Influence of EGF on phosphorylation level of p44/42 MAPK in HM-SMCs and HL-SMCs.

### 3.2. PD98059's effect on cell proliferation and p44/42 MAPK phosphorylation induced by EGF

Figure 2a showed that EGF could significantly promote the proliferation only in HL-SMCs ( $P < 0.05$ ). However, PD98059 could inhibit this promotion effect ( $P < 0.05$ ). Besides, PD98059 could inhibit the increase of the phosphorylation level of p44/42 MAPK in both cells induced by EGF (Figure 2b).

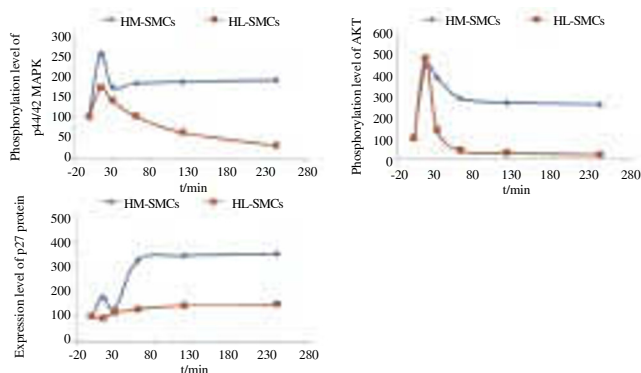


**Figure 2.** Influence of PD98059 on phosphorylation of p44/42 MAPK and proliferation rate in both SMCs.

Note: \*: compared with control group,  $P < 0.05$ ; #: compared with EGF group,  $P < 0.05$ .

### 3.3. EGF affected phosphorylation of p44/42 MAPK and AKT and p27 expression

When the processing time of EGF was over 15min, the phosphorylation level of p44/42 MAPK and AKT in both SMCs decreased. The decreasing amplitude in HL-SMCs was more obvious than that in HM-SMCs, the phosphorylation rate in HL-SMCs was close to 0 (Figure 3). P27 expression level in HM-SMCs induced by EGF increased significantly while the increase in HL-SMCs was little.



**Figure 3.** Phosphorylation level of p44/42 MAPK and AKT and expression level of p27 in HM-SMCs and HL-SMCs after being processed by EGF.

## 4. Discussion

EGF was a kind of strong mitogen, which could stimulate the division and proliferation of multi-type cells and participated in the regulation of multiple physiological and pathological functions[10,11]. In the study, EGF obviously raised the phosphorylation level of p44/42 MAPK in both SMCs and cell proliferation in HL-SMCs, while PD98059 could inhibit this up-regulation. These results implied that p44/42 MAPK, as the downstream signal factor of EGFR-MEK signal pathway, played an important role in the regulation of DNA synthesis in HL-SMCs[12,13]. With the processing time passed, the inhibitory effect on the activation of p44/42 MAPK in HL-SMCs was becoming more and more serious, but this inhibitory effect in HM-SMCs was quite weak.

A role of EGF in leiomyoma growth was supported by the fact that the selective EGF-R blocker AG1478 was able to block leiomyoma cell proliferation[14]. Mesquita *et al*[15] firstly proved in their study that reactive oxygen species (ROS) produced by NADPH oxidase system of LSMCs participated in the regulation of transduction pathway of growth factor signals, and they thought LSMCs produce ROS in response to EGF. Our study showed PD98059 could inhibit DNA synthesis in HL-SMCs as well as the increase of p44/42 MAPK phosphorylation level induced by EGF in both SMCs[16]. In the study of molecular mechanism by which genistein induced the

excessive proliferation of uterine leiomyoma cells, Di *et al*[9] found that after the processing of genistein, the trans-activation of estrogen receptor, the expression of mRNA of estrogen responsive gene, progesterone receptor, IGF-1 all increased while the use of PD98059 can inhibit the up regulation and the excessive proliferation of uterine leiomyoma cells caused by genistein. In the study, after the processing of EGF, the expression level of p27 in HM-SMCs was drastic and steady while it rose a little in HL-SMCs. Therefore, we speculated that in HM-SMCs, through promoting the activation of p44/42 MAPK sharply and steadily, the expression level of p27 raised sufficiently and thus ensured the cell cycle to function well[17]. But in HL-SMCs, EGF led the phosphorylation level of p44/42 MAPK or AKT to be close to 0, which possibly led to the above-mentioned occurrences happened. Eventually, the cell cycle could not function well in HL-SMCs[18–20].

Currently, there are many therapies inhibit the development of tumors through preventing the activation of EGFR[21–24]. Though we certified the role of EGF in the pathogenesis of uterine leiomyomas *in vitro*, there is a need for testifying its role *in vivo*.

In summary, EGF could not cause the activation of EGFR because of the dephosphorylation of p44/42 MAPK or AKT in HL-SMCs, which caused p27 expression insufficiently and cell cycle dysregulation.

## Conflict of interest statement

We declare that we have no conflict of interest.

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