Quantitative Transcriptional Control of ErbB Receptor Signaling Undergoes Graded to Biphasic Response for Cell Differentiation*

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ErbB receptor ligands, epidermal growth factor (EGF) and heregulin (HRG), induce dose-dependent transient and sustained intracellular signaling, proliferation, and differentiation of MCF-7 breast cancer cells, respectively. In an effort to delineate the ligand-specific cell determination mechanism, we investigated time course gene expressions induced by EGF and HRG that induce distinct cellular phenotypes in MCF-7 cells. To analyze independently the effects of ligand dosage and time for gene expression, we developed a statistical method for estimating the two effects. Our results indicated that signal transduction pathways convey quantitative properties of the dose-dependent activation of ErbB receptor to early transcription. The results also implied that moderate changes in the expression levels of a number of genes, not the predominant regulation of a few specific genes, might cooperatively work at the early stage of the transcription for determining cell fate. However, the EGF- and HRG-induced distinct signal durations resulted in the ligand-oriented biphasic induction of proteins after 20 min. The selected gene list and HRG-induced prolonged signaling suggested that transcriptional feedback to the intracellular signaling results in a graded to biphasic response in the cell determination process and that each ErbB receptor is inextricably responsible for the control of amplitude and duration of cellular biochemical reactions.

The different kinetics displayed by extracellular signal-regulated kinase (ERK)3 activation often results in distinct cellular phenotypes of mammalian cells. In PC12 cells, the epidermal growth factor (EGF)-stimulated transient activation of ERK induces cell proliferation, whereas a nerve growth factor-stimulated sustained activation of ERK induces differentiation (1, 2). Similarly, different growth factor ligands cause distinct kinetics of ERK activation in human breast cancer cells (3, 4). ERK and Akt/protein kinase B are deterministic kinases that control the activation of nuclear transcription factors (5–7); therefore, it is expected that the activation kinetics of these kinases might affect the following gene expression profiles. However, the question concerning gene expression dynamics induced by kinetically different kinase activities and its effect on cell determination mechanisms remains unsolved.

In this study we focused on the dose-dependent time course analysis of early transcription induced by two ligands of the ErbB family receptor, EGF and heregulin (HRG), which induce distinct kinase activity patterns and phenotypes of MCF-7 cells. Although many studies attempted to delineate the biochemical characteristics of ErbB ligands and receptors, no systematic study has been reported concerning the analysis of ErbB receptor-mediated cell fate control.

MCF-7 cells endogenously express all family members of the ErbB protein-tyrosine kinase receptors (EGFR/ErbB1, ErbB2, ErbB3, and ErbB4 receptors). EGF preferably binds to an EGFR, whereas HRG first binds to either the ErbB3 or ErbB4 receptor and then induces trans-activation of ErbB2 (8). ErbB receptors tend to form heterodimers in response to ligand binding when different ErbB receptors are co-expressed in the same cell. In particular, ErbB2 is the preferred heterodimerization partner among the receptor family (9), and it functions as an oncogenic unit through heterodimer formation with ErbB3 (10). EGFR activation induces self-down-regulation of the receptor by recruitment of the Cbl ubiquitin ligase (11, 12), and the activation of ErbB3 strongly evokes phosphatidylinositol 3’-kinase (PI3K) activation (13, 14). Activation of ErbB receptor is often accompanied by activation of Shc-ERK and PI3K-Akt pathways. These two pathways often cross-talk or inhibit each other (15, 16) thereby resulting in distinct activity patterns pertaining to intracellular signaling that activate various types of transcription factors (17–19). In addition, many studies attempted to identify specific gene expression induced by different ErbB ligands that show signaling diversities and distinct biological outcomes (20, 21); however, earlier results using platelet-derived growth factor (PDGF)-β receptor mutants indicated that diverse signaling pathways induce broadly overlapping transcription (22).
Our quantitative transcriptional analysis indicated that EGF and HRG also induced overlapping early transcription, however, with a large difference in their expression levels. Our results showed that ligand-specific dose-dependent activation of signal transduction pathways is precisely transmitted to early transcription, which was initially graded, induced ligand-oriented biphasic induction of c-Fos proteins after 20 min. For this mechanism, the duration of intracellular signaling, which was prolonged by ErbB receptor and, possibly, early transcription products, seemed to be responsible for this event. Our analysis indicated that MCF-7 cells utilize dose-dependent graded responses and transcriptional feedback to maintain signal duration to induce a biphasic response for cell determination and that expression of the ErbB receptor species is the most critical parameter to control the quantitative aspects of cellular processes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For growth factor treatment, cells were serum-starved for 16–24 h and then EGF or HRG-β176–246 was added (10 nM concentration was used unless otherwise indicated). For each inhibitor analysis, the inhibitors (AG1478, 100 nM; U0126, 200 nM; cycloheximide, 10 μM) were added 20 min prior to growth factor treatment.

**Gene Expression Analysis**—Cells were stimulated with 0.1, 0.5, 1, or 10 nM of either EGF or HRG for 5, 10, 15, 30, 45, 60 (HRG only), or 90 min. Cells untreated with growth factor were used as the control. Total RNA was isolated using TRIzol reagent (Invitrogen) and then purified using the Qiagen RNeasy mini kit. RNA quality was assessed using a Bioanalyzer (Agilent Technologies). GeneChip (Affymetrix U133A version 2) experiments were carried out according to the manufacturer’s protocol, and signals were processed according to the GeneChip expression analysis algorithm (GCOS version 1.2).

**Protein Phosphorylation Analysis**—For phosphorylation and protein measurements of Akt and ERK, the Bio-Plex Suspension Assay System (Bio-Rad) and Western blotting were used. For Western blotting, antibodies for detecting phospho-p44/42 ERK, ERK, phospho-Akt (Ser-473), and Akt (Cell Signaling Technology) were used. To detect ErbB receptor phosphorylation, immunoprecipitation (using corresponding anti-ErbB receptor antibody) followed by detection using anti-phosphotyrosine antibody (PY20) or anti-phospho-EGFR (pY1068) (Cell Signaling Technology) and anti-phospho-ErbB2 (pY1248) antibodies (Upstate) were used. Protein band intensities were quantified using a densitometer. Finally, the ratio of phosphorylation to total protein was calculated and normalized against the control.

**Cell Differentiation Assay**—To monitor differentiation, lipid droplets accumulated in MCF-7 cells were visualized with Oil Red. MCF-7 cells were incubated in the absence or presence of growth factor in serum-free Dulbecco’s modified Eagle’s medium. Follow-
ing cultivation for 14 days, cells were washed with phosphate-buffered saline and fixed with 10% formalin in phosphate-buffered saline for 1 h. After washing with water, the cells were stained with Oil Red O dye (0.6% in isopropanol water (6:4).

Statistical Analysis of Gene Expression Data—We developed a statistical model to divide gene expression changes into two components as follows: time course profiles and dose-dependent profiles (see supplemental Methods S1 for details). Gene expression (22,277 gene probes on an array) following each treatment (i.e. EGF or HRG) was modeled independently in the following manner. The log expression level for a gene following treatment is given by

\[ A(i) \times B(j) + C(j) + \text{error} \]

where \( A(i) \) and \( B(j) \) are dose levels, \( i = 1, \ldots, I \) and \( j = 1, \ldots, J \). For inhibitor data, a different stimulus was assigned to a different dose level (i.e., EGF only, HRG only). This model represents a generalization of the multiplicative decomposition model, where the time course profile is \( A(1), \ldots, A(I) \), and the dose-dependent profile is \( B(1), \ldots, B(J) \). The base line \( C(j) \) is allowed to depend on \( j \). These three components are estimated by fitting the model to the log-expression profile data. The constraint \( (B(1) + \cdots + B(J))/J = 1 \) is applied to resolve arbitrariness up to the constant factor, and smoothness of change in the time course and dose-dependent profiles is assumed. The weighted least squares method was used for model fitting. Estimated values are automatically derived from the model for missing values (at 60 min for 10 nM EGF). For gene selection, we used the log-fold change value defined as the maximum of the absolute values of \( A(i) \times B(j), \ldots, A(i) \times B(J) \). For inhibitor analysis, this value was calculated for each time course observed under a different stimulus, and the maximum value of these was used. We applied the normal approximation test to the log-fold change using the standard deviation estimated by a version of the bootstrap method (the resampling residuals methods). The \( p \) value is calculated to determine whether gene expression levels show more than a 1.5-fold change from the base line (for the inhibitor data analysis this value was set to 2.0). We selected genes with \( p \) values < 0.0001 so that the expected number of false discoveries is, at most, 22,277 \times 0.0001 \approx 2. Finally, we applied the PCA to the selected genes for visualization.

The time course profile is defined as \( A(1) \times B(j), \ldots, A(I) \times B(1) \) (i.e., fold changes from the base line for 10 nM), and the dose-dependent profile as \( A(i_{\text{max}}) \times B(1), \ldots, A(i_{\text{max}}) \times B(J) \), where \( i_{\text{max}} \) is the time at which the maximum change occurs (i.e., fold changes from the base line at the time of maximum change).

Hill Coefficient Analysis—Using EGF-activated ERK phosphorylation as an example, we assume that the response of \( y \) to EGF concentration \([\text{EGF}]\) is adequately approximated by a Hill equation (Equation 1),

\[ y = \frac{[\text{EGF}]^N}{K_{0.5}^N + [\text{EGF}]^N} \quad (\text{Eq. 1}) \]

\[ y = \frac{(\text{pERK} - \text{pERK}_{\text{basal}})}{\text{pERK}_{\text{max}}} \]

where \( y \) denotes the normalized concentration of doubly phosphorylated ERK as represented by \( y = (\text{pERK} - \text{pERK}_{\text{basal}})/(\text{pERK}_{\text{max}} - \text{pERK}_{\text{basal}}) \) with the basal level of phosphorylated ERK \( \text{pERK}_{\text{basal}} \) and the maximum level of phosphorylated ERK \( \text{pERK}_{\text{max}} \). \( K_{0.5} \) represents the concentration of [EGF] at which \( [\text{pERK}] \) is half-maximal and \( N \) represents a Hill coefficient.

FIGURE 1—continued
Quantitative Transcriptional Control by ErbB Receptor

A

Log-Expression Profile Data  Estimated Profile  Time-Course Profile  Dose-Dependent Profile

FOS (egf)  Model Fitting

FOS (egf)  dose

Multiplicative Decomposition Model

B

DUSP1 (hrg)  FOS (hrg)  EGR1 (hrg)  EGR2 (hrg)

DUSP1 (hrg) time  FOS (hrg) time  EGR1 (hrg) time  EGR2 (hrg) time

HCRP1 (hrg)  SPRED2 (hrg)  EGR4 (hrg)

HCRP1 (hrg) time  SPRED2 (hrg) time  EGR4 (hrg) time

F2RL1 (hrg)

F2RL1 (hrg) time

HBEFG (hrg)  PHLD1 (hrg)

HBEFG (hrg) time  PHLD1 (hrg) time

ERK  AKT

FIGURE 2
nonlinear fitting function of the Mathematica software program was used for data fitting.

RESULTS

**EGF and HRG Induce Different Kinetics of Intracellular Signaling Activities**—Incubation of EGF and HRG resulted in proliferation and differentiation (accumulation of lipid droplets in the cell) of MCF-7 cells, respectively (Fig. 1A). EGF and HRG induced phosphorylation of all ErbB receptors to some extent (Fig. 1B). In general, a receptor dimer containing EGFR tends to show a transient pattern of the receptor phosphorylation, because an activated EGFR is down-regulated by Cbl ubiquitination and lysosomal degradation (11, 23). It is known that mutation of the Cbl-binding site at Tyr-1045 on the EGFR abolishes the ubiquitination and enhances rapid recycling of the receptor (23). In our study, HRG-induced phosphorylation of EGFR and ErbB2 was initially transient and subsequently sustained. This pattern is thought to be caused by the consecutive
formation of ErbB1–3 and ErbB2–3 heterodimers (25). Because EGF mainly causes activation of EGFR as HRG does for ErbB2 and ErbB3 receptors (10), we measured EGFR phosphorylation for EGF-induced ErbB activation, and ErbB2 phosphorylation for HRG-induced activation to observe ligand dose-dependent receptor activation. Overall, EGF and HRG induced a transient and sustained dose-dependent phosphorylation of receptors, respectively, followed by similar activation kinetics of Akt and ERK (Fig. 1C). Thus, distinct kinetics of kinase activities seemed to result in different cell fate in MCF-7 cells in a similar manner as reported in PC12 cells (1, 2).

Ligand Dose-dependent Early Transcription Induced by EGF and HRG—Having determined the kinetic properties of intracellular signaling, a gene expression analysis was performed using a microarray in the presence of different concentrations (0.1, 0.5, 1 or 10 nM) of EGF or HRG for 5–90 min. It was hypothesized that transcription that is directly modulated by upstream pathways should be controlled in a dose-dependent manner. In an effort to identify such genes, we employed a statistical method, based on a multiplicative decomposition model (Fig. 2A) followed by gene selection (>1.5-fold change, \( p < 0.0001 \)) to analyze the effect of ligand dosage and time independently (see “Experimental Procedures” and supplemental Method S1). As a result, 63 EGF- and 251 HRG-regulated genes were selected, and 62 of the genes overlapped in the two groups (supplemental Table S1). Gene transcription was initiated very soon after ligand stimulation. Early responsive genes such as \( FOS, EGR, \) and \( DUSP \) were expressed just after 5 min, when the phosphorylation level of ERK was highest. Even in this short time, the time course profiles of each gene were quite distinct, suggesting the different regulation mechanisms for the transcription start for the different cluster of genes.

**FIGURE 2.** Ligand dose-dependent early transcription and ErbB signaling. *A*, multiplicative decomposition of a gene expression profile into time course and dose-dependent profiles. An example is shown for the \( FOS \) gene following EGF treatment. The multiplicative model was fitted to the log expression levels for seven time points (\( t1 = 5, t2 = 10, t3 = 15, t4 = 30, t5 = 45, t6 = 60, \) and \( t7 = 90 \) min) and four dose levels (\( d1 = 0.1, d2 = 0.5, d3 = 1, \) and \( d4 = 10 \) nM). B, PCA of time course profiles. The first (PC1) and second (PC2) principal components are plotted only for 62 genes, showing 1.5-fold significant changes following both EGF (red circle) and HRG (blue triangle) treatment, and each corresponding gene pair is indicated by green lines. Additionally, points for the phosphorylation profiles are plotted for ERK (black rectangle) and Akt (black circle). The relative position, particularly the direction, of the points from the base-line profile (solid) indicates major patterns of time course profiles. Representative expression profiles are shown around the graph. Larger views of A and B are also shown in supplemental Fig. S1. C, PCA of dose-dependent profiles. D, ligand dose-dependent phosphorylation of the ErbB receptor (at 2 min) and ERK and Akt (at 5 min). Fold increase of phosphorylation was determined by normalizing each value with total protein and then the control value. Two independent analyses were performed for each experiment. Mean and range are indicated in the graphs. \( K_{0.5} \) represents the concentration of EGF at which phosphorylated ERK is half-maximal, and \( N \) represents a Hill coefficient. Representative figures of the Western blots are shown in the upper part of the graph.
Furthermore, we found that apparent HRG-specific 189 genes were also modulated by EGF, even though the modulation levels were not statistically significant (supplemental Fig. S1). It meant that EGF-regulated genes are substantially contained in the HRG-induced gene groups, and both ligands do not induce qualitative difference in the early transcription. Thus, the phosphorylation levels of upstream kinases and gene expression triggered by growth factor-induced signal transduction pathways are highly regulated quantitatively but not qualitatively.

To find out kinetic features in the expression profiles of the 62 selected genes that were common to EGF and HRG treatment, we employed the PCA for visualization (Fig. 2, B and C, and supplemental Fig. S1). In the PCA plots, the genes are placed at positions according to similarities in the profiles. We are interested in which directions these points are placed relative to the base-line point because the direction reflects the profile shape pattern. In Fig. 2B, ERK and Akt show rapid increase in early time points followed by DUSP1, FOS, and JUN, and the profile peak shifts to later time points as the points of the PCA plot move in the clockwise direction. EGF-regulated genes are, in general, closer to the base line (i.e. smaller expression averages) than HRG-regulated genes; however, the directions (profile shapes) are almost the same for the connected gene pairs (Fig. 2B). The result implies that sustained upstream kinase activity does not induce a change in the duration of transcription (mRNA induction). In Fig. 2C, we observed that the expression change becomes gradually larger as the dose concentration increases in the EGF treatment, but it changes steeply in the HRG treatment. In summary, the result showed that although the effect of time for early transcription was relatively similar for the EGF- and HRG-regulated genes, the effect of the dosage was distinct. HRG-induced ErbB2 phosphorylation might be facilitated by high affinity receptor dimer than the receptor monomer (27), and HRG-induced ErbB2 phosphorylation revealed a positive cooperative mechanism in the signaling network system with higher Hill coefficients (HRG, n 1.87, and EGF, n 1.48 for receptor phosphorylation; HRG, n 2.19, and EGF, n 1.27 for ERK phosphorylation; and HRG, n 2.11, and EGF, n 1.31 for Akt phosphorylation) (Fig. 2D).

Ligand Dose-dependent Property of Early Transcription Is Determined by the Membrane Receptor—To explain ligand-dependent quantitative profiles of early transcription, we analyzed the ligand-dose phosphorylation responses of ErbB receptor, ERK and Akt (Fig. 2D). Analysis of the phosphorylation response of these kinases to the HRG concentration revealed a positive cooperative mechanism in the signaling network system with higher Hill coefficients (HRG, n 1.87, and EGF, n 1.48 for receptor phosphorylation; HRG, n 2.19, and EGF, n 1.27 for ERK phosphorylation; and HRG, n 2.11, and EGF, n 1.31 for Akt phosphorylation) (Fig. 2D). Similar cooperative phosphorylation of the ErbB2 receptor has been observed for antibody-induced tyrosine phosphorylation of p185 neu (26). ErbBligand tends to show high affinity for the receptor dimer than the receptor monomer (10, 28).

Effect of Kinase Inhibitors on ERK and Akt Phosphorylation—Although duration of the upstream kinase activity does not cause any effect for duration of transcription, our results suggest that the quantitative characteris-
tics of dose dependence (or sensitivity) of the receptor phosphorylation responding to the growth factor have a large effect for early transcription. A comparison of the 62 EGF- and HRG-regulated overlapping genes revealed that whereas the expression level of particular genes was almost identical regardless of ligand types, HRG often markedly regulated the intensity of gene expression more than EGF did (supplemental Table S1). ERK and Akt cooperatively facilitate cellular transformation (29, 30) and often have overlapping downstream transcription factor targets (18); therefore, it is most likely that both kinases cooperatively contribute toward downstream gene expression. In MCF-7 cells, HRG showed higher Akt phosphorylation than EGF did (1.5–2-fold), whereas both ligand-induced ERK phosphorylations reached almost the same levels (Fig. 1C). Consequently, HRG-triggered high Akt activity might result in a higher transcriptional induction.

To confirm the contribution of ErbB receptor-mediated ERK and Akt activities to early transcription, the effect of kinase inhibitors, U1026 (a MEK inhibitor) and AG1478 (an EGFR kinase inhibitor), was examined in the presence of HRG. We could not use a PI3K inhibitor to suppress Akt activity in our study because the inhibitor also lowered ERK phosphorylation (data not shown), probably because of the presence of cross-talk between the MAPK and PI3K-Akt pathways in MCF-7 cells. Therefore, we decided to compare expression levels of the genes obtained from the cells treated with the EGFR kinase inhibitor (to suppress both ERK and Akt activities) or the MEK inhibitor (to suppress only ERK) in the presence and absence of HRG. If the Akt contributed more for HRG-induced gene expression than did ERK, the gene that showed larger transcriptional change after HRG stimulation (high HRG/EGF transcription ratio) should show higher sensitivity to the EGFR kinase inhibitor.

Following the transcriptional assay, 124 genes were selected as U0126-regulated genes. Of these, 89 genes were overlapped in the 251 gene set selected for the HRG dose-dependent experiment. As for AG1478, 144 genes were selected as AG1478-regulated genes, and 92 genes were overlapped with the same 251 gene set (supplemental Table S2). A reason for the lower number of selected genes resulted from a higher threshold used for gene pre-screening (1.5-fold change to 2.0-fold change), and the decreased overlapping resulted from the presence (for dose-dependent) or absence (for inhibitor test) of zero adjustment for the control (see supplemental Method S1). The result (Fig. 3 and supplemental Fig. S2) showed that most of the genes (89 for U0126-regulated genes and 92 for AG1478-regulated genes) were more or less equally regulated by both AG1478 and U0126. However, further quantitative analysis indicated that genes with higher expression levels in the HRG-treated cells compared with the EGF-treated cells were likely to be more AG1478-sensitive (Fig. 3, B and C, and supplemental Fig. S2). It could be that the higher HRG induced gene expression levels less dependent on ERK and perhaps more dependent on Akt activity.

**Ligand-oriented Biphasic c-Fos Production**—Thus far, this study has indicated that EGF- and HRG-induced activation of ErbB receptor signaling and early transcription differed quantitatively but not qualitatively. Why then is it that only HRG allows MCF-7 cells to be differentiated?

We examined protein induction and observed HRG-oriented biphasic induction of c-Fos after 20 min (Fig. 4A). Previous studies using Swiss 3T3 fibroblasts showed that PDGF-stimulated sustained ERK activity could stabilize c-Fos protein through DEF (FXFP) domain-mediated phosphorylation and therefore induced sustained induction of c-Fos (31, 32). In
MCF-7 cells, both HRG-induced sustained and EGF-induced transient ERK activation resulted in transient production of c-Fos (Fig. 4B). Although the EGF- and HRG-induced FOS transcription levels were almost identical in this study (Fig. 2B), only HRG yielded a higher induction of c-Fos protein. Our analyses on induction of Myc protein, which also possesses the DEF domain, showed the same ligand-oriented biphasic response, whereas c-Jun (no DEF domain) did not show such a response (data not shown). The result was consistent with an earlier study (31, 33); consequently, HRG-induced sustained ERK activity is thought to be responsible for the stabilization of c-Fos protein. In our sequence analysis, 22 genes (including gene coding for transcription factors, DUSPs, PPP1R15A (GADD34), and PPP1R3D (PPP1R6)) out of the 251 HRG-modulated genes were identified to possess the DEF sequence (supplemental Table S3). Thus, biphasic induction of transcription factors and signaling proteins stabilized by HRG-induced prolonged ERK activity could serve for the next transcriptional circuit.

**Ligand-induced Early Transcription in MCF-7 Cells May Induce Feedback to Upstream Signaling**—It was noted that both EGF and HRG were able to regulate genes that code for autocrine ligands (34–36) and genes that have been reported to play a role in signal transduction pathways (supplemental Table S1). EPHA2 and SPRED2 have been shown to negatively regulate Ras and Raf activity, respectively (37–39). Genes that were significantly modulated by HRG included an ErbB2 receptor inhibitory protein (TOB2) (40), autocrine growth factors (TGFβ2 and VEGF) (36, 41), kinases (MAPK2K3 and MAP2K8) (42), and phosphatases (DUSP4, DUSP8, and DUSP10) (43–45) that regulate the MAPK cascade. The HRG also regulated genes that code for regulatory subunits of protein phosphatase 1 (PPP1R15A, PPP1R3C (PPP1R5), and PPP1R3D), AKAP9 (46–49), which promotes dephosphorylation of ErbB2-mediated phosphorylation of Akt (49), and PIP5K1A, an Akt activator (50). Thus, early transcription induced a number of genes that are able to regulate ErbB receptor-stimulated signaling.
Consistent with this observation, treatment with a protein synthesis inhibitor (cycloheximide) altered the protein and phosphorylation levels of ERK and Akt in HRG-treated cells after 20 min but not in EGF-treated cells (Fig. 5B). HRG-induced ERK phosphorylation (not total ERK protein) was strongly up-regulated by cycloheximide, indicating a suppression of ERK dephosphatases (DUSPs). Akt phosphorylation seemed to be indirectly controlled by positive (up-regulation of PIP5K1A and PPP1R15A) and negative (down-regulation of PPP1R3C, PPP1R3D, and AKAP9) regulation of PP1 (49). Both ERK and Akt protein levels decreased following cycloheximide treatment. MAPKs were listed in our data set, but not a gene encoding Akt. Accordingly, it was assumed that the Akt protein level is controlled by an alternative mechanism. Overall, our analysis suggested that the HRG-induced sustained intracellular signaling pathway is stabilized by early transcription products to maintain its activity.

**DISCUSSION**

This study on the ErbB receptor-mediated early transcription indicated that the quantitative feature of transcriptional regulation was determined by receptor activation kinetics. Although previous gene expression studies using M-CSF-stimulated M-CSF-PDGF receptor chimeras showed that diverse signaling pathways resulted in broadly overlapping early gene expression (22), another experimental setup using similar PDGF receptor chimeras, which possess different ligand affinity and duration of ERK activity, showed divergent embryological functions in mice (51). Taken together, our data suggested that a property of various ligand-induced ErbB receptor signaling is to control cells by means of quantitative magnitude, not specificity (52) at the early stage of transcription for cell fate determination.

To confirm whether or not the genes significantly modulated in our analysis are commonly observed for other experimental setups, we compared the gene lists that appeared in the previous studies using the PDGF-stimulated T98G human glioblastoma cells (18) and M-CSF-stimulated NIH3T3 cells that express the M-CSF-PDGF receptor chimera (22). For comparison, GenBank™ accession numbers in the original papers were mapped to the Entrez Gene ID using Gene Data Base. Genes without the Entrez Gene ID, which were mostly derived from dbEST, were then searched against the UniGene Data Base. For mouse genes in the NIH3T3 cells, the HomoloGene Data Base was also utilized. As a result, 28 transcripts were overlapped between 62 EGF/HRG common transcripts observed in MCF-7 datasets and 74 transcripts in the PDGF-stimulated T98G datasets (45.2% of the MCF-7 transcripts), and 33 transcripts were overlapped (within 66 transcripts) in the M-CSF-stimulated NIH3T3 datasets (53.2% of the MCF-7 transcripts), respectively, regardless of differences in ligands, cell types, and analytical criteria. Therefore, although we used MCF-7 human breast cancer cells to attempt to delineate the growth hormone-induced cell fate control mechanism, our current results might indicate a general trend of gene expression regulated by the ligand-stimulated receptor signal transduction pathways.

In addition, ligand-induced early transcription may have an
effect on controlling duration of intracellular ERK activity to stabilize the transcriptional product. HRG induced a greater level of gene expression in general, and therefore might facilitate more potent and stable feedback to prolong intracellular signaling activity (Fig. 6). It is noted that this quantitative feedback strategy utilized in MCF-7 differentiation resembles that observed during various development events. For example, with EGF signaling during Drosophila development, cells receive multiple feedback by effectors such as ARGOS (an EGFR signaling inhibitor), Sprouty (SPRED2-related protein, Ras suppressor), Kekkon1 and Rhomboid-1 (activator of ligand production), and Vein (a ligand for EGFR) (24), where receptor concentration (or ligand affinity) appears to be the most critical parameter. We speculated that this kind of graded-and-biphasic strategy might be universally utilized in higher organisms to switch cellular states.

Interestingly, a ligand-stimulated receptor activation kinetics is converted into amplitude and duration of intracellular kinase activity, and then this two-dimensional information was converted solely to the amplitude and duration of intracellular ERK and Akt activities induced by the ligands. Based on this assumption, we constructed a mathematical model in order to estimate how the intensity of gene expression is controlled by EGF- and HRG-initiated Ras-MAPK and PI3K-Akt pathways (supplemental Method S2). Our simulation analysis indicated that the model well explains our experimental data and that not only the intensity but also the duration of phosphorylation of both ERK and Akt may have a substantial influence on the intensity of the gene expression. If this is the case, it is estimated that the sensitivity, in terms of the system gain of phosphorylated Akt, is 4.8 times larger than that of ERK when expression intensity of a gene in the HRG-treated cells is 3.27 times larger than in the EGF-treated cells.

In MCF-7, HRG induced graded signaling and early transcription, followed by auto-induction of multiple positive/negative feedback mechanisms, and prolongation of signaling activity might switch cells irreversibly. We hypothesize that this biphasic supply of transcription factors and signaling mediators acts to change secondary gene expression for HRG-induced cellular differentiation of MCF-7 cells. Indeed, we could identify a time-series interaction of several transcription factors whose expression levels were significantly altered by HRG, but not by EGF, as a result of up to 72 h exposure of the ligands with the MCF-7 cells (the mechanism is under investigation). In the case of the short term and long term of gene expression analyses, the initial activation kinetics of the ErbB receptor seemed to be the first and most critical factor in controlling transcription for proceeding with cell differentiation. We believe that further quantitative integrated analysis of ErbB receptor signaling will prove this hypothesis.

REFERENCES

1. Kao, S., Jaiswal, R. K., Kolch, W., and Landreth, G. E. (2001) J. Biol. Chem. 276, 18169–18177
2. York, R. D., Molliver, D. C., Grewal, S. S., Stenberg, P. E., McCleskey, E. W., and Stork, P. J. (2000) Mol. Cell. Biol. 20, 8069–8083
3. Neve, R. M., Holbro, T., and Hynes, N. E. (2002) Oncogene 21, 4567–4576
4. Thottassery, J. V., Sun, Y., Westbrook, L., Rentz, S. S., Manuvakhova, M., Qu, Z., Samuel, S., Upshaw, R., Cunningham, A., and Kern, F. G. (2004) Cancer Res. 64, 4637–4647
5. Bouchard, C., Marquardt, J., Bras, A., Medema, R. H., and Eilers, M. (2004) EMBO J. 23, 2830–2840
6. Pandey, S. K., He, H. J., Chesley, A., Juhaszova, M., Crow, M. T., and Bernier, M. (2002) Endocrinology 143, 375–385
7. Yang, S. H., Whitmarsh, A. J., Davis, R. J., and Sharrocks, A. D. (1998) EMBO J. 17, 1740–1749
8. Carraway, K. L., III, Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C., and Cerione, R. A. (1994) J. Biol. Chem. 269, 14303–14306
9. Tzahar, E., Waterman, H., Chen, X., Levkovitz, G., Karunagaran, D., Lavi, S., Ratziuk, B. J., and Yarden, Y. (1996) Mol. Cell. Biol. 16, 5276–5287
10. Holbro, T., Beeri, R. R., Maurer, F., Koziczak, M., Barbas, C. F., III, and Hynes, N. E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 8933–8938
11. Baulida, J., Kraus, M. H., Alimandi, M., Di Fiore, P. P., and Carpenter, G. (1996) J. Biol. Chem. 271, 5251–5257
12. Levkovitz, G., Klapper, L. N., Tzahar, E., Freywald, A., Sela, M., and Yarden, Y. (1996) Oncogene 12, 1117–1125
13. Hellyer, N. J., Kim, M. S., and Koland, J. G. (2001) J. Biol. Chem. 276, 42153–42161
14. Ram, T. G., Hosick, H. L., and Ethier, S. P. (2000) J. Cell. Physiol. 183, 301–313
15. Moelling, K., Schad, K., Bosse, M., Zimmermann, S., and Schwener, M. (2002) J. Biol. Chem. 277, 31099–31106
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16. Mograbi, B., Bocciardi, R., Bourget, J., Busca, R., Rochet, N., Farahi-Far, D., Juhel, T., and Rossi, B. (2001) J. Biol. Chem. 276, 45307–45319
17. Du, K., and Montminy, M. (1998) J. Biol. Chem. 273, 32377–32379
18. Tullai, J. W., Schaffer, M. E., Mullenbrock, S., Kasif, S., and Cooper, G. M. (2004) J. Biol. Chem. 279, 20167–20177
19. Xing, J., Ginty, D. D., and Greenberg, M. E. (1996) Science 273, 959–963
20. Sweeney, C., Fambrough, D., Huard, C., Diamonti, A. J., Lander, E. S., Cantley, L. C., and Carraway, K. L., III (2001) J. Biol. Chem. 276, 22685–22698
21. Amin, D. N., Tuck, D., and Stern, D. F. (2005) Exp. Cell Res. 309, 12–23
22. Fambrough, D., McClure, K., Kazlauskas, A., and Lander, E. S. (1999) Cell 97, 727–741
23. Grovdal, L. M., Stang, E., Sorkin, A., and Madshus, I. H. (2004) Exp. Cell Res. 300, 388–395
24. Freeman, M. (2000) Nature 408, 313–319
25. Graus-Porta, D., Beerli, R. R., Daly, J. M., and Hynes, N. E. (1997) EMBO J. 16, 1647–1655
26. Yarden, Y. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2569–2573
27. Uyemura, T., Takagi, H., Yanagida, T., and Sako, Y. (2005) Biophys J. 88, 3720–3730
28. Slawkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., III (1994) J. Biol. Chem. 269, 14661–14665
29. Hayashi, K., Takahashi, M., Kimura, K., Nishida, W., Saga, H., and Sobue, K. (1999) J. Biol. Chem. 274, 727–740
30. Li, W., Zhu, T., and Guan, K. L. (2004) J. Biol. Chem. 279, 37398–37406
31. Murphy, L. O., Smith, S., Chen, R. H., Fingar, D. C., and Blenis, J. (2002) Nat. Cell Biol. 4, 556–564
32. Murphy, L. O., Mackeigan, J. P., and Blenis, J. (2004) Mol. Cell. Biol. 24, 144–153
33. Mackeigan, J. P., Murphy, L. O., Dimitri, C. A., and Blenis, J. (2005) Mol. Cell. Biol. 25, 4676–4682
34. Johnson, G. R., Kannan, B., Shoyab, M., and Stromberg, K. (1993) J. Biol. Chem. 268, 2924–2931
35. Riese, D. J., Kim, E. D., Elenius, K., Buckley, S., Klagsbrun, M., Plowman, G. D., and Stern, D. F. (1996) J. Biol. Chem. 271, 20047–20052
36. Semino, C. E., Kamm, R. D., and Lauffenburger, D. A. (2006) Exp. Cell Res. 312, 289–298
37. Davy, A., Aubin, J., and Soriano, P. (2004) Genes Dev. 18, 572–583
38. Macrae, M., Neve, R. M., Rodriguez-Viciana, P., Haqq, C., Yeh, J., Chen, C., Gray, J. W., and McCormick, F. (2005) Cancer Cell 8, 111–118
39. Wakioka, T., Sasaki, A., Kato, R., Shouda, T., Matsumoto, A., Miyoshi, K., Tsuneoka, M., Komiya, S., Baron, R., and Yoshimura, A. (2001) Nature 412, 647–651
40. Matsuda, S., Kawamura-Tsuzuku, I., Ohsugi, M., Yoshida, M., Emi, M., Nakamura, Y., Onda, M., Yoshida, Y., Nishiyama, A., and Yamamoto, T. (1996) Oncogene 12, 705–713
41. Wenner, C. E., and Yan, S. (2003) J. Cell. Physiol. 196, 42–50
42. Das, S., Cho, J., Lambertz, I., Kellilher, M. A., Eliopoulos, A. G., Du, K., and Tsichlis, P. N. (2005) J. Biol. Chem. 280, 23748–23757
43. Bennett, A. M., and Tonks, N. K. (1997) Science 278, 1288–1291
44. Guan, K. L., and Butch, E. (1995) J. Biol. Chem. 270, 7197–7203
45. Yin, Y., Liu, Y. X., Jin, Y. J., Hall, E. J., and Barrett, J. C. (2003) Nature 422, 527–531
46. Armstrong, C. G., Browne, G. J., Cohen, P., and Cohen, P. T. (1997) FEBS Lett. 418, 210–214
47. Greenberg, C. C., Meredith, K. N., Yan, L., and Brady, M. J. (2003) J. Biol. Chem. 278, 30835–30842
48. Hollander, M. C., Pooja-Kella, S., and Fornace, A. J., Jr. (2003) Oncogene 22, 3827–3832
49. Xu, W., Yuan, X., Jung, Y. J., Yang, Y., Basso, A., Rosen, N., Chung, E. J., Trepel, J., and Neckers, L. (2003) Cancer Res. 63, 7777–7784
50. Carricaburu, V., Lamia, K. A., Lo, E., Favereaux, L., Payrastre, B., Cantley, L. C., and Rameh, L. E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 9867–9872
51. Klinghoffer, R. A., Mueting-Nelsen, P. F., Faerman, A., Shi, M., and Soriano, P. (2001) Mol. Cell 7, 343–354
52. Jones, R. B., Gordus, A., Kralli, J. A., and MacBeath, G. (2006) Nature 439, 168–174