Biotechnology and Bioprocess Engineering **2005**, 10: 444-450

DNA Microarray Analysis of Immediate Response to EGF Treatment in Rat Schwannoma Cells

Min-Kyu Oh[1,2]*, Daniel R. Scoles[3], and Stefan-M. Pulst[3]

[1] Department of Chemical and Biological Engineering, Korea University, Seoul 136-713, Korea
[2] Institute of Nanoscience, Korea University, Seoul 136-713, Korea
[3] Rose Moss Neurogenetics Laboratory for Parkinson and Related Diseases, CSMS Burns and Allen Research Institute, Division of Neurology, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Los Angeles, CA 90048, USA

Abstract Epidermal growth factor (EGF) activates many intracellular effector molecules, which subsequently influence the expression levels of many genes involved in cell growth, apoptosis and signal transduction, *etc.* In this study, the early response of gene expressions due to EGF treatment was monitored using oligonucleotide DNA microarrays in rat schwannoma cell lines. An immunoblotting experiment showed the successful activation of EGF receptors and an effector protein, STAT5, due to EGF treatment. The microarray study showed that 35 genes were significantly induced and 2 were repressed within 60 min after the treatment. The list of induced genes included early growth response 1, suppressor of cytokine signaling 3, c-fos, interferon regulatory factor 1 and early growth response 2, *etc.* According to the microarray data, six of these were induced by more than 10-fold, and showed at least two different induction patterns, indicating complicated regulatory mechanisms in the EGF signal transduction.

Keywords: oligonucleotide microarray, expression profiling, immediate response, epidermal growth factor (EGF), schwannoma cell line

INTRODUCTION

Cell stimulation due to growth factors triggers the activations of many intracellular effector molecules, which consequently influence many cellular processes, including cell growth, survival, differentiation and metabolism. The research on the growth factor stimulation has been mainly focused on the signaling pathways of a handful of biomolecules. Examples include the cascades of protein phosphorylation [1], protein-protein interactions [2] and dynamics of the receptor signaling [3]. These activities have greatly advanced biochemical knowledge related to the functions of growth factors. However, attempts to understand the signaling network on a large scale or to study the interactions between the signaling pathways have been scarce.

A member of the EGF receptor family, ErbB1, binds to EGF and induces homo- or hetero-oligomerization with ErbB1, ErbB2 and ErbB4. The oligomerization of receptors induces their intracellular protein tyrosine kinase (PTK) activities, which attach phosphoryl groups to the receptors as well as other effector proteins [1]. The phosphorylation of the receptors is crucial for recruitment and activation of various signaling molecules that have SH2 (Src homolog 2) or PTB (Phosphotyrosine binding) domains. The EGF subsequently activates Ras/MAP kinase, signal transducers and activators of transcriptions (STATs), protein kinase C (PKC) and phosphoinositol-3 kinase signaling pathways [4].

Some of the effector molecules activate transcriptional factors (TFs) through cascade phosphorylation of several proteins. For example, the Ras/MAP kinase pathway activates c-fos, c-jun and Sp1 [5]. These TFs are known to induce the expression of a group of genes essential for cell division. The mechanism of this pathway was examined with several genes as model systems [5,6]. Other effector molecules, such as STAT3 or STAT5, are TFs by themselves, which are tyrosine-phosphorylated, dimerized, and then migrate into the nucleus. They directly bind to the specific sequence of DNA and induce the transcription of a set of genes, including those that protect the cell from apoptosis [7].

Recently, a proteomic analysis of EGF signaling has been attempted with a mass spectrometry-based approach using isotope labeling [8]. Using three different sets of stable arginine isotopes, the analyses identified the levels of tyrosine phosphorylated proteins and their closely associated binders at 1, 5, 10, and 20 min after EGF treatment in HeLa cell lines. The experiments demonstrated that EGFR and its binders, Esp15 and c-Cbl, were immediately phosphorylated at 1 min, but 5 min after the treatment were slowly dephosphorylated. The phosphorylation of other proteins involved in the receptor trafficking, such as Hrs and STAM/STAM2, were slightly delayed, but became

***Corresponding author**
Tel: +82-2-3290-3308 Fax: +82-2-926-6102
e-mail: mkoh@korea.ac.kr

fully activated at 10 to 15 min after stimulation [8].

The phosphorylations of the proteins in the signal transduction influence the levels of gene expression. Here, we present the global gene expression level changes after EGF treatment in rat schwannoma cells. Using oligonucleotide microarrays, the transcript level changes of rat RT4 schwannoma cells were monitored after EGF treatment. Schwannoma cells are a model cell line of a benign brain tumor. In this experiment, the gene expression profiling in the first sixty minutes after EGF stimulation was monitored using the Affymetrix rat genome chip 230A.

MATERIALS AND METHODS

Cell Culture Condition

The construction and maintenance of inducible Tet-On RT4 cell lines have been described previously [9]. The cell line was maintained in a 100 mm diameter Petri dish with 10 mL DMEM (Invitrogen, San Diego, CA, USA), containing 10% Tet-approved FBS (Clontech, Palo Alto, CA, USA), 100 μg/mL streptomycin, 100 μg/mL G418 and 250 μg/mL Hygromycin B, in a humid 5% CO_2 incubator. The cells were split when 30% confluent and grown for a day. The cells were washed three times with 10 mL DMEM without serum and antibiotics, and then cultured for 18 h in 5 mL DMEM to make them quiescent. To stimulate the cells, 100 μg/mL of epidermal growth factor (Sigma, St. Louis, MO, USA) was added to the media. As the reference, pure water, with no EGF, was added to the other set of cell cultures.

RNA Preparation and cDNA Probe Synthesis

RNA's were purified from the EGF treated cell lines at 10, 30 and 60 min after the treatment as well as from water-treated cell lines at 0, 10 and 30 min. For RNA purification, cells were washed twice with ice-cold saline solution (Invitrogen), and 1.5 mL of Trizol solution (Invitrogen) then applied to each dish. After scraping, the cells were mixed with the solution by pipetting. The solution was transferred to an Eppendorf-type tube, and chloroform added to the solution. After vortexing and centrifugation, the aqueous solution was taken and transferred to a new tube. RNA was purified from the solution using an RNeasy Mini Kit (Qiagen, Courtaboeuf, France), according to the manufacture's protocol. The organic phase of the Trizol solution was saved for an immunoblotting experiment. The quality and quantity of RNA were measured using an Agilent 2100 Bioanalyzer. Microarray experiments were conducted with 10 μg of RNA using the Affymetrix rat chip 230A, according to the manufacturer's protocol, at the Cedars Sinai Medical Center microarray core facility. The microarray experiment was duplicated independently.

Data Analysis

After the DNA microarray experiment, the array images (CEL files) were smoothed using the MAS 5.0 program (Affymetrix, Santa Clara, CA, USA). The image files were imported into the DCHIP program, which can calculate the expression levels using a model-based method. After normalization using a rank invariant protocol, the method computes the affinity of each probe by comparing the intensity pattern of the probe sets across several microarray chips. Using the calculated affinities, the program can filter the outliers that do not follow the overall pattern of the intensities. Because these outliers originate due to contaminated spots or bad probes, they must be removed when computing gene expression levels [10]. After the gene expression levels were computed, differentially expressed genes were filtered based on the percentage of 'present' call (80%), fold changes (2-fold) and p-value (< 0.01) using the 'compare samples' menu in the DCHIP program [11].

Immunoblotting

The proteins were purified from the organic phase of the Trizol solution, according to the manufacturer's protocol. Briefly, ethanol was added to the solution and the DNA removed by centrifugation. The supernatant was transferred to a new tube, and protein precipitated by centrifugation after the addition of isopropanol. The precipitant was washed three times with 0.3 M guanidine hydrochloride in 95% ethanol. The precipitant was dissolved in 1% SDS solution and loaded on 4~15% SDS-PAGE gradient gels (BioRad, Labs, Hercules, CA, USA). The protein was transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech., Piscataway, NJ, USA). The immunoblot was detected by anti-phospho-STAT5 and anti-phospho-EGFR monoclonal antibodies (Santa Cruz Biotech., Santa Cruz, CA, USA). The blot was re-probed with anti-actin monoclonal antibody AC40 (Sigma) to normalize the protein loading. The primary antibodies were re-probed with horseradish peroxidase (HRP)-labeled secondary antibodies, with the level monitored using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech.).

RESULTS AND DISCUSSIONS

The Quality of the Chip Experiment

Total RNA's were purified at three time points for both the EGF treated and untreated samples, respectively. Therefore, six RNA samples were purified, labeled and hybridized to the chips for expression profiling. Each experiment was duplicated to increase the reliability of the data. After image analysis, the image files (CEL files) were imported into a model based analysis program, DCHIP [11]. While calculating expression levels using the PM/MM difference model, the program excluded "array outliers" and "single outliers" (Table 1). "Array outliers" are those probe sets that did not match the overall pattern of intensities observed with other chips and "single outliers" are those probes that did not match the intensity

Table 1. The Affymetrix chips used for expression profiling of rat schwannoma cell lines treated with or without EGF

Array Names	Median ntensity	P call %[b]	Array outlier %[c]	Single outlier %[d]
0 min_EGF(-)_1[a]	160	58.35	0.100	0.062
10 min_EGF(-)_1	146	57.53	0.013	0.021
30 min_EGF(-)_1	146	54.52	0.075	0.074
10 min_EGF(+)_1	168	57.92	0.006	0.022
30 min_EGF(+)_1	173	55.98	0.069	0.056
60 min_EGF(+)_1	146	55.80	0.025	0.039
0 min_EGF(-)_2	147	57.02	0.075	0.046
10 min_EGF(-)_2	189	57.33	0.025	0.060
30 min_EGF(-)_2	147	54.76	0.038	0.047
10 min_EGF(+)_2	158	57.50	0.094	0.033
30 min_EGF(+)_2	145	56.42	0.069	0.047
60 min_EGF(+)_2	148	55.20	0.038	0.031

[a] The experiment was duplicated and indicated with numbers at the end of the array names.

[b] P call % represents the percentage of the probes called 'present' in the array by the DCHIP program.

[c] Array outliers represent the probe sets that do not follow the overall pattern of the intensities calculated by the model based expression tags in DCHIP.

[d] Single outliers represent single probes that do not follow the intensity pattern of the respective probe in other sets. High percentage of array and single outliers (> 5%) represents high background noise of the chip image.

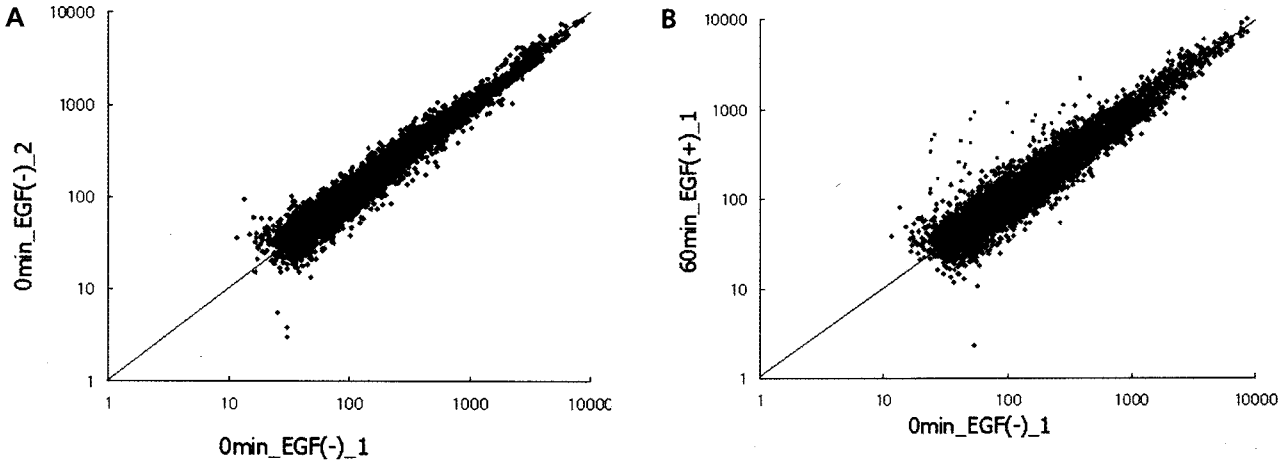

Fig. 1. The comparisons of the intensity levels of 9,000 probe sets between two quiescence cell lines (A) and between quiescent and stimulated cell lines (B), drawn on log-log charts. The 32 significantly regulated genes were determined using the statistical model within the DCHIP program, with 2-fold and a p-value < 0.01, which are marked with red spots.

pattern of the respective probe on other chips. In general, the outlier percentage is inversely correlated with the image quality [12]. According to the outlier percentages of the set of chips, the experimental images had very low background noise (Table 1).

For determination the background noise in the chip experiments, the expression levels of quiescent cell lines were compared with each other. The Affymetrix rat genome chip 230A has more than 15,000 probe sets, of which about 9,000 showing intensities above a threshold (100) were selected. The expression profiles of these probe sets were compared between quiescent cell lines. On applying the 2-fold and p-value = 0.01 criteria, none of the comparisons filtered out any significantly regulated genes. The expression levels of the 9,000 probe sets of the two chips from quiescent cell lines were plotted on a log-log scale (Fig. 1A). Only a few probe sets with low expression levels showed differential levels, but these did not show statistical significance with the model used by DCHIP.

Conversely, the comparison between the quiescent and stimulated cell lines clearly showed regulated genes (Fig. 1B). The red dots in Fig. 1B represent significantly regulated genes of more than 2-fold at a p-value = 0.01 ac-

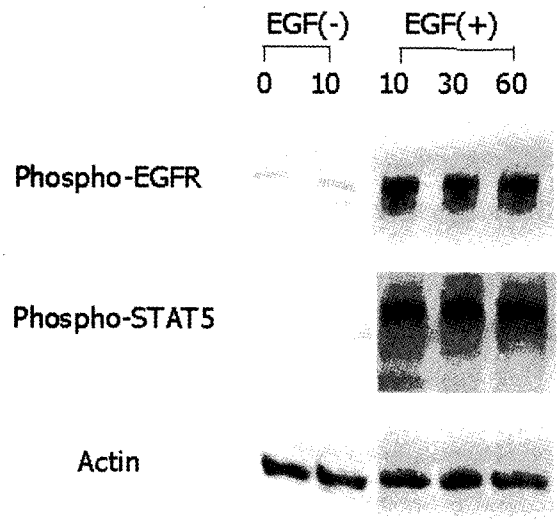

Fig. 2. Immunoblot detection of the tyrosine phosphorylation levels of the EGF receptor and STAT5 protein in RT4 cell lines. Cells were incubated in the medium without serum for 18 hrs to become quiescent, and then activated by the addition of 100 μg/mL EGF. Both proteins were phosphorylated immediately after the stimulation, in accordance with Blagoev *et al.* [8]. As a loading control, blots were re-probed with an anti-actin monoclonal antibody, AC40 (Sigma).

cording to the statistical model. The other comparisons between quiescent samples or quiescent and stimulated samples showed similar results to Fig. 1A and 1B, respectively (data not shown). These results suggest the microarray experiments were well controlled for identifying the genes induced by EGF treatment.

Activation of EGF Receptor and STAT5

To examine the activation of the EGF receptors and the signaling molecules, the phosphorylation levels of the EGF receptor (ErbB1) and STAT5 were monitored using immunoblotting methods. The protein samples were prepared as previously described. The total protein from 0 and 10 min samples without EGF treatment, and 10, 30 and 60 min samples with EGF treatment, were loaded onto polyacrylamide gel, blotted, and probed with anti-phospho-EGFR and anti-phospho-STAT5 antibodies.

As expected, the EGF treatment immediately induced levels of tyrosine phosphorylation of the EGF receptor (ErbB1) (Fig. 2). Within 10 min after EGF treatment, the cell line showed a high level of phosphorylation of the EGF receptor, while the level of phosphorylation was noticeably low in quiescent samples. This result was in accordance with a previous report [8], which showed high levels of phosphorylation within 1 min. A similar trend of phosphorylation was shown when phosphor-STAT5 was probed from the blotted proteins. No STAT5 phosphorylation was observed in the quiescent samples, while in the EGF treated cells significantly high levels of phosphorylation were shown The phosphorylation level did not change until 60 min after EGF treatment, proving that the schwannoma cells produced enough EGFR (ErbB1) to mediate EGF signaling, with successful activation of the downstream pathways of the EGF signaling. The time points for gene expression profiling, 10, 30 and 60 min, were chosen considering the time period taken from the protein phosphorylation to the gene expression regulations via signal transduction pathways.

Immediately Regulated Genes by EGF Treatment

To minimize the experimental errors, the expression profiles of the duplicated experiments were combined. First, the six data sets for the quiescent cell lines were averaged, and then used as a reference. The expression levels of the EGF-treated samples were then averaged at each time point, 10, 30 and 60 min, and compared with those of the reference. The significantly regulated genes were filtered using the 'compare samples' option in the DCHIP program, with a 2-fold increase and a p-value <0.01 used as the statistical criteria [11]. The numbers of regulated genes were 2, 12 and 36 at 10, 30 and 60 min after EGF treatment, respectively. Many of the regulated genes overlapped, so that 37 genes were significantly regulated by at least one time point. The genes and the changes in their level of expression at different time points are listed in Table 2. Interestingly, most of the regulated genes were induced, while only two genes were repressed. This may have been because EGF signaling pathways are mainly involved in the induction of gene expressions.

The most up-regulated genes coded transcription factors, including early growth response 1 (Egr1), c-fos gene, interferon regulatory factor 1 (IRF1), early growth response 2 (Egr2) and the jun-B proto oncogene. All five genes were up-regulated more than 5-fold at 30 minutes. IRF1 is regulated by various cytokines and induces the expression levels of many genes involved in host defense, including interferons, interleukins and inducible nitric oxide synthase [13]. Egr2, a homolog of Krox-20, is a TF that is important for myelination of Schwann cell [14] and Egr1 is involved in blocking angiogenesis [15]. Both Egr1 and 2 are known to be induced immediately after treatment with growth factor. The components of activating protein-1 (AP-1), c-fos and jun-B were also significantly induced by EGF stimulation. AP-1 is responsible for cell growth due to cytokine stimulation. Although their activation was expected, their induction patterns were quite different. The c-fos gene was fully induced in the sample at 30 min, while junB was slowly induced, showing a maximum level at 60 min. Other up-regulated transcription factors in this experiment were zinc finger protein 36, immediate early gene transcription factor (NGFI-B) and activating transcription factor 3.

Another group of significantly up-regulated genes coded proteins for turning off the EGF signals. An example is the mouse ortholog of the suppressor of cytokine signaling 3 (Socs3), which was up-regulated more than 15-fold at 30 min after stimulation. The Socs3 protein

Table 2. The expression level changes of 37 significantly regulated probe sets in EGF stimulated compared to the quiescent cells

Probe set #	Gene name	Fold changes		
		10 min	30 min	60 min
1368321_at	Early growth response 1	3.36	22.50	9.34
1377092_at	Mouse suppressor of cytokine signaling 3 (Socs3) ortholog	1.57	11.20	15.80
1375043_at	c-fos gene	2.64	14.30	3.58
1368073_at	Interferon regulatory factor 1	1.17	5.13	14.00
1387306_a_at	early growth response 2	1.40	7.16	12.86
1389402_at	Unknown	1.06	8.83	10.90
1387788_at	junB proto-oncogene	2.16	7.68	8.80
1373035_at	Unknown	1.13	2.12	8.76
1373093_at	Similar to human mitogen-inducible gene 6	1.36	2.12	8.50
1372389_at	Unknown	1.80	8.50	3.56
1368223_at	a disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS-1)	1.28	2.61	5.44
1369182_at	Coagulation factor III (thromboplastin, tissue factor)	1.13	1.60	4.96
1369067_at	nuclear receptor subfamily 4, group A, member 3	1.66	2.11	4.84
1387870_at	zinc finger protein 36	1.72	4.76	3.14
1377064_at	dual specificity phosphatase 6 (Dusp6)	1.58	3.13	4.60
1374864_at	latent transforming growth factor beta binding protein 1	1.45	2.52	4.33
1373403_at	Unknown	1.18	2.61	4.15
1388587_at	immediate early response 3 (Ier3)	1.19	3.48	3.82
1368124_at	dual specificity phosphatase 5 (Dusp5)	1.35	1.69	3.70
1382778_at	dual specificity phosphatase 6 (Dusp6)	1.27	2.50	3.70
1374157_at	Phosphodiesterase 4B	0.95	1.24	3.67
1386935_at	immediate early gene transcription factor NGFI-B	1.25	2.88	3.41
1376151_a_at	Unknown	1.16	1.15	3.11
1371091_at	Unknown	1.04	1.78	3.05
1369268_at	Activating transcription factor 3	1.23	2.32	2.77
1368308_at	v-myc avian myelocytomatosis viral oncogene homolog	1.05	1.78	2.76
1377103_at	Unknown	1.34	2.01	2.65
1369958_at	rhoB gene	1.11	1.68	2.53
1372510_at	Unknown	1.04	1.36	2.47
1369415_at	basic helix-loop-helix domain containing, class B2	1.07	1.33	2.31
1368144_at	regulator of G-protein signaling protein 2	0.89	1.57	2.16
1387024_at	dual specificity phosphatase 6 (Dusp6)	1.14	1.79	2.12
1367802_at	serum/glucocorticoid regulated kinase	0.98	1.46	2.08
1383519_at	Hexokinase 2	0.96	1.28	2.08
1371960_at	Unknown	1.20	1.13	2.04
1368025_at	DNA damage inducible transcript 4	1.18	1.21	0.48
1372622_at	Unknown	1.13	0.83	0.41

having an SH-2 domain is known to be activated by EGF or prolactin treatment [16], which has the function of inhibiting the STAT5 signaling pathway activated by the growth factor treatment. Within 120 min after EGF treatment, the level of STAT5 phosphorylation was significantly decreased in our experiment (data not shown); therefore, Socs3 may be responsible for the inactivation of the STAT5 [16].

When EGF stimulates the MAP kinase pathway, the expression of several tyrosine phosphatases are also up-regulated, which will turn off the pathway [17]. In this experiment, dual specificity phosphatase 5 (Dusp5) and dual specificity phosphatase 6 (Dusp6), two of the eight known tyrosine phosphatases, were significantly up-regulated. Three probe sets coded for dual specificity phosphatase 6 (Dusp6) in the rat 230 chip, all of which showed significant up-regulation in the sample at 60 minutes. Another gene for turning off the EGF signaling is phosphodiesterase 4B (PDE4B), which was also up-regulated in the sample at 60 min. This gene is known to hydrolyze cyclic adenosine monophosphate (cAMP) to abort the signal made by this secondary messenger.

A few other signal transduction proteins, including serum/glucocorticoid regulated kinase, regulator of G-protein signaling protein 2, Immediate early response 3 (Ier3) and Ras homolog protein B (rhoB), were also up-regulated. The only metabolic gene regulated significantly was hexokinase 2, while the gene coding DNA damage inducible transcript 4 was significantly down-regulated. Among the 37 significantly regulated genes, 11 remain to

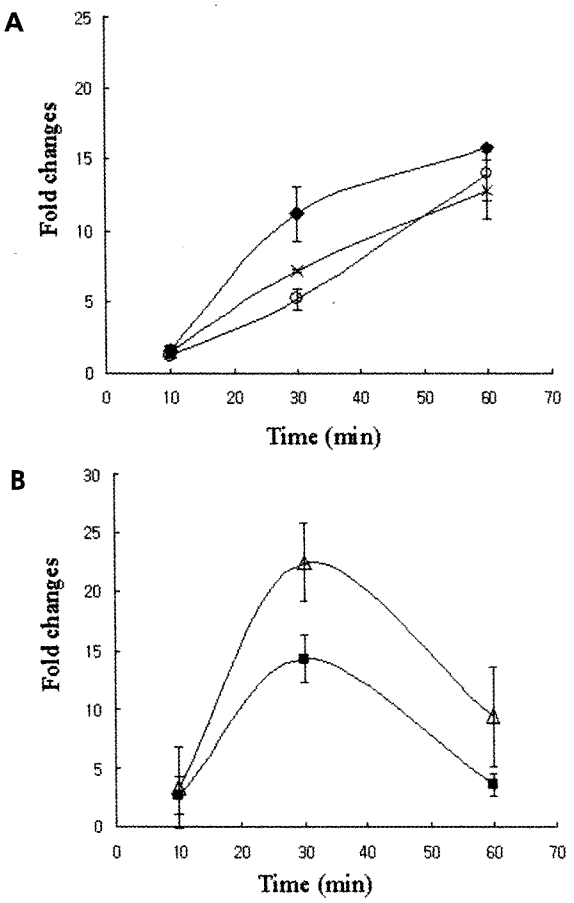

Fig. 3. Transcript level changes of the five genes most significantly regulated within 60 min after EGF treatment. The symbol ◆ represents mouse suppressor of cytokine signaling 3 (Socs3) ortholog, ○ interferon regulatory factor 1, X early growth response 2 (Egr2), △ early growth response 1 (Egr1) and ■ c-fos gene, respectively. The expression regulation patterns of the first three genes (A) are distinct from the last two genes (B). The error bars represent the standard deviations of the experimental data.

be functionally identified. Therefore, the results in Table 2 will provide important information for the functional analysis of some of the unidentified genes.

Induction Pattern of Up-Regulated Genes

In the phosphor-proteomics experiment, there were time gaps between the phosphorylation levels of the effector proteins [8]. The phosphorylation level of most effector proteins peaked at 5 min after EGF treatment. However, a few proteins, such as Hrs, STAM2 and p38, showed fully activated levels around 15 min after the treatment. The time gap of the protein phosphorylation possibly occurred because of the receptor trafficking of the phosphorylated EGFR, which can take between a few seconds to several minutes.

Similar to the phosphorylation levels, those of the mRNA also showed a different pattern of induction. In this experiment, most of the up-regulated genes gradually increased from 10, 30 and through to 60 min. However, four genes showed their full induction around 30 min after the treatment, but the expression levels significantly decreased at 60 min, including early growth response 1 (Egr1), c-fos and zinc finger protein 36, as well as one unknown gene The gene expression patterns of the first two genes are illustrated in the Fig. 3B, and compared with three other significantly up-regulated genes (Fig. 3A). The Egr1 and c-fos genes are known as members of immediate-early response genes in the brain [18]. The differential pattern of induction of immediately response genes will require further investigation of its mechanisms. As the differential phosphorylation pattern of the proteins can provide information about the direct or indirect modification of the EGF receptor, the detailed pattern of gene expressions can be an effective tool for studying the induction or repression mechanisms of various transcription factors following EGF stimulation.

CONCLUSION

The large scale investigation of cellular phenotypes has been enabled by the development of high-throughput technology. These methods have already generated a large quantity of data, which may contain valuable information about the function and mechanism in the living system. The recent proteomic analysis of the EGF signaling pathway has reported dozens of proteins that were tyrosine phosphorylated within 20 min after EGF treatment. The microarray experiment to study the EGF signaling also reported 37 genes that were significantly regulated within 60 min in rat schwannoma cells. Systematic analyses of the phosphor-proteomic and DNA microarray study, as well as their interactions, must be performed for a clearer understanding of the phenomena of the cellular responses to growth factor stimulations. This dynamic expression profile will offer a good starting point for the biological systems of growth factor stimulation of the cell. These studies must be followed by mathematical as well as molecular models, which will provide a more comprehensive picture of cell growth and signal transduction inside the cell.

Acknowledgements The authors thank Cedars Sinai Medical Center microarray core facility for performing the microarray experiment. This work was supported by the Korea Research Foundation, KRF-2004-005-D00057, and the Carmen and Louis Warschaw endowment for neurology, grants DAMD 17-99-1-9548 and 01-01-0703 from the USAMRAA, and grant R01 NS37883.

REFERENCES

[1] Schlessinger, J. (2000) Cell signaling by receptor tyrosine kinases. *Cell* 103: 211-225.

[2] Pawson, T. and P. Nash (2003) Assembly of cell regulatory systems through protein interaction domains. *Science* 300: 445-452.
[3] Wiley, H. S., S. Y. Shvartsman, and D. A. Lauffenburger (2003) Computational modeling of the EGF-receptor system: a paradigm for systems biology. *Trends Cell Biol.* 13: 43-50.
[4] Schlessinger, J. (2004) Common and distinct elements in cellular signaling via EGF and FGF receptors. *Science* 306: 1506-1507.
[5] Chen, B. K. and W. C. Chang (2000) Functional interaction between c-Jun and promoter factor Sp1 in epidermal growth factor-induced gene expression of human 12(S)-lipoxygenase. *Proc. Natl. Acad. Sci. USA* 97: 10406-10411.
[6] Wang, Y. N. and W. C. Chang (2003) Induction of disease-associated keratin 16 gene expression by epidermal growth factor is regulated through cooperation of transcription factors Sp1 and c-Jun. *J. Biol. Chem.* 278: 45848-45857.
[7] Paukku, K. and O. Silvennoinen (2004) STATs as critical mediators of signal transduction and transcription: lessons learned from STAT5. *Cytokine Growth Factor Rev.* 15: 435-455.
[8] Blagoev, B., S. E. Ong, I. Kratchmarova, and M. Mann (2004) Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. *Nat. Biotechnol.* 22: 1139-1145.
[9] Gutmann, D. H., A. C. Hirbe, and C. A. Haipek (2001) Functional analysis of neurofibromatosis 2 (NF2) missense mutations. *Hum. Mol. Genet.* 10: 1519-1529.
[10] Irizarry, R. A., B. M. Bolstad, F. Collin, L. M. Cope, B. Hobbs, and T. P. Speed (2003) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.* 31: e15.
[11] Li, C. and W. H. Wong (2001) Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc. Natl. Acad. Sci. USA* 98: 31-36.
[12] Schadt, E. E., C. Li, C. Su, and W. H. Wong (2000) Analyzing high-density oligonucleotide gene expression array data. *J. Cell. Biochem.* 80: 192-202.
[13] Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka (2001) IRF family of transcription factors as regulators of host defense. *Annu. Rev. Immunol.* 19: 623-655.
[14] Parkinson, D. B., S. Dickinson, A. Bhaskaran, M. T. Kinsella, P. J. Brophy, D. L. Sherman, S. Sharghi-Namini, M. B. Duran Alonso, R. Mirsky, and K. R. Jessen (2003) Regulation of the myelin gene periaxin provides evidence for Krox-20-independent myelin-related signalling in Schwann cells. *Mol. Cell. Neurosci.* 23: 13-27.
[15] Khachigian, L. M. (2004) Early growth response-1: blocking angiogenesis by shooting the messenger. *Cell Cycle* 3: 10-11.
[16] Tonko-Geymayer, S., O. Goupille, M. Tonko, C. Soratroi, A. Yoshimura, C. Streuli, A. Ziemiecki, R. Kofler, and W. Doppler (2002) Regulation and function of the cytokine-inducible SH-2 domain proteins, CIS and SOCS3, in mammary epithelial cells. *Mol. Endocrinol.* 16: 1680-1695.
[17] Mandl, M., D. N. Slack, and S. M. Keyse (2005) Specific inactivation and nuclear anchoring of extracellular signal-regulated kinase 2 by the inducible dual-specificity protein phosphatase DUSP5. *Mol. Cell. Biol.* 25: 1830-1845.
[18] Farivar, R., S. Zangenehpour, and A. Chaudhuri (2004) Cellular-resolution activity mapping of the brain using immediate-early gene expression. *Front. Biosci.* 9: 104-109.

[Received July 22, 2005; accepted October 5, 2005]