CANCER GENOMICS & PROTEOMICS 5: 161-168 (2008)

Phosphoproteome and Transcriptome Analyses of ErbB Ligand-stimulated MCF-7 Cells

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Abstract. *Cellular signal transduction pathways and gene expression are tightly regulated to accommodate changes in response to physiological environments. In the current study, molecules were identified that are activated as a result of intracellular signaling and immediately expressed as mRNA in MCF-7 breast cancer cells shortly after stimulation of ErbB receptor ligands, epidermal growth factor (EGF) or heregulin (HRG). For the identification of tyrosine-phosphorylated proteins and expressed genes, a SILAC (stable isotopic labeling using amino acids in cell culture) method and Affymetrix gene expression array system, respectively, were used. Unexpectedly, the overlapping of genes appeared in two experimental datasets was very low for HRG (43 hits in the proteome data, 1,655 in the transcriptome data, and 5 hits common to both datasets), while no overlapping gene was detected for EGF (15 hits in the proteome data, 211 hits in the transcriptome data, and no hits common to both datasets). The HRG overlapping genes included ERBB2, NEDD9, MAPK3, JUP and EPHA2. Biological pathway analysis indicated that HRG-stimulated molecular activation is significantly related to cancer pathways including bladder cancer, chronic myeloid leukemia and pancreatic cancer ($p<0.05$). The proteome datasets of EGF and HRG contain molecules that are related to Axon guidance, ErbB signaling and VEGF signaling at a high rate.*

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Key Words: SILAC, transcription, phosphorylation, proteome, breast cancer, heregulin, epidermal growth factor, ErbB, MCF-7.

Cells respond to external stimuli and immediately activate intracellular signal transduction pathways to adapt to the surrounding environment (1-3). These pathways consist of protein phosphorylation cascades followed by *de novo* gene expression. The purpose of gene expression is to supply and replenish necessary cellular proteins that impact on cell fate in terms of proliferation, differentiation or apoptosis. Therefore, if an existing protein is phosphorylated and its corresponding mRNA immediately expressed, such a protein should play a critical role in maintaining requisite activation pathways within the cell until cellular transition is made. When this is not the case, such phosphorylated proteins should be recycled through dephosphorylation under conditions where the signaling activity persists. Additionally, new stimulus-responsive gene expression products should possess functions that enable cells to adapt to altered physiological conditions (4).

In this study, our aim was to identify protein molecules that are activated and immediately expressed as mRNA in MCF-7 breast cancer cells shortly after growth hormone stimulation. Epidermal growth factor (EGF) and heregulin (HRG) were used as growth hormone stimulants; both are known to be ErbB receptor ligands. EGF and HRG induce distinct signaling durations, transient and persistent, accompanied by different cellular effects in MCF-7 cells, comprising cell proliferation and differentiation, respectively (5, 6).

For the determination of activated signaling proteins, a SILAC (stable isotopic labeling using amino acids in cell culture) method was employed and proteins modified by tyrosine phosphorylation were identified (7). This method allows us to identify dozens of known and unknown proteins that become phosphorylated following ligand stimulation at specific time points. For the identification of newly synthesized genes, the Affymetrix gene expression array system was used.

Based on these two kinds of high throughput data, a series of genes that appeared in the proteome, transcriptome or both datasets were obtained together with their relationship to biological functions.

Materials and Methods

Cell culture. The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and routinely maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Githersburg, MD, USA) supplemented with 10% fetal bovine serum. For the SILAC analysis, cells were labeled using stable isotopes of L-arginine or L-[U-13C6,14N4] arginine as described elsewhere (8). Prior to the growth factor treatment, cells were serum-starved for 16-24 hours, 10 nM EGF (PeproTech House, London, UK), or HRG β176-246 (R&D Systems, Inc., Minneapolis, MN, USA) was then added and the cells were incubated for a further 5 min. Non-stimulated cells were used for control.

SILAC-encoded phosphoproteome analysis. After washing three times with PBS, the harvested cells were lysed in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% Na-deoxycholate, 1 mM Na-orthovanadate, protease inhibitors (Complete Mini, Roche Diagnostics, Basel, Switzerland). Each lysate was quantified using the BioRad DC protein assay reagent (BioRad Laboratories, Hercules, CA, USA) and mixed in equal ratios. Immunoprecipitation of the tyrosine-phosphorylated protein complex was performed as described elsewhere (8), and the elution of target molecules was achieved using 25 mM phenyl phosphate. The eluted protein complex was digested with trypsin directly in the solution, desalted using ZipTip C-18™ (Millipore, Billerica, MA, USA), and finally concentrated to a volume of ~20 μl which was then injected into a nanoLC system. After applying the peptide mixture to a C-18 column (800 μm ID×3 mm long, KYA technologies, Tokyo, Japan), reversed-phase separation of the captured peptides was performed on a column (150 μm ID×75 mm long) filled with HiQ sil C-18 (3-μm particles, 120-Å pores; KYA technologies, Tokyo, Japan) using a direct nanoflow LC system (Dina; KYA technologies). The peptides were eluted with a linear 5-65% gradient of acetonitrile containing 0.1% formic acid over 120 min at a flow rate of 200 nl/min and then sprayed into a quadrupole time-of-flight tandem mass spectrometer (Q-Tof-2; Micromass, Manchester, UK/Q-STAR elite; Applied Biosystems, Foster City, CA, USA) (9). The acquired MS/MS spectra were then processed against the RefSeq (NCBI) protein database (10) using the Mascot algorithm (Matrix Science, London, UK) with the following parameters: variable modifications, oxidation (Met), *N*-acetylation, pyroglutamination (Gln), phosphorylation (Ser, Thr, Tyr), stable isotopes of arginine-13C6 (Arg); maximum missed cleavages, 3; peptide mass tolerance, 500 ppm; MS/MS tolerance, 0.5 Da. Protein identification was based on the criterion of at least one MS/MS data with a Mascot score >35.

Data analysis.

Phosphoproteome. Regarding the peptides with a Mascot score >25, the extent of activation at 5 min following growth factor stimulation was automatically calculated and integrated for each protein through AYUMS (11) for the Q-Tof-2 data and MSQuant (12) for the Q-STAR elite data. Proteins that showed more than a 1.5-fold increase or decrease were regarded as ligand-regulated molecules.

Transcriptome analysis. Part of the gene expression data previously obtained and analyzed (5) using the Affymetrix Human Genome U133A 2.0 array platform containing 14,500 well-characterized genes was used. For the current analysis, only the following time course datasets were used: MCF-7 cells non-stimulated (control) or stimulated with 10 nM EGF or HRG for 5, 10, 15, 30, 45, 60 and 90 min (EGF lacks 60 min data). Prior to gene selection, probe sets were filtered using the following criteria: i) maximum expression level smaller than 100, ii) the number of present calls less than 2 and iii) probe sets have no Entrez Gene ID. Probe sets showing larger than 2-fold changes against the control at two consecutive time points were then regarded as growth hormone-induced genes.

Gene ontology (GO) analysis. Gene ontology terms and generic GO slim terms were utilized to investigate the functional properties of selected genes. RefSeq protein accession numbers and probe set IDs were converted to Entrez Gene IDs using NCBI's accession mapping table (gene2accession) and the manufacturer's annotation dataset (NetAffx Release #24), respectively. The relationship between the GO ID and gene ID was then extracted from NCBI's annotation (gene2go). All GO IDs were mapped to the second level of generic GO slim terms and the number of genes was counted for each GO category.

Pathway and functional annotation analysis. The KEGG pathway database was utilized to analyze the enrichment of biological pathways for the selected molecules. Selected proteins and probe sets were converted to Entrez Gene IDs in the same way as for the GO analysis. Pathway enrichment was tested using Fisher's exact test followed by Bonferroni's correction.

To investigate gene function and its relation to the ErbB signaling pathway of ligand-induced phosphorylated proteins, protein interaction dataset provided as part of the Entrez Gene database was used in addition to KEGG and GO. Those interactions where both gene IDs are derived from the human gene were extracted from the NCBI interaction dataset. All binary relationships, except enzyme–compound and protein–protein relationships with subtype value 'state', 'compound' and 'indirect', were extracted from the KEGG pathway database. An interaction network was then constructed from these binary relationships using i) both the KEGG and NCBI datasets and ii) only the KEGG dataset. A protein which was associated with a path to EGFR, ErbB2, ErbB3 and ErbB4 in the reconstructed network was regarded as an ErbB signaling pathway-related protein. Furthermore, proteins associated with the MAPK signaling pathway and ErbB signaling pathway were also regarded as ErbB signaling-related. These proteins are shown by the black box in the top three rows in the lower panel of Figure 1 and are labeled 'ErbB1/2/3/4(N+K)', 'ErbB1/2/3/4(K)' and 'KEGG(MAPK/ErbB)', respectively. Other criteria were also employed in an effort to identify proteins involved in signal transduction pathways other than the ErbB or MAPK signaling pathways. Proteins associated with signal transducer activity (GO:0004781) and signal transduction (GO:0007165), are shown by the black box in the 5th row (labeled as 'signal transduction') in the lower panel of Figure 1. In the same way, a protein is highlighted in the phosphorylation row if associated with phosphorylation (GO:0016310). Proteins are highlighted in the row labeled as kinase if associated with kinase activity (GO:0016301), kinase regulator activity (GO:0019207) and protein protein relationship with subtype value phosphorylation of the KEGG pathway. The label 'phosphatase' was assigned to those proteins annotated as 'protein phosphatase activity' (GO:0004721), 'phosphatase regulator activity' (GO:0016208) and 'dephosphorylation' of the KEGG pathway.

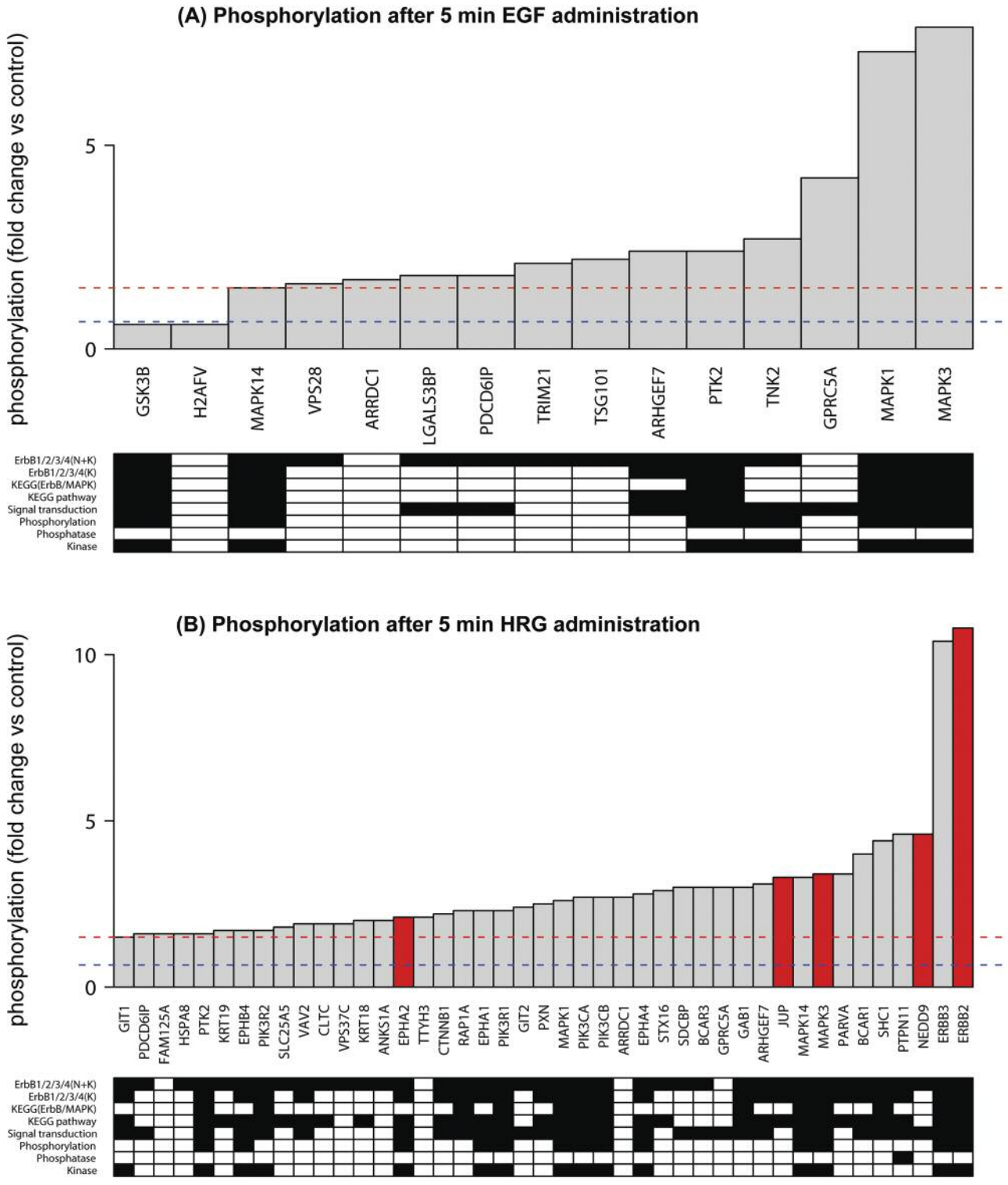

Figure 1. *Changes in phosphorylation induced by growth hormone stimulation. The relative ratio of phosphorylated proteins following 5 minutes of EGF and HRG treatment compared with the control (without growth hormone stimulation) are shown in (A) and (B), respectively. The upper and lower panels in each figure represent the fold change in phosphorylation and gene function in relation to the ErbB signaling pathway, respectively. In the upper panel, red and blue dashed lines along the horizontal axis represent a 1.5-fold increase and decrease, respectively. Those genes whose expression level increased following growth hormone stimulation are designated by red bars. The lower part of the figure is a graphical representation of gene function and its relation to the ErbB signaling pathway compiled from public databases (see Materials and Methods for details of the annotation method).*

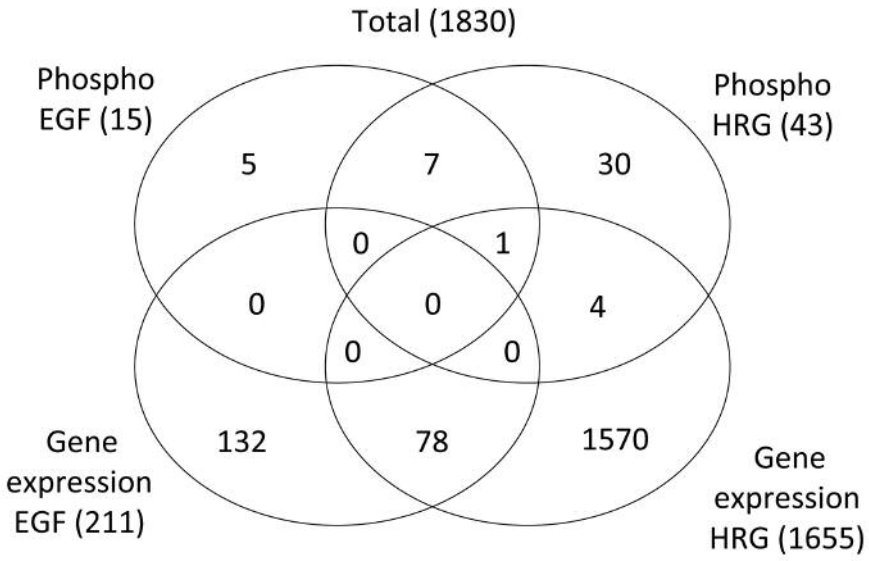

Figure 2. *The number of growth hormone-induced genes. The number of genes detected by proteome and transcription analysis of EGF- or HRG-stimulated MCF-7 cells is shown in the Venn diagram. Given that 4 common genes between phospho (EGF) and gene expression (HRG), and 1 common gene between phospho (HRG) and gene expression (EGF) could not be drawn in the figure, the sum of the numbers shown in the Venn diagram is smaller than the actual total.*

Results

Initially, gene IDs taken from the transcriptome and phosphoproteome data of EGF- or HRG-stimulated MCF-7 cells were compared. HRG showed larger data sizes compared with EGF in the two experimental datasets (Table I). Additionally, HRG stimulation induced a higher percentage of up-regulated molecules in terms of phosphorylation (EGF, 13/15=86.7%; HRG, 43/43 100%) and gene expression (EGF, 155/211=73.5%; HRG, 1449/1655=87.6%) compared with EGF. However, genes in both the proteome and transcriptome datasets showed minimal overlap (Figure 2). Overlapping genes appeared in different experimental datasets to a small extent for both EGF (15 hits in the proteome data, 211 hits in the transcriptome data, and no hits common to both datasets) and HRG (43 hits in the proteome data, 1,655 hits in the transcriptome data, and 5 hits common to both datasets). Furthermore, there was no overlap in both two experimental datasets for both EGF and HRG.

Next, we investigated the gene ontology (GO) of selected genes from each analysis in an effort to see the picture of the overall cellular state (Figure 3) (13). Graphical patterns showing molecular function ontology (Figure 3A) and biological process ontology (Figure 3B) were almost identical for EGF and HRG. However, EGF showed a larger increase in phosphoproteins related to transport and response to biotic stimulus (Figure 3B). On the other hand, HRG stimulation showed increases in phosphoproteins related to structural molecule activity (Figure 3A) and multicellular organismal development (Figure 3B). Differences between the proteome and transcriptome data were also observed. As expected, a higher percentage of genes related to signal transducer activity (Figure 3A) and cell communication (Figure 3B) was found in both proteome datasets. On the other hand, genes related to transcription regulator activity (Figure 3A) were more frequently found in the transcriptome data. A large number of protein molecules related to binding, metabolic process and regulation of biological process were phosphorylated and expressed for EGF and HRG, suggesting a rapid turnover of these processes regardless of ligand species.

Table I. *The number of genes induced by growth hormone stimulation.*

	Up-regulated	Down-regulated	Total
Phospho			
(EGF)	13	2	15
(HRG)	43	0	43
Gene expression			
(EGF)	155	56	211
(HRG)	1449	222	1655

A detailed pathway enrichment analysis was then performed using public pathway databases for each experimental dataset. Table II shows results obtained using the KEGG pathway database (14). For both ligands, a large number of signaling and cancer-related pathways showed

significant changes in protein phosphorylation following ligand stimulation. In particular, the ErbB signaling pathway and MAPK signaling pathway, as expected, and Axon guidance were significant ($p<0.00005$), whereas only one common pathway (MAPK signaling pathway) observed for EGF-induced protein phosphorylation and gene expression, bladder cancer, chronic myeloid leukemia and pancreatic cancer-related pathways was common in the HRG-treated datasets in addition to the MAPK signaling pathway ($p<0.05$).

Integration of the proteome and gene expression datasets indicated that molecules such as ERBB2, NEDD9, MAPK3, JUP and EPHA2 showed over 2-fold increases in protein phosphorylation and gene expression in response to HRG, while such an increase was not significant for EGF (Figure 1). Of these, *ERBB2, NEDD9, MAPK3* and *EPHA2* have been shown to have potential connections with cancer progression (15-18), and *JUP* has been shown to act as a tumor suppressor gene (19). *ERBB2* and *MAPK3* are the main players involved in ErbB signaling pathways (20). The aforementioned result is consistent with the fact that HRG induces persistent activation of the ErbB signaling pathway for several hours and that the protein molecules involved should be recycled or supplied *de novo*. All of the genes associated with HRG-induced altered levels of protein phosphorylation and expression play significant roles in the onset and progression of cancer.

Finally, pathway and reference information was gathered for each molecule in the EGF and HRG phosphoproteome datasets (lower columns in Figure 1). This information indicated that the phosphoproteins identified in our analysis have been shown to be directly or indirectly involved in the ErbB receptor signal transduction pathway. However, molecules such as ARRDC1 (arrestin domain containing 1), TTYH3 [tweety homolog 3 (Drosophila)], FAM125A (family with sequence similarity 125, member A) and H2AFV (H2A histone family, member V) appeared in either EGF, or HRG, or both datasets, thus indicating the rare finding that these proteins are associated with signal transduction pathways.

Discussion

In this study, an integrated analysis of two high throughput datasets was performed for the first time: protein tyrosine phosphorylation analyzed by LC/MS (phosphoproteome) and immediate transcription analyzed by a GeneChip array (transcriptome) on EGF- or HRG-stimulated MCF-7 cells. Genes associated with altered levels of protein phosphorylation or expression following ligand stimulation were annotated using public databases or references. EGF and HRG induce distinct cell fates (5), and the overlapping of genes in each dataset in the current analysis was shown to be low. On the other hand, both EGF and HRG are ErbB receptor ligands and are known to stimulate the ErbB receptor signaling pathway (20). In fact, both ligands showed significant changes in protein tyrosine phosphorylation levels associated with the ErbB signaling pathway as analyzed by the KEGG database. Additionally, utilizing other reference annotation studies also indicated that the majority of the proteins which appeared in our EGF and HRG proteome data are directly or indirectly linked to the ErbB receptor signaling pathway.

Although we initially expected to find a certain proportion of overlapping genes between tyrosine phosphorylation and transcription, these genes were few in number. This indicated that phosphorylated proteins are not immediately expressed to replenish cellular needs, but may be recycled through dephosphorylation processes. HRG is known to induce persistent signaling activities associated with the MAPK cascade and PI3K/Akt pathways up to several hours (5). Our results indicated that the levels of most of the signaling molecules related to these pathways are maintained by a rapid turnover of phosphorylation and dephosphorylation processes, except for ERBB2 and MAPK3. Additionally, the overlap between the EGF and HRG datasets was found to be significantly low.

Taken together, the current analysis indicated that the main response of EGF and HRG concerns activation of the ErbB signaling pathway, and that the specificity of each ligand response may be characterized by subsequent protein phosphorylation and gene expression. Although the current study investigated tyrosine phosphorylation in the proteome analysis, an investigation of serine/threonine phosphorylation should provide information relating to the overall cellular flux.

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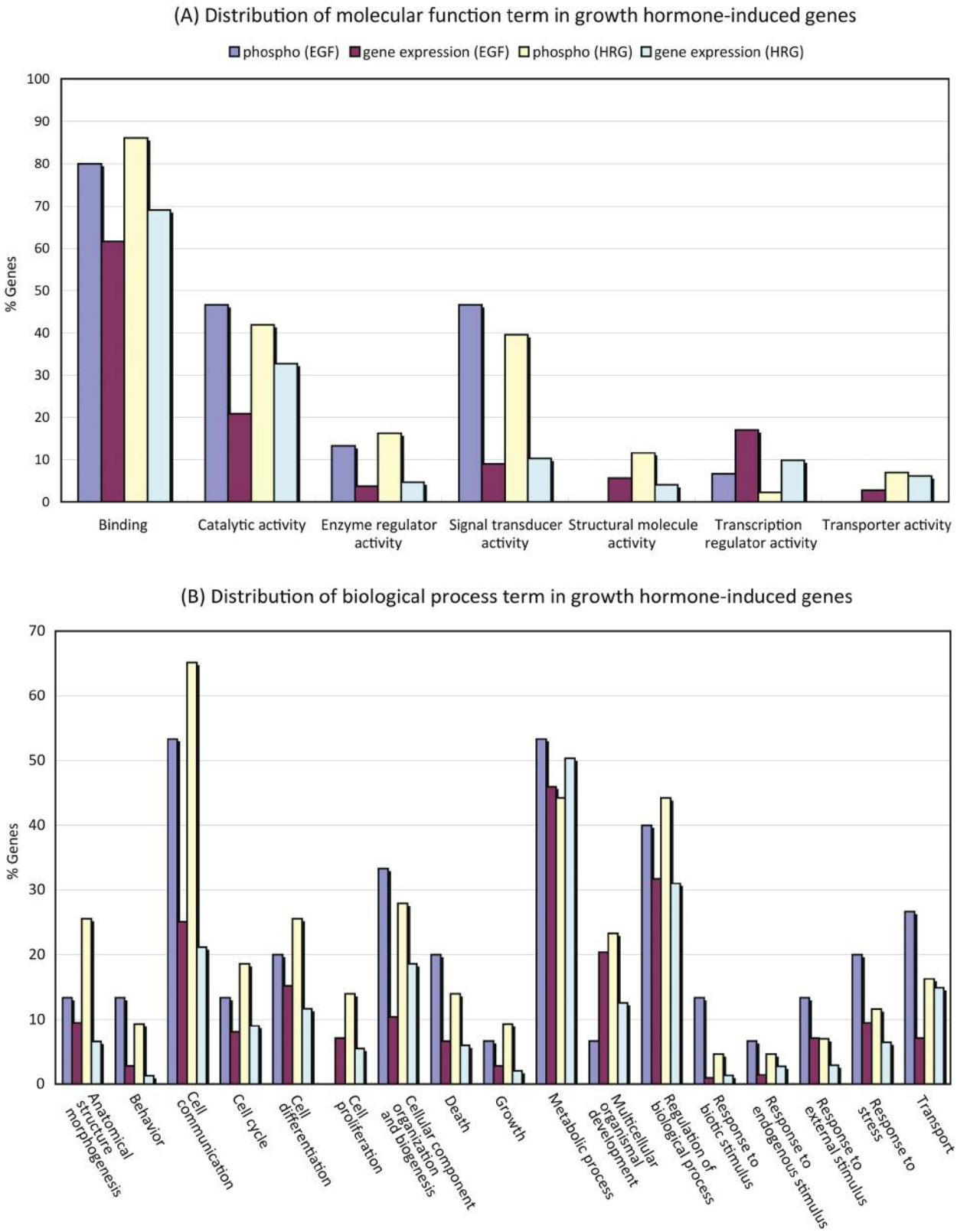

Figure 3. *Functional properties of growth hormone induced genes. Relative ratios of generic GO terms in molecular function ontology (A) and biological process ontology (B) for 4 gene sets are plotted. All GO terms were mapped to the second level of generic GO slim terms, and GO terms without any gene association were not plotted. Those GO terms which were associated with more than 5% of the genes in at least one dataset are shown.*

Table II. *Enriched KEGG pathways*.

KEGG pathway	*P*-value			
	Phospho		Gene expression	
	EGF	HRG	EGF	HRG
Acute myeloid leukemia	**0.01852**	***<0.00001***	>0.05	0.05316
Adherens junction	**0.03702**	***0.00042***	>0.05	0.32738
Apoptosis	n.d.	***0.00067***	>0.05	>0.05
Axon guidance	***0.00003***	***<0.00001***	>0.05	0.09992
B cell receptor signaling pathway	>0.05	***<0.00001***	0.23742	>0.05
Bladder cancer	**0.01162**	***0.00262***	>0.05	***0.00043***
Chronic myeloid leukemia	**0.03800**	***<0.00001***	>0.05	***0.00046***
Colorectal cancer	***0.00068***	***<0.00001***	0.84332	0.21757
Dorso-ventral axis formation	***0.00512***	***0.00076***	n.d.	>0.05
Endometrial cancer	***0.00015***	***<0.00001***	>0.05	0.34640
Epithelial cell signaling in *Helicobacter pylori* infection	>0.05	**0.01109**	>0.05	>0.05
ErbB signaling pathway	***0.00001***	***<0.00001***	0.16865	0.09921
Fc epsilon RI signaling pathway	***0.00047***	***<0.00001***	>0.05	>0.05
Focal adhesion	***0.00020***	***<0.00001***	>0.05	0.08645
Glioma	**0.02699**	***<0.00001***	>0.05	0.09897
GnRH signaling pathway	***0.00101***	**0.03152**	>0.05	>0.05
Insulin signaling pathway	***0.00271***	***<0.00001***	>0.05	0.08760
Jak-STAT signaling pathway	n.d.	***0.00032***	>0.05	>0.05
Leukocyte transendothelial migration	0.08331	***<0.00001***	>0.05	>0.05
Long-term depression	**0.03800**	0.38488	n.d.	>0.05
Long-term potentiation	**0.03136**	**0.01158**	n.d.	>0.05
MAPK signaling pathway	**0.01809**	**0.04947**	**0.00804**	***0.00008***
Melanogenesis	***0.00117***	**0.03649**	>0.05	>0.05
Melanoma	**0.03319**	***<0.00001***	>0.05	0.93685
Natural killer cell mediated cytotoxicity	0.11309	***<0.00001***	>0.05	>0.05
Non-small cell lung cancer	**0.01923**	***<0.00001***	>0.05	0.17234
Pancreatic cancer	**0.03508**	***<0.00001***	>0.05	**0.03514**
Phosphatidylinositol signaling system	n.d.	***0.00050***	>0.05	>0.05
Prostate cancer	***0.00073***	***<0.00001***	>0.05	0.05255
Regulation of actin cytoskeleton	***0.00024***	***<0.00001***	>0.05	0.16971
Renal cell carcinoma	**0.03136**	***<0.00001***	>0.05	>0.05
Small cell lung cancer	>0.05	***0.00002***	>0.05	>0.05
T cell receptor signaling pathway	n.d.	***0.00003***	>0.05	>0.05
Thyroid cancer	***0.00550***	***0.00085***	>0.05	>0.05
Toll-like receptor signaling pathway	***0.00117***	***<0.00001***	>0.05	>0.05
Type II diabetes mellitus	**0.01275**	***<0.00001***	>0.05	>0.05
VEGF signaling pathway	***<0.00001***	***<0.00001***	>0.05	>0.05
mTOR signaling pathway	**0.01456**	***<0.00001***	>0.05	>0.05

n.d.: Not determined. *P*-values smaller than 0.01 and 0.05 are shown in bold italic face and italic bold face, respectively.

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Received March 3, 2008
Revised April 17, 2008
Accepted April 21, 2008