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Roles of EGFR Signaling Pathway in Silica-induced Epithelial-mesenchymal Transition in Human A549 cells*

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ABSTRACT Objective: Silica exposure results in an initially acute lung inflammatory response followed by the proliferation of fibroblasts. The epidermal growth factor receptor (EGF-R) signaling pathway maintains a balance between cell proliferation, differentiation and apoptosis, and thus it is imagined that EGF-R signaling pathways play an important role in the development and progression of Pulmonary fibrosis. To investigate silica-induced Epithelial-mesenehymal transition (EMT) in human A549 cells and the roles of EGFR signaling pathway in silica-induced EMT in Human A549 cells in vitro. **Methods:** A549 cells were cultured and stimulated with indicated doses of silica (0, 50, 100, 200 ug/ml). The cells morphology changes were observed under phase-constrast microscope. In addition, The mRNA level of E-cadherin and a-SMA were also evaluated by Real-time PCR(qRT-PCR). The protein level of E-cadherin, a-SMA and EGFR were assessed by immunnoflurescence staining. **Results:** The data showed that silica-induced A549 cells with epithelial cell characteristics to undergo EMT in a dose-dependent manner. After exposure to silica, A549 cells induced EMT characterized by cells morphological changes, such as displayed a spindle-shape, fibroblast-like morphology. Compared with the control, the expression of E-cad mRNA and protein in silica-induced A549 cells was significantly down-regulated; The expression of a-SMA mRNA and protein in silica-induced A549 cells was significantly up-regulated. Compared with the control group, the activation of EGFR was obvious in silica-stimulated A549 cells ($P<0.05$). **Conclusion:** Silica might induce EMT in human A549 cells indirectly, and the mechanism is most likely associated with the activation of EGFR signaling pathway.

Key words: EGFR; SiO_2; A549 cell; Epithelial-mesenehymal transition

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Introduction

Silicosis is a chronic pulmonary fibrotic disease caused by the persistent aspiration of dust containing crystalline silica. The sequence of events leading to lung fibrosis involves inflammation and disruption of the normal tissue architecture, followed by tissue repair with mesenchymal cell accumulation and excess extracellular matrix (ECM) production [1]. Therefore, the proliferation of fibroblasts and fibrotic scars to replace the damaged lung tissue caused by silica-induced pathologic Processes[2,3]. Traditional theory is that alveolar macrophage is considered one of the most important target cell in silica-induced lung fibrosis. Alveolar macrophages could release a variety of fibrogenic factors and cytokines after inhaled silica particle [4,5]. There is evidence that human bronchial epithelial cell line has direct changes when exposes to silica in vitro[6-8]. The alveolar epithelial cells(AECs) through a process termed "epithelial mesenchymal transition" (EMT), has only recently received consideration to involve the process of pulmonary fibrosis [9]. In addition, the signaling pathways involved in EMT are very important fields for research in fibrotic diseases. There is an evidence than the activation of EGFR can induce dedifferentiation EMT [10]. Based on these findings, we should better understand the role of EMT in lung fibrosis and observe whether the silica-stimulated A549 cells associated with EGFR signaling pathway. Using Real-Time PCR (qRT-PCR) and immunnoflurescence staining analysis, we tested the mRNA expression of E-cad, a-SMA and the protein expression of E-cad, a-SMA, EGFR on a human A549 cell line that was exposed to silica in vitro.

1 Materials and methods

1.1 Materials

The following antibodies were used in this study: Monoclonal antibodies against E-cadherin(ZSGB-BIO, Beijing, China); Monoclonal antibodies against a-SMA (ZSGB-BIO, Beijing, China); Monoclonal antibodies against EGFR(ZSGB-BIO, Beijing, China); FITC-conjugated secondary antibodies labeled with goat anti-mouse IgG(ZSGB-BIO, Beijing, China); The fluorescent images were obtained by confocal laser scanning microscope (Olympus, BX40BXFLA3,Japan); Silica crystallization type standard (sigma company, USA, 99 % purity, particle diameter between 0.5-10 um,

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80 % between 1-5 um. Sterilized at 180 ℃ dry roasted for 6 h, with serum-free culture medium DMEM match the original liquor concentration into 3 g/L, 4 ℃ storage for later use).

1.2 Cell and cell culture

The human A549 cell line was purchased from the Typical culture preservation commission cell bank of Chinese academy of sciences (CCTCC) (Shanghai, China) and saved according to the CCTCC instructions. All cells were cultured in Dulbecco's Modified Eagle's Medium(DMEM) (Hyclone, Logan, Utah, USA) supplemented with 10 % foetal bovine serum(FBS) (Hyclone, Logan, Utah, USA) and antibiotics(100 units/ml penicillin and 100 μg/ml streptomycin). The cells were maintained at 37 ℃ in 5 % CO_2 and 95 % air. The culture medium was exchanged for fresh medium every 2 days.

1.3 Light microscope to observe the cell morphology

When the cells reached 70% -80% , the cultures were trypsinized using 0.25% trypsin/ethylenediamine tetraacetic acid (EDTA; Gibco BRL, China). For experiments cell were seeded in 6 well-plates at 1×10^6 /ml in serum-free culture medium, incubated for 24 h, and then exposed to silica at 0, 50, 100, 200 μg/ml for 48 h in duplicate wells. To observe the cell morphology and take pictures under light microscope.

1.4 Quantitative RT-PCR(qRT-PCR)

To determine the gene expression levels of E-cadherin and a-SMA, silica-stimulated (0-200 μg/ml) A549 cell were trypsinized, and the total RNA was extracted by RNAiso Plus (Takara, Japan) and quantity was assessed by absorbance at 260 and 280 nm (BEACKMAN DU640, America). The RNA was reverse transcribed into cDNA by PCR instrument(ABI 2720, America) using PrimeScript™ RT reagent Kit(Takara, Japan). Real-time PCR was performed with SYBR Green Premix Ex Taq™ II kit (Takara, Japan) using the Real-time PCR instrument(LightCycler 480Ⅱ, Germany). Total reaction mixture (25 μl) containing cDNA, SYBR Green Supermix, 10 uM forward primer and reverse primer, and Rnase free water was prepared. The following oligonucleotide 5' and 3' primer sequences were used: GAPDH-sense: 5' -ggtggtctcctctgacttcaacag-3' ;antisense: 5' -gttgctctagccaaattcgttgt-3' ; (amplification product: 179 bp); E-cadherin-sense: 5' -ctcacatttcccaactcctctc-3' ; antisense: 5' -agccatcctgtttctctttcaa-3' ; (amplification product: 224 bp); a-SMA-sense: 5' -gctgttttcccatccattgt-3' ; antisense: 5' -ctcttttgctctgtgcttcgt-3' ; (amplification product: 103 bp); The real-time PCR reaction procedures were as follows: 95 ℃ for 5 min (UDG inactivation and DNA polymerase activation), 40 PCR cycles consisting of denaturation for 30 s at 95 ℃ , annealing and extension for 30 s at 60 ℃ and primer extension at 72 ℃ for 30 s. The relative transcript abundance of the target gene is expressed in △Ct values (△Ct=Ct reference-Ct target). Relative changes in transcript levels compared with controls are expressed as △△Ct values(△△Ct=△Ct treated-△Ct control).

1.5 Immunofluorescence studies

Cells were seeded in 24 well-plates at 4×10^5/ml in serum-free culture medium, incubated for 24 h, and then exposed to silica at 0, 50, 100, 200 ug/ml for 48 h. The cells were washed three times, fixed 4% paraformaldehyde for 20 min, and then soaked in PBS containing 0.3% Triton X-100 for 10 min to increase their permeability to antibodies. After washing the cells three times for 5 min in PBS and then blocked for 1 h with 10 % goat serum in PBS. Following overnight incubation at 4 ℃ with anti-E-cadherin (1:100), anti-a-SMA (1:100),and anti-EGFR (1:200) in 10% goat serum respectively. A 1:200 dilution of FITC-labeled anti-mouse IgG was added for 1 h in the dark. The fluorescent images were obtained by fluorescence confocal microscopy (488 nm).

1.6 Statistical analysis of data

Data were analyzed with SPSS software (Version16.0 for Windows, USA) and expressed as mean± SD error of the mean (SEM), For statistical analysis, an unpaired t-test was used for pair-wise comparisons and ANOVA. P values less than 0.05 were considered as statistically significant.

2 Results

2.1 A549 cells underwent EMT phenotype changes on exposure to silica

In this study, after exposure to silica(50-200 μg/ml) for 48 h, we found obvious changes in the cell morphology of the A549 cells compared with the control group(0 ug/ml), especially in high silica dose group (200 μg/ml). All cells changed to the mesenchymal phenotype, as evidenced by an elongated appearance, irregular shape, spindle, pseudopodia, and loose of cell-cell junctions(Fig. 1).

2.2 Silica influenced the mRNA and protein expression of E-cadherin and a-SMA in A549 cells

Apart from changes in the cell morphology, the changes in the mRNA expression of some EMT markers, such as E-cadherin and a-SMA, are also used to ensure the mesenchymal phenotype. Following stimulation by silica (50-200 μg/ml) for 48 h, we tested two typical molecular markers of A549 cells through Real-time P CR. The results show that the epithelial E-cadherin was markedly down-regulated (0.837 ± 0.119, 0.478 ± 0.062, 0.223 ± 0.044, $F=146.877$, $P<0.05$), and the mesenchymal marker a-SMA was markedly up-regulated (2.123 ± 0.167, 2.817 ± 0.128, 4.142 ± 0.149, $F=42.996$, $P<0.05$) compared to the untreated A549 cells (Fig. 2, $P<0.05$). Furthermore, the results of immunofluorescence reveal similar results, with A549-EMT cells demonstrating an almost complete absence of the green E-cadherin expression, while the green a-SMA and EGFR epression was much greater than untreated A549 cells(Fig. 3).

3 Discussion

Many recent studies have focused on the ability of alveolar macrophages when expouse to silica. Ingestion of silica by alveo-

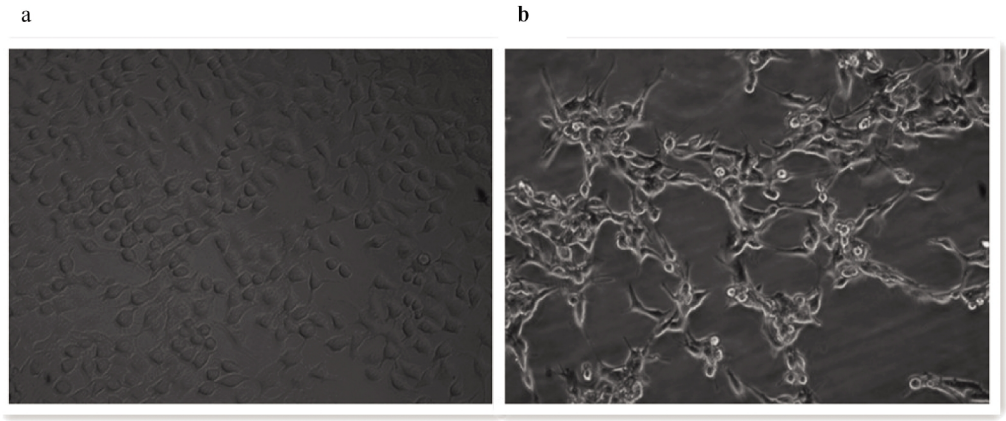

Fig. 1 Effects of silica on cell morphology in A549 cells. Light microscope showing A549 cells appeared to have a spindle morphology after the stimulation of silica(b) compared with the control group(a) (magnification =200×)

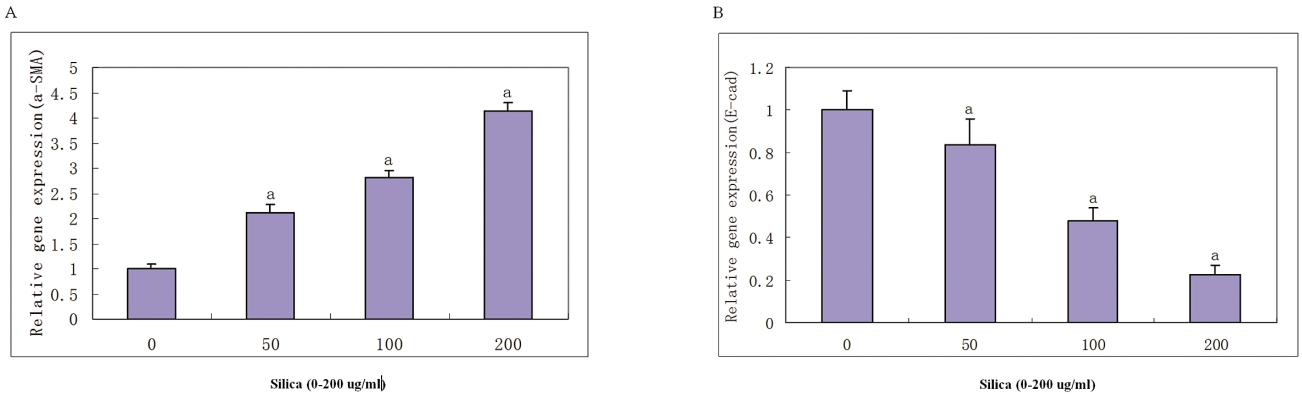

Fig. 2 E-cad and a-SMA gene expression in EMT by Real-time PCR. A: Expression of a-SMA, which is a mesenchymal maker, is up-regulated after silica stimulated(Compared with the control group, $^{a}P<0.05$). B: the epithelial E-cad was markedly down-regulated (Compared with the control group, $^{a}P<0.05$)

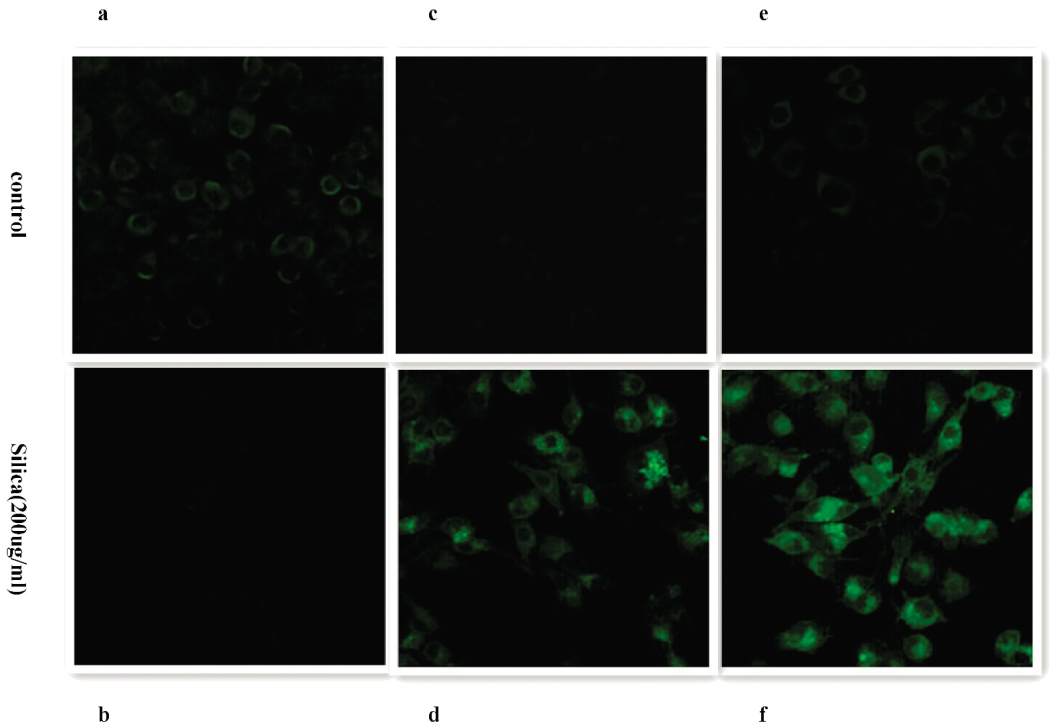

Fig. 3 Immunofluorescence detection of E-cad(a,b), a-SMA(c,d) and EGFR(e,f) was performed after silica(200 ug/ml) induced for 48 h (magnification =200×)

lar macrophages leads to cell activated, and release all kinds of inflammatory cytokines, which involved in the process of pulmonary fibrosis [11, 12]. Some scholars believe that inflammation is neither necessary nor sufficient to induce fibrosis. The origin of the primary effector cell of fibrosis in the lung, the myofibroblast, is not clearly established[9].

EMT is a process by which fully differentiated epithelial cells undergo phenotypic transition to fully differentiated mesenchymal cells, often fibroblasts and myofibroblasts [13]. It is clear that the well-described phenomenon of epithelial-mesenchymal transition (EMT) plays a pivotal role in embryonic development, wound healing, tissue regeneration, organ fibrosis and cancer progression. EMT is characterized by loss of epithelial markers E-cad and transition to a spindle-shaped morphology concomitant with expression of three mesenchymal markers, α-SMA, Vim and FN [14, 15]. In recent years, alveolar epithelial cells (AECs) have been viewed as one of the key participants in fibrosis. AECs themselves may acquire a mesenchymal phenotype and serve as an important source of fibroblasts and myofibroblasts through a process of EMT[13]. But whether SiO_2 as a special kind of fibrosis damage lung epithelial cells and lead to EMT, which the specific mechanism is still unclear. One key piece of study found that the activation of epidermal growth factor(EGF) receptor signalng pathway not only stimulate epithelial to mesenchymal cells, down-regulate the expression of E-cad, but differentiation of mesenchymal cells can extend survival time, promote the level of pulmonary fibrosis[16]. The epidermal growth factor receptor (EGF-R) is a 170-kd, 1186-amino acid long transmembrane receptor belonging to a family of receptor tyrosine kinases that includes, in addition to EGFR, three other members (ErbB2/HER-2, ErbB3/HER-3, and ErbB4/HER-4)[17-19]. EGFR is over-expressed in a wide variety of human carcinomas, including non-small cell lung, breast, head and neck, bladder, ovarian and prostate cancer [20,21]. The activation of ErbB receptor, generally need a number of ligand bind to EGFR such as EGF, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC) and transforming growth factor-alpha (TGF-α) [22-25]. Activation of EGFR can induce dedifferentiation EMT which is accompanied by over-expression of mesenchymal markers such as vimentin, fibronectin and N-cadherin[10]. Some researches have found that the activation of ErbB signaling pathway can result in increased activity of transcriptional repressors, such as Snail, Zeb, and Twist, which repress expression of cell adhesion molecules like E-cadherin[26].

In this report, the epithelial and mesenchymal characteristics of silica-induced A549 cell lines were examined by Real-time PCR and Immunofluorescence. It is clear that silica can result in the down-regulated expression of E-cad and the up-regulated expression of α -SMA in a dose-dependent manner, thus indicating a transition from an epithelial phenotype to a mesenchymal phenotype. This research has also shown that the signs of myofibroblast-like differentiation under a light microscope, such as the appearance of irregular shape, spindle, pseudopodia, and loose of cell-cell junctions. On the basis of this experiment, the key signaling events underlying silica-induced EMT has also been identified. It is obvious that EGFR activity increased significantly in silica-treated A549 cells by Real-time PCR and Immunofluorescence. Therefore, under the conditions of in vitro, lung epithelial cells may directly induce EMT after exposed to silica, then mesenchymal cells increased and its mechanism may be associated with EGFR signaling pathway. But the specific mechanism of EGFR influence pulmonary fibrosis is still not very clear.

In conclusion, this study preliminarily shows lung epithelial cell is an important target cell in the process of pulmonary fibrosis. Silica can directly stimulate the lung epithelial cells or by stimulating alveolar macrophages, and release a series of active cytokines, led to the epithelial phenotype to a mesenchymal phenotype. The signal pathway may be associated with EGFR. Therefore, the research to explore the intervention of EMT or some targeted drugs that regulate the activity of EGFR may become a new way for the treatment of pulmonary fibrosis.

References

[1] Nkyimbeng T, Ruppert C, Shiomi T, et al. Pivotal role of matrix metalloproteinase 13 in extracellular matrix turnover in idiopathic pulmonary fibrosis[J]. PLoS One, 2013, 8(9): 1-14

[2] Vietti G, Ibouraadaten S, Palmai-Pallag M, et al. Towards predicting the lung fibrogenic activity of nanomaterials: experimental validation of an in vitro fibroblast proliferation assay [J]. Part Fibre Toxicol, 2013, 10(1): 52-66

[3] Barbarin V, Arras M, Misson P, et al. Characterization of the effect of interleukin-10 on silica-induced lung fibrosis in mice[J]. Am J Respir Cell Mol Biol, 2004, 31(1): 78-85

[4] Driscoll KE, Lindenschmidt RC, Maurer JK, et al. Pulmonary response to silica or titanium dioxide: inflammatory cells, alveolar macrophage-derived cytokines, and histopathology [J].Am J Respir Cell Mol Biol, 1990, 2(4): 381-390

[5] Orfila C, Lepert JC, Gossart S, et al. Immunocytochemical characterization of lung macrophage surface phenotypes and expression of cytokines in acute experimental silicosis in mice [J]. Histochem J, 1998, 30(12): 857-867

[6] Magnan A, Frachon I, Rain B, et al. Transforming growth factor beta in normal human lung: preferential location in bronchial epithelial cells[J]. Thorax, 1994, 49(8): 789-792

[7] Levine SJ. Bronchial epithelial cell-cytokine interactions in airway inflammation[J]. J Invest Med, 1995, 43(3): 241-249

[8] Maria Bodo, Tiziano Baroni, Silvia Bellocchio, et al. Bronchial Epithelial Cell Matrix Production in Response to Silica and Basic Fibroblast Growth Factor[J]. Molecular Medicine, 2001, 7(2): 83-92

[9] Willis BC, duBois RM, Borok Z. Epithelial origin of myofibroblasts during fibrosis in the lung [J]. Proc Am Thorac Soc, 2006, 3 (4): 337-382

[10] Ala-Eddin Al Moustafa, Amal Achkhar, Amber Yasmeen. EGF-receptor signaling and epithelial-mesenchymal transition in human

carcinomas[J]. Frontiers in Bioscience S4, 2012, 1(1): 671-684

[11] Mossman BT, Churg A. Mechanisms in the pathogenesis of asbestosis and silicosis[J]. Am J Respir Crit Care Med, 1998, 157(5 Pt 1): 1666-1680

[12] Boucherat O, Boczkowski J, Jeannotte L, et al. Cellular and molecular mechanisms of goblet cell metaplasia in the respiratory airways[J]. Exp Lung Res, 2013, 39(4-5): 207-216

[13] Kage H, Borok Z. EMT and interstitial lung disease: a mysterious relationship[J]. Wolters Kluwer Health, 2012, 18(5): 517-523

[14] Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition[J]. Cell Res, 2009, 19(2): 156-172

[15] Tuo Y, Miaomiao C, Tieying S. Simvastatin Attenuates TGF-β 1-Induced Epithelial-Mesenchymal Transition in Human Alveolar Epithelial Cells[J]. Cell Physiol Biochem, 2013, 31(6): 863-874

[16] Docherty NG, O'Sullivan OE, Healy DA, et al. TGF-beta1-induced EMT can occur independently of its proapoptotic effects and is aided by EGF receptor activation[J]. Am J Physiol Renal Physiol, 2006, 290 (5): 1202-1212

[17] Burgess AW. EGFR family: structure physiology signalling and therapeutic targets[J]. Growth Factors, 2008, 26(5): 263-274

[18] Arkhipov A, Shan Y, Kim ET, et al. Her2 activation mechanism reflects evolutionary preservation of asymmetric ectodomain dimers in the human EGFR family[J]. Elife, 2013, 7(2): 1-14

[19] Sibilia M, Kroismayr R, Kroismayr BM, et al. The epidermal growth factor receptor: from development to tumorigenesis [J]. Differentiation, 2007, 75(9): 770-787

[20] Grandal MV, Madshus IH. Epidermal growth factor receptor and cancer: control of oncogenic signalling by endocytosis [J]. J Cell Mol Med, 2008, 12(5A): 1527-1534

[21] Mitsudomi T, Yatabe Y. Epidermal growth factor receptor in relation to tumor development: EGFR gene and cancer[J]. FEBS J, 2010, 277 (2): 301-308

[22] Harari D, Tzahar E, Romano J, et al. Neuregulin-4:a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase [J]. Oncogene, 1999, 18(17): 2681-2689

[23] Di Marco E, Pierce JH, Fleming TP, et al. Autocrine interaction between TGF alpha and the EGF-receptor: quantitative requirements for induction of the malignant phenotype [J]. Oncogene, 1989, 4(7): 831-838

[24] Carraway KL 3rd, Weber JL, Unger MJ, et al. Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases [J]. Nature, 1997, 387(6632): 512-516

[25] Fuller SJ, Sivarajah K, Sugden PH. ErbB receptors, their ligands, and the consequences of their activation and inhibition in the myocardium [J]. J Mol Cell Cardiol, 2008, 44(5): 831-854

[26] Hugo HJ, Wafai R, Blick T, et al. Staurosporine augments EGF-mediated EMT in PMC42-LA cells through actin depolymerisation, focal contact size reduction and Snail1 induction-a model for cross-modulation[J]. BMC Cancer, 2009, 15(9): 235-250

EGFR 信号通路在 SiO_2 诱导肺上皮细胞间质转化中的作用机制 *

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摘要 目的：在二氧化硅（SiO_2）刺激下可引起肺部一系列的炎症反应及其伴随相关的成纤维细胞增殖，然而 EGFR 信号通路可维持细胞增殖、分化和凋亡的平衡，因此，我们可以设想 EGFR 信号通路是否在肺纤维化的发生发展中起到重要的作用。本实验探讨 SiO_2 是否能诱导人肺上皮细胞（A549）发生上皮间质转化，并且研究 EGFR 信号通路在矽肺纤维化中的作用机制。**方法：**以 A549 为研究对象，用 0（对照组）、50、100、200 μg/ml SiO_2 孵育 A549，作用 48h 后于倒置显微镜观察细胞形态学改变，并收集不同时段细胞，采用实时荧光定量 PCR（RT-PCR）检测 E- 钙黏蛋白(E-cadherin) 和 α- 平滑肌肌动蛋白(α-SMA) mRNA 表达变化，细胞免疫荧光方法检测 E-cadherin、α-SMA 及信号转导蛋白 EGFR 表达的变化。**结果：**倒置显微镜观察 A549 经 SiO_2 处理后细胞形态由鹅卵石状转变为纺锤型或梭型，形态似成纤维细胞，随着 SiO_2 浓度的升高，E-cad mRNA 和蛋白表达逐渐下调，在 200 μg /ml 组表达最低，α-SMA mRNA 和蛋白表达逐渐上调，200 μg /ml 组 α-SMA 表达最高；EGFR 蛋白表达上调；50、100、200 μg/ml 与对照组的差异具有统计学学意义（$P<0.05$）。**结论：**SiO_2 可诱导肺上皮细胞向间质细胞转化，其机制可能与 EGFR 信号通路有关。

关键词：表皮生长因子受体；矽尘；A549 细胞；上皮间质转化

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