

Primary research

## Protein kinase ERK contributes to differential responsiveness of human myeloma cell lines to IFN $\alpha$

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**Keywords:** IFN $\alpha$ , myeloma, ERK, STAT

### Abstract

**Background:** Despite IFN $\alpha$  has been used extensively in the treatment of multiple myeloma (MM), there are also several reports suggesting that IFN $\alpha$  may aggravate disease in some MM patients. That means the effect of IFN $\alpha$  on the growth of myeloma cells in vivo may be different. In this study, we selected two human myeloma cell lines that vary remarkably in response to IFN $\alpha$  and focused on elucidating the mechanism of differential IFN $\alpha$  responsiveness.

**Results:** Sko-007 is a myeloma cell line whose growth is arrested by IFN $\alpha$ ; however, IFN $\alpha$  promoted the proliferation of the other myeloma cell line U266. We observed that the growth-stimulation effect of IFN $\alpha$  on U266 cells did not result from up-regulation of the IL-6 receptors on cell surface; while IFN $\alpha$  treatment on Sko-007 cells significantly reduced gp130 expression. Moreover, the transcription factors STAT3 and STAT1, which are involved in the JAK/STAT signal transduction pathway, can be activated in both IFN $\alpha$ -stimulated and -inhibited myeloma cell lines; while the activation of the protein kinase ERK, which is involved in the Ras/MAPK signal transduction pathway, can be down-regulated in IFN $\alpha$ -arrested Sko-007 cells and up-regulated in IFN $\alpha$ -stimulated U266 cells. In addition, both IFN $\alpha$ -induced growth-stimulation effect and the up-regulated activation of ERK in U266 cells were efficiently inhibited by PD98059, the specific inhibitor of MAPK/ERK kinase (MEK).

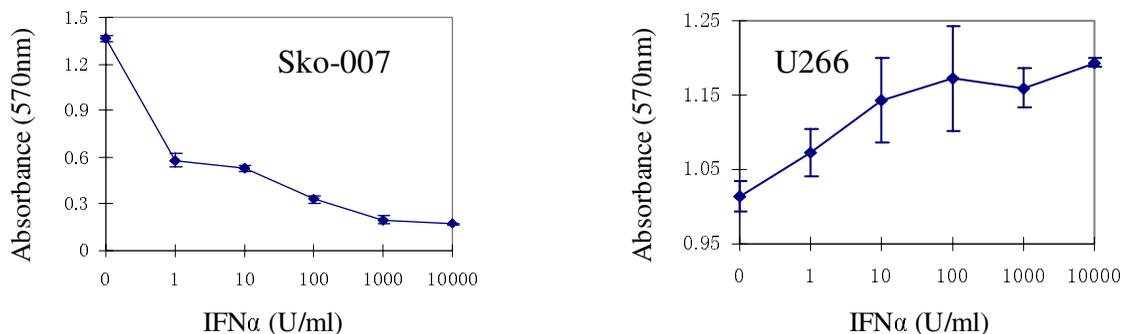
**Conclusion:** Myeloma cells responsiveness to IFN $\alpha$  is heterogeneous and the activation state of ERK in the Ras/MAPK signalling pathway mainly contributed to this difference.

### Background

The typically growth-inhibitory action of Interferon $\alpha$  (IFN $\alpha$ ) has made it a commonly used therapeutic agent in the treatment of a wide variety of human malignancies, including multiple myeloma (MM) [1]. However, although a considerable number of the clinical trials have addressed the effectiveness of IFN $\alpha$  on MM therapy [2], other reports also showed that in some MM patients,

IFN $\alpha$  can promote the proliferation of myeloma cells in vivo and result in the development of aggressive plasma cell leukemia (PCL) [3]. Therefore, the action of IFN $\alpha$  on MM cell growth is a matter of debate, and the mechanism of this discrepancy is the topic of this study.

Interleukin-6 (IL-6) is the major growth and survival factor for myeloma cells. IFN $\alpha$  and IL-6 clearly use distinct



**Figure 1**

**Effect of IFN $\alpha$  on the growth of Sko-0007 and U266 cells.**  $2 \times 10^3$  Sko-0007 or U266 cells/well were cultured in 96-well tissue culture plates in the absence or presence of increasing concentrations of IFN $\alpha$ . After incubation for 48 h at 37 $^\circ$ , MTT was mixed into each well and the incubation was prolonged for another 6 h, then the dark blue crystal were dissolved in 100  $\mu$ l of 10% SDS in 0.01 N HCl. Absorbance was measured at 570 nm.

receptor complexes; however, both cytokines have been shown to use two common signal transduction pathways to mediate their biological activities. One is the janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway which results to the activation of the protein tyrosine kinases JAKs and the transcription factors STAT3 and STAT1; the other is the Ras-dependent mitogen-activated protein kinase (Ras/MAPK) pathway which involves the subsequent activation of a series of serine/threonine kinases including extracellular signal-regulated kinase (ERK) [4,5]. In the previous report, we have investigated in detail the mechanism of IL-6 signal transduction in different human myeloma cell lines [6]. In this study, we analyzed IFN $\alpha$ -induced activation of STAT3, STAT1 and ERK in Sko-007 and U266 human myeloma cell lines, which displayed overall different response to IFN $\alpha$ . And we demonstrated the evidence that the activation state of the Ras/MAPK in stead of the JAK/STAT signal transduction pathway accounted mainly for the differential effect of IFN $\alpha$  on myeloma cells.

## Results

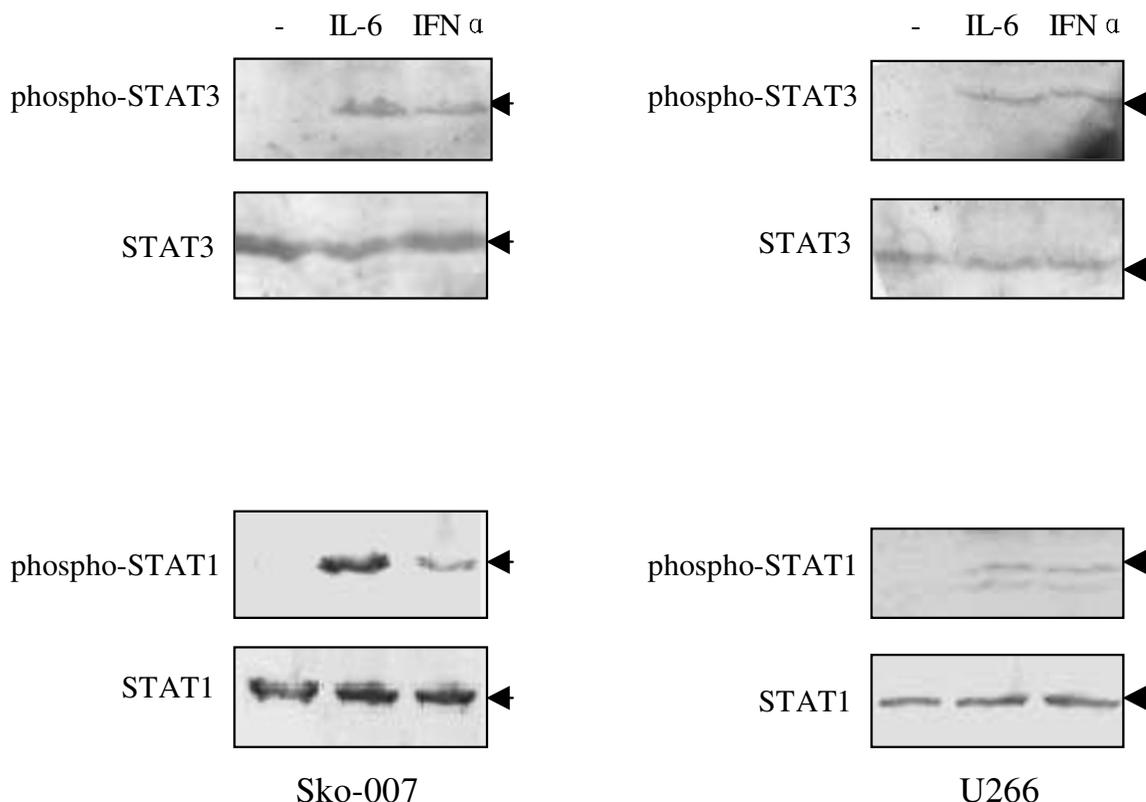
### **Myeloma cell lines are heterogeneous in response to IFN $\alpha$**

To determine the effect of IFN $\alpha$  on the growth of myeloma cells, MTT assay was firstly performed on four human myeloma cell lines-Sko-007, U266, KM-3 and XG-7 [7]. The growth of three myeloma cell lines (Sko-007, KM-3 and XG-7) were significantly inhibited in the presence of IFN $\alpha$ ; whereas IFN $\alpha$  moderately promoted the proliferation of the fourth myeloma cell line U266. Fig. 1 displayed a concentration-dependent growth-inhibition and -stimulation effect of IFN $\alpha$  on Sko-007 and U266 cells, respec-

tively. This result supported the conclusion that there are two distinct patterns of IFN $\alpha$  response in human myeloma cells.

### **Effect of IFN $\alpha$ on the expression of two IL-6 receptor chains on myeloma cell surface**

IL-6 is the major growth factor for myeloma cells. It has been reported that the effect of many other growth-stimulating or growth-inhibitory factors on myeloma cells is a secondary consequence that is mediated by the regulation on the expression or the activity of IL-6 or its receptors [4]. To determine whether a similar mechanism accounted for the different effect of IFN $\alpha$  on Sko-007 and U266 cells, IFN $\alpha$ -treated and untreated cells were incubated respectively with the specific antibodies to the two IL-6 receptor chains, gp80 (IL-6R) and gp130, then FACS analysis was performed. As shown in Table 1, the expression level (represented by both the average strength of fluorescence on cell surface and the percentage of the positive cells) of gp130 was down-regulated significantly in Sko-007 cells with the treatment of IFN $\alpha$  for 48 h, while there was no obvious changes on IL-6R level at the same conditions. This result indicated that growth-inhibition effect of IFN $\alpha$  on Sko-007 cells was partially mediated by down-regulated expression of gp130 on cell surface. In contrast, growth-stimulation of U266 cells in the presence of IFN $\alpha$  did not involve any induction of IL-6R and gp130 expression. Taken these results together, we concluded that IFN $\alpha$ -induced regulation of IL-6 receptors expression on cell surface is not a common mechanism to mediate the effect of IFN $\alpha$  on myeloma cells.

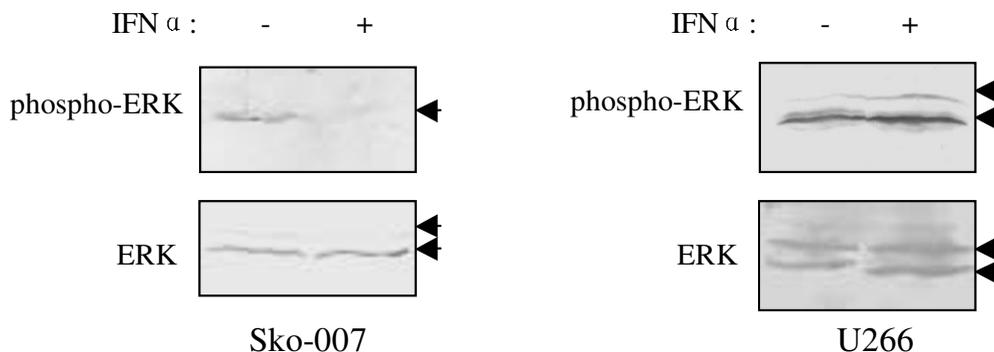


**Figure 2**  
**Activation states of STAT3 and STAT1 in Sko-007 and U266 cells in the absence or presence of IL-6 or IFN $\alpha$ .**  $2 \times 10^6$  Sko-007 or U266 cells were left untreated or treated with IL-6 (10 ng/ml) or IFN $\alpha$  (100 U/ml) for 10 min and lysed in cell lysis buffer. Then 30  $\mu$ g cytoplasmic proteins were separated by 10% polyacrylamide-SDS gel and transferred electrophoretically to Immobilon. After blocking with 5% dry milk, the membranes were blotted with anti-STAT3, STAT1, phospho-STAT3 or phospho-STAT1 antibodies and the relevant HRP-labeled second antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham, USA).

**Table 1: Expression of IL-6R and gp130 on the surface of Sko-007 and U266 cells in the absence or presence of IFN $\alpha$ .**

	IFN $\alpha$ (-)		IFN $\alpha$ (+)	
	%	mean	%	mean
IL-6R Sko-007	13.00 $\pm$ 1.21	20.57 $\pm$ 2.74	10.11 $\pm$ 0.96	19.80 $\pm$ 0.47
Gp130	99.06 $\pm$ 1.01	60.68 $\pm$ 1.70	65.77 $\pm$ 3.19*	34.21 $\pm$ 1.31*
IL-6R U266	1.50 $\pm$ 0.33	50.44 $\pm$ 2.09	0.92 $\pm$ 0.36	50.09 $\pm$ 4.28
Gp130	8.66 $\pm$ 0.64	29.19 $\pm$ 2.13	9.33 $\pm$ 1.05	26.66 $\pm$ 0.63

$2 \times 10^5$  or cells were treated with or without IFN $\alpha$  (100 U/ml) for 48 h and then subsequently incubated with anti-IL-6R or anti-gp130 antibodies for 30 min and with the relevant FITC-labeled second antibodies for 20 min. Cell samples were harvested and subjected to FACS analysis equipped with ModFitLT software. "mean" represents the average strength of fluorescence. "%" represents the percentage of the cells expressing target antigen (positive cells). The *p* values were determined by paired t-test. \*significantly different (*p* < 0.01) from the corresponding values in cultures without IFN $\alpha$ .



**Figure 3**

**Activation state of ERK in Sko-007 and U266 cells in the absence or presence of IFN $\alpha$ .**  $2 \times 10^6$  Sko-007 or U266 cells were left untreated or treated with IFN $\alpha$  (100 U/ml) for 48 h and lysed in cell lysis buffer. Then cytoplasmic proteins were extracted and the expressions of phospho-ERK and ERK were determined by immunoblot assay as similar as in Fig. 2 except using anti-ERK or phospho-ERK antibodies.

#### **Activation of the transcription factors STAT3 and STAT1 in myeloma cells stimulated by IFN $\alpha$**

JAK/STAT pathway is one of two common signal transduction pathways used by both IL-6 and IFN $\alpha$  to mediate their activities [4,5]. To address whether there exists any difference in the activation of the JAK/STAT pathway in Sko-007 and U266 cells stimulated by IFN $\alpha$ , immunoblot assay was performed using anti-phospho-STAT3 and phospho-STAT1 antibodies to detect the activation of the transcription factors STAT3 and STAT1 in the JAK/STAT pathway. As shown in Fig. 2, there was no activation of STAT3 and STAT1 in unstimulated Sko-007 and U266 cells, while IL-6 and IFN $\alpha$  can activate both two transcription factors in the both two cell lines. Anti-STAT3 and STAT1 antibodies blotting showed equal amount of STAT3 and STAT1 proteins in untreated and IL-6 or IFN $\alpha$ -treated cells. These results indicated that the activation of the JAK/STAT pathway did not account for the different effect of IFN $\alpha$  on Sko-007 and U266 cells.

#### **Activation state of the protein kinase ERK in myeloma cells stimulated by IFN $\alpha$**

Ras/MAPK pathway is the other common signal transduction cascade used by both IL-6 and IFN $\alpha$  [4,5]. In the previous report, we have proved that the survival of Sko-007 and U266 cells were mediated by the constitutive activation of this pathway in the presence of the autocrine IL-6 [7]. To analyze whether the activation of this pathway was different in above two cell lines in the presence of IFN $\alpha$ , a similar immunoblot assay was performed using anti-phospho-ERK antibody to detect the activation state of the

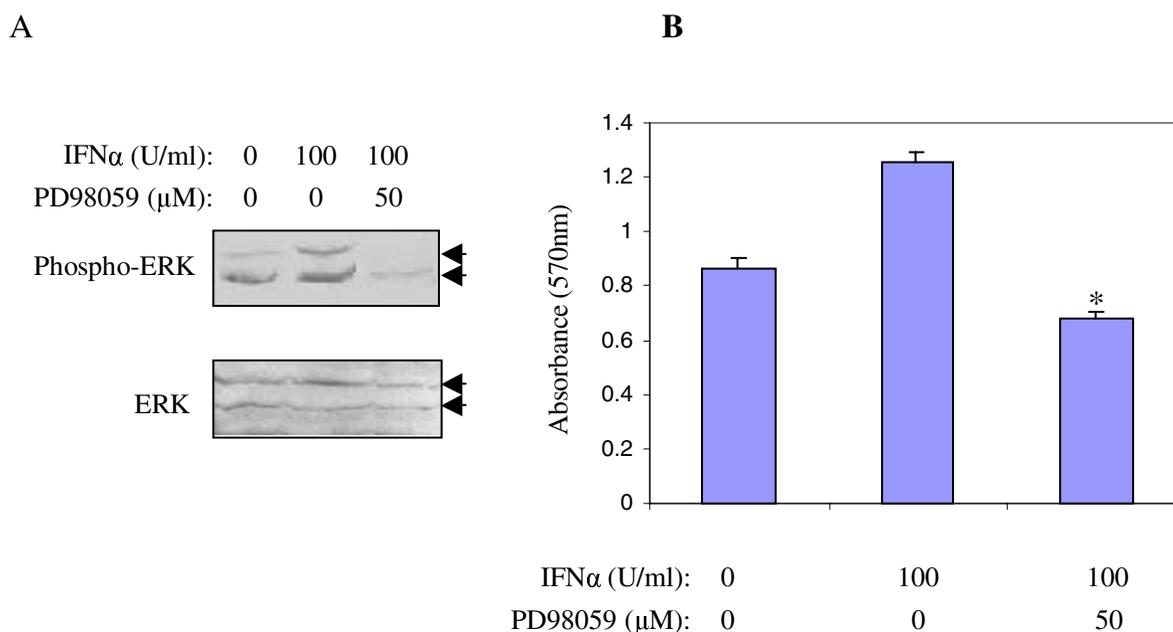
protein kinase ERK, the most important kinase in the Ras/MAPK pathway. As shown in Fig. 3, constitutive activation of ERK can be observed in both Sko-007 and U266 cells in the absence of IFN $\alpha$ ; and IFN $\alpha$  simulation down-regulated ERK activation in Sko-007 cells and up-regulated ERK activation in U266 cells. This result strongly suggested that different regulation of the protein kinase ERK in the Ras/MAPK signal transduction pathway might play a key role in responding to IFN $\alpha$  in Sko-007 and U266 cells.

#### **PD98059 inhibited IFN $\alpha$ -induced growth-stimulation effect and up-regulation of ERK activation in U266 cells**

To further confirm the result from Fig. 3, we next analyzed whether the regulation on ERK activation can result to the corresponding changes on the activity of IFN $\alpha$  on myeloma cells. As shown in Fig. 4, when IFN $\alpha$ -induced increase of ERK activation in U266 cells was efficiently inhibited by treatment of PD98059, the specific inhibitor of the upstream kinase of ERK, MAPK/ERK kinase (MEK), in the Ras/MAPK pathway, IFN $\alpha$ -mediated growth-stimulation effect was also decreased at the same conditions. This finding confirmed the result that protein kinase ERK in the Ras/MAPK pathway mainly contributed to the differential responsiveness of myeloma cells to IFN $\alpha$ .

#### **Discussion**

Up till now, the clinical value of IFN $\alpha$  on multiple myeloma therapy remains debatable due to the conflicting in vivo results reported by different clinical groups [2,3]. Therefore in this study, we analyzed the responsiveness of

**Figure 4**

**PD98059 inhibited IFN $\alpha$ -induced growth-stimulation effect and increase of ERK activation in U266 cells.** (A) U266 cells were left untreated or treated with IFN $\alpha$  (100 U/ml) or pre-treated with PD98059 (50  $\mu$ M) before IFN $\alpha$  was added. Then cytoplasmic proteins were extracted and the expressions of phospho-ERK and ERK were determined by immunoblot assay as same as in Fig. 3. (B) U266 cells ( $2 \times 10^6$  /well) were cultured in 96-well tissue culture plates in the absence or presence of IFN $\alpha$  (100 U/ml) alone or IFN $\alpha$  together with PD98059 (50  $\mu$ M). MTT assay was performed as described in Fig. 1. The  $p$  values were determined by paired  $t$ -test. \*significantly different ( $p < 0.01$ ) from the corresponding values in cultures with IFN $\alpha$  alone.

a panel of human myeloma cell lines to IFN $\alpha$  in vitro and the mechanism of myeloma cells heterogeneous response to IFN $\alpha$ .

The results in this study showed that IFN $\alpha$  exerted different effects on the growth of different myeloma cell lines. The growth-inhibition effect of IFN $\alpha$  was significant while the growth-stimulation effect was moderate (Fig. 1). This result is in accordance with the fact that IFN $\alpha$  usually exerts a strongly inhibitory or a slowly stimulatory effect on the proliferation of myeloma cells in different patients with MM [2,3].

To elucidate the mechanism responsible for the different activity of IFN $\alpha$  on myeloma cells, we firstly examined the regulation of IL-6R and gp130 expression on cell surface in the presence of IFN $\alpha$ , which may be one of the reasons for the heterogeneous IFN $\alpha$  responsiveness according to the previous reports [8]. Interestingly, we only detected down-regulated expression of gp130 in IFN $\alpha$ -arrested Sko-007 cells, while not any changes on two IL-6 receptor chains level in IFN $\alpha$ -stimulated U266 cells. Therefore, the

regulation of IL-6 receptors expression may not mainly account for the different effect of IFN $\alpha$  on myeloma cells.

In the further study, we focused on investigating the activation states of IFN $\alpha$ -induced signal transduction pathways in myeloma cells. We observed that both STAT3 and STAT1 could be activated in the both two myeloma cell lines irrespective of growth outcome upon IFN $\alpha$  stimulation; while the activation state of ERK correlated tightly with the growth effect of IFN $\alpha$  (Fig. 2, 3 and 4). That means, the Ras/MAPK in stead of the JAK/STAT signal transduction pathway mainly contributed to the different response of myeloma cells to IFN $\alpha$ . Identification of the target gene(s) regulated by protein kinase ERK in the Ras/MAPK pathway in IFN $\alpha$ -arrested and stimulated myeloma cells will be helpful to further elucidate the mechanism of IFN $\alpha$  on myeloma cells.

### Conclusions

Our data in this study clearly demonstrated that IFN $\alpha$  can exert growth-stimulatory or -inhibitory effect on different

myeloma cells in vitro and protein kinase ERK played a key role in myeloma cells in response to IFN $\alpha$ .

## Materials and methods

### Reagents

Human recombinant IFN $\alpha$  was provided by Institute of Biotechnology (Beijing, China). Anti-human IL-6R and gp130 antibodies were prepared in our lab. Anti-ERK and phospho-ERK antibodies were purchased from Santa Cruz Biotechnology (USA). Anti-STAT3, STAT1, phospho-STAT3 and phospho-STAT1 antibodies were purchased from NEB Biotechnology (USA).

### Human myeloma cell lines

Human myeloma cell line U266 was kindly provided by Prof. Xueguang Zhang (Suzhou University, China). Sko-007 myeloma cell line was obtained from Medical School of Stanford University (USA). The cells were maintained in RPMI1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin and 80 U/ml streptomycin.

### Effect of IFN $\alpha$ on the growth of myeloma cells

The proliferation of myeloma cells in the presence of IFN $\alpha$  was measured by MTT assay as described previously [7].

### Expression of two IL-6 receptor chains (IL-6R and gp130) on myeloma cell surface

$2 \times 10^5$  myeloma cells were treated with or without IFN $\alpha$  (100 U/ml) for 48 h and then incubated with anti-IL-6R or anti-gp130 antibodies for 30 min and with the relevant FITC-labeled second antibodies for 20 min. Cell samples were harvested and subjected to FACS analysis.

### Activation of the protein kinase ERK and the transcription factors STAT3 and STAT1 in myeloma cells induced by IFN $\alpha$

$2 \times 10^6$  myeloma cells were treated with IFN $\alpha$  (100 U/ml) for 10 min (to detect the activation of STAT3 and STAT1) or 48 h (to detect the activation of ERK) and then lysed in 100  $\mu$ L lysis buffer (10 mM Tris-Cl pH 7.6, 150 mM NaCl, 0.5% NP40, 5 mM EDTA, 1 mM PMSF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 2  $\mu$ g/mL aprotinin). After 30 min on ice, cell lysates were cleared by centrifugation at 12000  $\times$  g for 30 min at 4°. The concentrations of the cytoplasmic proteins were measured by Bradford assay [9]. Then 30  $\mu$ g cytoplasmic proteins were separated by 10% polyacrylamide-SDS gel and transferred electrophoretically to Immobilon (Millipore). After blocking with 5% dry milk, the membranes were blotted with anti-ERK, STAT3, STAT1 or anti-phospho-ERK, phospho-STAT3, phospho-STAT1 antibodies and the relevant HRP-labeled second antibodies. Immunoreactive proteins were visualized with an en-

hanced chemiluminescence (ECL) detection system (Amersham, USA).

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## References

1. Grandt D: **How does Interferon-alpha exert its antitumor activity in multiple myeloma?** *Acta Oncol* 2000, **39**:801-805
2. Browman GP, Bergsagel D, Sicheri D, O'Reilly S, Wilson KS: **Randomized trial of interferon maintenance in multiple myeloma: a study of National Cancer Institute of Canada clinical trials group.** *J Clin Oncol* 1995, **13**:2354-2360
3. Sawamura M, Murayama K, Ui G, Matsushima T, Tamura J, Hurakami H, Naruse T, Tsuchiya J: **Plasma cell leukemia with alpha-interferon therapy in myeloma.** *Br J Haematol* 1992, **82**:631-636
4. Teron SP, Anderson S: **IL-6 in multiple myeloma and related plasma cell dyscrasias.** *Curr Opin Hematol* 1998, **5**:42-48
5. Hawley R, Berger L: **Growth control mechanism in multiple myeloma.** *Leuk Lymphoma* 1997, **29**:465-475
6. Song L, Shen B, Li Y: **Mechanism of IL-6 signal transduction in human myeloma cells.** *Cell Bio Int* 2001, **25**:1065
7. Song L, Shen B, Li Y: **Activation of IL-6 signal transduction pathways in different human myeloma cell lines.** *U S Chin J Microbiol & Immunol* 2001, **3**:42-46
8. Schwabe M, Brini AT, Bosco MC, Rubboli F, Egawa M, Zhao J, Princler GL, Kung H: **Disruption by interferon-alpha of an autocrine interleukin-6 growth loop in IL-6-dependent U266 myeloma cells by homologous and heterologous downregulation of the IL-6 receptor  $\alpha$  and  $\beta$  chains.** *J Clin Invest* 1994, **94**:2317-2325
9. Ausibel FM, Brent R, Kingston RE: *Short protocols in molecular biology. 3rd ed.* John Wiley & Sons, Inc. 1994

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