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表皮生長因子感受器負向調控成骨細胞分化的機制

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中文摘要：

這個實驗的目的是要探討表皮生長因子感受器如何來負向調控成骨細胞分化的機轉, 透過檢視表皮生長因子感受器在成骨細胞分化時期的訊息傳遞, 我們選用 MC3T3-E1 成骨前驅細胞和 ROS17/2.8 成骨細胞來檢測那一種 MAPK 訊息路徑扮演負向調控成骨細胞分化的角色, 並使用兩種特效的 kinase 抑制因子(U0126 和 SB203580)來分別審察 MAPK 訊息路徑, 包括 Erk42/44 和 p38 MAPK 在調控成骨細胞分化時所扮演的角色。

這個研究所獲得的資訊將使我們更明瞭表皮生長因子感受器調控的 Ras-MAPK 訊息傳遞路徑在成骨細胞分化過程中的角色, 同時也能更清楚的了解這個感受器是如何來負向調控成骨前驅細胞分化的機轉, 並經由調控成骨細胞分化的機轉和成骨前驅細胞如何的保持其未分化的表現型態, 期望在不久的將來能發展出以分子為基礎的治療策略, 透過成骨前驅細胞的生長周期, 來不斷的分裂增殖補充成骨前驅細胞的數量和更進一步的增強骨形成的能力。

背景及目的：

It is well known that osteoblastic differentiation and activities are regulated by numerous systemic hormones (reviewed in Martin *et al.,* 1987) and locally produced cytokines as well as growth factors (Krane *et al.,* 1988; Canalis *et al.,* 1989). The analysis of their effects on osteoblastic differentiation *in vivo* therefore has been very difficult to achieve (Nijweide *et al.,* 1988; Aubin *et al.,* 1993; Aubin and Liu 1996). Although the cell culture system has been extremely useful in the understanding of their effects and has generally generated more meaningful data, the outcomes from these studies have often been variable due partly to the marked heterogeneity of osteogenic cell populations in primary cultures in particular and also to the disparate stages of differentiation of the target osteogenic cell (Aubin *et al.,* 1993). Another major problem is the inability to isolate osteogenic cells at different stages of osteoblastic differentiation (Aubin *et al.,* 1993). Consequently, the selection and use of appropriate cell lines have been a very critical element in the understanding of the function of EGF-R. In this study, we have used two well-characterized osteogenic cell

lines, MC3T3-E1 and ROS17/2.8 cells that possess all the characteristics of preosteoblastic cells and fully differentiated osteoblast-like cells, respectively. MC3T3-E1 cells have numerous high affinity EGF binding sites (Uneno *et al.,* 1989), but do not express osteoblast-specific markers, although they synthesize collagen type I and osteonectin (Kodama *et al.,* 1982; Quarles *et al.,* 1992). On the other hand, as they differentiated into osteoblasts, they lost their ability to express EGF-R, and started to produce osteoblast markers. In other word, MC3T3-E1 cells are capable of differentiation into osteoblasts and formation of mineralized bone matrix when grown in the presence of β-glycerophosphate and ascorbic acid, and thus lost the ability to express EGF-R (Chien *et al.,* 2000). Therefore, MC3T3-E1 cells may serve as a useful model to study the function of EGF-R and the signaling cascades in osteoblastic differentiation since these cells display a time-dependent and sequential expression of osteoblast characteristics similar to *in vivo* bone formation. Conversely, ROS17/2.8 cells, which possess the characteristics similar to those of fully differentiated osteoblasts, expressed few EGF-R (Matsuda *et al.,* 1993), but high levels of osteoblast markers that are characteristics of mature osteoblasts (Majeska *et al.,* 1978; McCabe *et al.,* 1994; Chien *et al.,* 2000). Therefore, ROS17/2.8 cell is a good model for transfection analysis of Ras protein in this study. These cells will be transfected with a constitutively active form of Ras gene (pSV/V_{12} Ras) to determine if activation of Ras abrogates the ability of cells to express biochemical markers of mature osteoblast as a result of activation of MAP kinase activity.

The earlier studies by Cho *et al.* (1988 and 1991) demonstrated the expression of numerous EGF receptors on preosteoblasts and prechondrocytes, a decrease in number during their differentiation, and a complete loss in fully differentiated osteoblasts and chondrocytes. The role of the EGF-R as a negative regulator in osteoblastic cell differentiation was recently explored by our laboratory using preosteoblastic MC3T3-E1 cells and osteoblastic-like ROS 17/2.8 cells. However, the signal transduction pathways of EGF-R in osteogenic cell growth and differentiation remain largely unknown. Recently, the Ras-MAP kinase pathway was proposed to be involved in both proliferation and differentiation of osteoblasts (Suzuki *et al.,* 2002). Currently, there are three major subfamilies of structurally related MAP kinases that have been identified in mammalian cells: the extracellular signal regulated kinase (Erk); the c-Jun N-terminal kinase (JNKs); and the p38 MAP kinase. In osteoblast-like cells, activation of Ras-Erk signaling seems to play an essential role in cell replication, whereas p38 MAP kinase is involved in the regulation of osteogenic cell differentiation (Suzuki *et al.,* 2002).

In the first year of this study, we have successfully determined which MAP kinases are involved during normal osteoblast development and which MAP kinases

are activated by EGF in down-regulation of osteogenic cell differentiation. We have used murine calvarial-derived MC3T3-E1 cells as an *in vitro* model to study EGF-R mediated signal transduction during osteoblastic differentiation by immunoblotting analysis. Furthermore, we have analyzed the role of Erk and p38 MAP kinase in mediating osteoblastic differentiation using specific kinase inhibitors (U0126 and SB203580). The roles of the Erk and p38 pathways were investigated with the kinase inhibitors U0126 and SB203580, respectively.

The purpose of this study is to examine the mechanisms by which EGF-R functions as a negative regulator in osteoblastic differentiation. The mechanisms were elucidated by investigation of EGF-R signaling transduction pathways during osteoblastic cell differentiation, and further by examination of the relationships between EGF-R signaling and the expression of Cbfa1 transcription factor in osteoblastic cells.

材料與方法：

Cell Culture:

(A). MC3T3-E1 cells, a clonal preosteoblastic cell line originated from newborn mouse calvaria (purchased from Riken Gene Bank, Tsukuba Science City, Japan), were plated at 7 x 10^5 cells/60 mm dish and grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (GIBCO/BRL Gland Island, NY, USA). Cultures will be grown at 37 °C in a humidified atmosphere of 5% CO_2 until further uses. When grown in the presence of β-glycerophosphate (GP) and ascorbic acid (AA), MC3T3-E1 cells are able to differentiate into osteoblasts and form mineralized bone matrix within 21 days (Chien *et al.,* 2000). Therefore, MC3T3-E1 cells may serve as a useful *in vitro* model of osteoblast development since these cells display a time-dependent and sequential expression of osteoblast characteristics similar to *in vivo* bone formation. To assess the effect of EGF on cell differentiation, MC3T3-E1 cells were cultured on 60-mm culture dishes (Becton Dickson Laboratory) with 10 mM GP and 50 μg/ml of AA in the presence or absence of 50 ng/ml EGF (Sigma, USA) for various time intervals.

(B). ROS17/2.8 cells (a gift kindly provided by Dr. L.T. Hou, National Taiwan University, Taiwan), a clonal rat osteosarcoma cells, were plated at 7 x 10^5 cells/60 mm dish and grown in DMEM medium supplemented with 10% fetal bovine serum and antibiotics. Cultures were grown at 37 °C in a humidified atmosphere of 5% CO_2 until further uses. To assess the effect of EGF on activation of MAPK, ROS17/2.8 cells were cultured on 60-mm

culture dishes with 10 mM GP and 50 μg/ml of AA in the presence or absence of 50 ng/ml EGF for various time intervals.

(C). To determine the effects of stimulatory dose of EGF on osteoblastic differentiation, MC3T3-E1 and ROS17/2.8 cells were incubated together with GP, AA and EGF (50ng/ml) for up to 8 hours. The effects of EGF on the activation of Erk1/2 or p38 proteins were investigated by immunoblotting analysis. To minimize the effects of growth factor in serum, cells were serum-starved for 12 hours before addition of the fresh culture medium. In order to clarify the involvement of Erk1/2 or p38 in the regulation of EGF-R mediated down-regulation of osteoblastic cell differentiation, the effects of the correspondent inhibitor U0126 or SB203580 were examined. In experiment aims at testing the effects of the MAPK inhibitors, either 50 μM of U0126 or SB203580 was added 1 hour prior to addition of EGF. A 20 mM stock solution of U0126 and SB203580 were prepared by dissolving these inhibitors in DMSO. Control incubations contain DMSO instead of U0126 or SB203580.

(D). Preparation of cell extracts and Western blot analysis.

After treatment, the cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) for three times, scraped and collected by centrifugation at 1000 x g for 10 min. Whole cell lysates were obtained by solubilizing the cells in lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupetin, 2 mM Na_3VO_4, 0.01 μM calyculin A, 0.1 μM mycrocystin LR, 1% NP-40, 1% sodium deoxycholate, and 1% SDS] for 1 hour at 4 °C as described previously (Suzuki *et al.*, 2002). The cell lysate were cleared by centrifugation at 6000 rpm for 30 min at 4℃. For immunoblotting analyses, an aliquot of the supernatant (30 μg protein) will be denatured and subjected to 12% SDS-PAGE gel. Proteins were then transferred to nitrocellulose membrane by electroblotting. For blocking unoccupied binding sites the membrane was incubated in Tris-buffered saline containing 0.1% Tween-20 with 1% BSA or 5% nonfat dry milk powder at room temperature for 1 hour. The phosphorylation of Erk1/2 and p38 was identified and quantified by immunoblotting analysis using anti-phospho-Erk1/2 and anti-phospho-p38, antibodies (New England Biolabs, USA) with appropriate dilution for 1 hour in room temperature or at 4℃ overnight with gentle motion. Polyclonal anti-Erk1/2 and anti-p38 antibodies (Santa Cruz Biotechnology, Inc., USA) were used to detect total amounts of these kinases. The membrane was then washes four times followed by incubation of the membrane with secondary

horseradish peroxidase-labeled secondary antibodies for 1 hour according to the manufacturer's instruction (Amersham Corp., USA). After washing the membrane four times, the immunoreactive bands were detected with enhanced chemoluminescence (ECL) Western blotting system (Amersham). For incubation with another antibody, the membrane was stripped by incubating in 62.5 mM Tris-HCl; pH6.7, 100 mM 2-mercaptoethanol, 2% SDS for 30 minutes at 50℃ and incubated in blocking buffer for 1 hour before adding the new antibodies.

結果與討論：

1. **EGF and GP+AA down-regulated Erk1/2 kinase activity in preosteoblastic MC3T3-E1 cells:** To assess the effect of EGF on the activation of Erk1/2 and p38, MC3T3-E1 cells were cultured on 60-mm culture dishes with 10 mM GP and 50 μg/ml of AA in the presence or absence of 50 ng/ml EGF for various time intervals. Cell lysates were analyzed for Erk1/2 and p38 activation using anti-phospho-specific Erk1/2 and anti-phospho-specific p38 antibodies, respectively. The ECL Western blot illustrated that EGF down-regulates the phosphorylation of Erk1/2 as indicated in Fig 1A (compare lane 1 with lane 2 and 4). The down-regulation effect of EGF on the Erk1/2 phosphorylation was completely abolished by the addition of the specific inhibitor U0126 (as shown in Fig 1A; compare lane 1 with lane 3 and 5). However, EGF did not induce the p38 activity in MC3T3-E1 cells as shown in Fig 1C. Our result suggests that the regulation of osteoblastic growth or differentiation by EGF may be associated with the declination of Erk1/2 phosphorylation, and not related to the p38.
2. **The Erk1/2 kinase activity was not influenced by EGF and GP+AA in mature osteoblast ROS17/2.8 cells:** To assess the effect of EGF on the activation of Erk1/2 and p38, ROS17/2.8 cells were cultured on 60-mm culture dishes with 10 mM GP and 50 μg/ml of AA in the presence or absence of 50 ng/ml EGF for various time intervals. Cell lysates were analyzed for Erk1/2 and p38 activation using anti-phospho-specific Erk1/2 and anti-phospho-specific p38 antibodies, respectively. EGF did not influence the Erk1/2 phosphorylation as indicated in Fig 2A (compare lane 1 with lane 2, 4 and 6). This result is in agreement with our previous finding that EGF receptors are not expressed on the mature osteoblast ROS17/2.8 cells, even with the stimulation of EGF. On the other hand, EGF did not induce the p38 activity in ROS17/2.8 cells as shown in Fig 2C. Our result shows that EGF does not change the phosphorylation of Erk1/2 in mature osteoblast, and is not able to activate the phosphorylation of p38 protein.
3. We have demonstrated that Erk1/2 signal transduction pathway is down-regulated

by the EGF in preosteoblast MC3T3-E1 cells; however, this pathway is not affected by EGF in mature osteoblast ROS17/2.8 cells. Results from the present study suggest that the mechanism of EGF on the down-regulation of osteoblastic cell differentiation is possibly mediated by the inactivation of the Erk1/2 pathway.

未來研究方向：

We will next investigate the roles of Erk and p38 pathways in controlling bone cell differentiation by analyzing the Erk and p38 at various stage of MC3T3-E1 cell differentiation. We will also examine the effects of the kinase specific inhibitors (U0126 and SB203580) on the expression of phenotypic markers of osteoblast. The osteoblastic phenotype will be characterized by examining the ability of cells to express mRNAs for type I collagen, alkaline phosphatase, bone sialoprotein, osteonectin and osteocalcin using RT-PCR analysis.

In order to better understand the role of Ras/MAPK in regulation of osteoblastic differentiation, we select ROS17/2.8 cells as an experimental cell line for transfection of these cells with a constitutively active form of Ras gene (pSV/V_{12} Ras). Currently, the Ras gene (pSV/V_{12} Ras) was successfully transfected into ROS17/2.8 cells in our laboratory. Expression of the transfected Ras gene and downstream components Erk1/2 will be validated by immunoblotting analysis. The mRNAs expression for osteoblast phenotype-related genes in these successfully transfected cells will be analyzed by RT-PCR. The U0126 inhibitor will also be used to test the role of Erk in the Ras transfected ROS17/2.8 cells.

The proposed study will help to better understand the role of EGF receptor in maintenance of bone cell phenotype and in control of self-renewal of osteoprogenitor cells. By understanding the regulatory mechanisms of osteoblastic differentiation and how progenitor cells remain as an undifferentiated phenotype, we may develop molecular based strategies to recruit and promote mitogenic growth of osteoprogenitor cells via the osteoprogenitor cell cycle, and further increase bone-forming activity.

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A.

Phospho p42/44 (Erk1/2)

B.

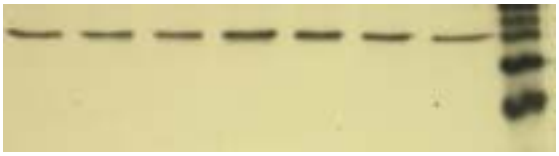
P42/44 (Erk1/2)

EGF	-	+	+	+	+	+	+	M
U0126	-	-	+	-	+	-	+	
Time (h)	0	1	1	2	2	4	4	

C.

Phospho-p38 MAP Kinase

D.



p38 MAP Kinase

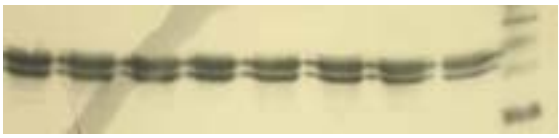
EGF	-	+	+	+	+	+	+	M
SB203580	-	-	+	-	+	-	+	
Time (h)	0	1	1	2	2	4	4	

Fig 1: EGF induces the activation of Erk but not p38 MAP kinase pathways in MC3T3-E1 cells. MC3T3-E1 cells were stimulated with EGF (50ng/ml) for different time intervals and cell lysates were subjected to 12% SDS-PAGE against each specific MAP kinase antibodies. The results are representative of triplicate independent experiments.

A.

Phospho p42/44 (Erk1/2)

B.



P42/44 (Erk1/2)

EGF	-	+	+	+	+	+	+	+	M
U0126	-	-	+	-	+	-	+	-	
Time (h)	0	1	1	2	2	4	4	8	

C.

Phospho-p38 MAP Kinase

D.

p38 MAP Kinase

EGF	-	+	+	+	+	+	+	+	M
SB203580	-	-	+	-	+	-	+	-	
Time (h)	0	1	1	2	2	4	4	8	

Fig 2: EGF induces the activation of Erk but not p38 MAP kinase pathways in ROS17/2.8 cells. ROS17/2.8 cells were stimulated with EGF (50ng/ml) for different time intervals and cell lysates were subjected to 12% SDS-PAGE against each specific MAP kinase antibodies. The results are representative of triplicate independent experiments.