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Prediction of Differentiation-ralated Pathways Induced by Rho-GDIγ *

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Abstract Rho-GDIγ, one of GDP-dissociation inhibitors (GDIs) in the GTPases Rho-subfamily, plays a key role in modulating the activation of GTPases, and contributes to reorganization of actin cytoskeleton, cell aggregation, cell-to-cell adhesion, membrane ruffling, cell motility, and so on. In this work, a functional linkage network that consists of genes differentially expressed between undifferentiated and differentiated neural stem cells (NSCs) is first constructed. Differentiation-ralated pathways induced by Rho-GDIγ are subsequently predicted from the functional linkage network. The results of the biological experiments indicated that overexpression of Rho-GDIγ in neural stem cell line C17.2 upregulates VCP (valosin-containing protein) gene. The results demonstrate that differentiation-related pathways predicted from functional linkage network are indeed reliable.

Keywords Gene expression, Differentiation-related pathway, Functional linkage network

1 Introduction

Neural stem cells (NSCs) are selfrenewing, and can differentiate into three lineages of cells, including neurons, astrocytes and oligodendrocytes [1, 2, 3]. Their properties of self-renewal and multipotential differentiation make NSCs an attractive and presumably donor source for cell replacement therapy to treat neurological disorders [4, 5, 6, 7]. Due to these properties, C17.2, a clone of neural stem cells, has been proved to be an ideal candidate for clinical purpose on cell replacement therapy recently [8]. C17.2 cells proliferate and differentiate into neurons, oligodendrocytes, and astrocytes in vitro and in vivo [9, 10, 11, 12, 13]. Generally, many internal and external signals affect proliferation and differentiation of neural stem cells (NSCs), and promote differentiation towards desired phenotypes [14]. However, little is known about the regulatory mechanisms underlying cell differentiation.

Based on microarray analysis of neural stem cell differentiation [16], it is found that Rho-GDIγ plays a key role in differentiation of NSCs into specific cells, and it is further

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confirmed by our previous results [15]. Rho-GDIγ is a member of Rho family of small GTPases. It acts as a molecular switch in signal transduction pathways that regulate a set of biological processes including cell proliferation, apoptosis, differentiation, cytoskeletal reorganization, and membrane trafficking [17, 18, 19]. It is found that all small GTP-binding proteins will show different biochemical activity between GDP-bound inactive state and GTP-bound active state [20]. There are three classes of GDIs that have been reported to act on proteins in Rho family: Rho-GDI1 (also called Rho-GDI or Rho-GDIα) [21], Rho-GDI2 (also named D4/Ly-GDI or Rho-GDIβ) [23, 24], and Rho-GDI3 (also named Rho-GDIγ) [25, 20]. Rho-GDIγ is preferentially expressed in brain tissues but also found in lung, kidney, and testis [25]. Our previous experimental results indicated that downregulation of Rho-GDIγ increases the tendency of neural stem cell line C17.2 to differentiate. Nevertheless, the differentiation-related pathways induced by Rho-GDIγ are still unclear.

In this work, a functional linkage network that consists of genes differentially expressed between undifferentiated and differentiated NSCs is first constructed. Differentiation-ralated pathways induced by Rho-GDIγ are subsequently predicted from the functional linkage network. The results of the biological experiments indicate that overexpression of Rho-GDIγ in C17.2 cells upregulates valosin-containing protein (VCP). The results demonstrate that differentiation-related pathways predicted from functional linkage network are indeed reliable.

2 Results

2.1 Prediction of differentiation-ralated pathways induced by Rho-GDIγ

The results of gene expression profiles [16] in neural stem cells differentiation and undifferentiation showed that a total of 1406 genes were differentially expressed, among which 148 genes exhibited more than two-fold differences. As a consequence, 61 genes were found three-fold change. Due to the scarcity of protein-protein interaction data, the functional protein association network for *Mus musculus* from STRING database is used to investigate the functional relationship among differentially expressed genes. After mapping the differentially expressed genes to the functional linkage network, we get a small network that only consists of differentially expressed genes. Furthermore, a number of differentiation related genes collected from literature are identified in the functional linkage network. Subsequently, the shortest paths between Rho-GDIγ and differentiation related genes were identified and assembled into a network. Since signal is transduced in a parsimonious way, the genes in the shortest path from Rho-GDIγ to differentiation related genes are more possibly putative genes involved in regulatory pathways by Rho-GDIγ. These shortest paths are regarded as putative differentiation related pathways induced by Rho-GDIγ here. Figure 1 shows the pathways predicted related to Rho-GDIγ.

In addition, we investigated the functions that are enriched in the predicted pathways. Table 1 lists the top 5 most enriched functions for the components in the predicted pathways. Pathway enrichment analysis found that genes in the functional network are enriched in "Focal adhesion" pathway from KEGG with P-value $5.1E-2$, and Role of "MAL in Rho-Mediated Activation of SRF" pathway from BIOCARTA with P-value 8.1E-2.

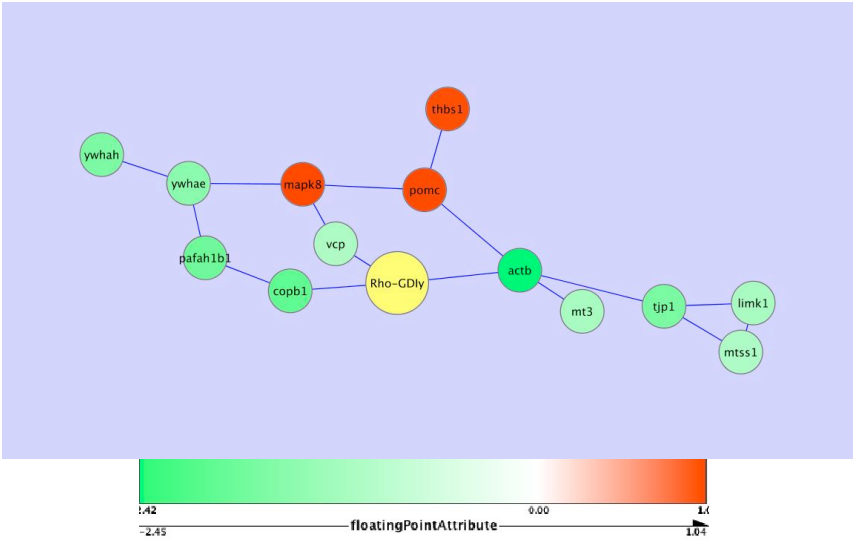

Figure 1: Differentiation related pathways induced by Rho-GDI-γ.

2.2 Verification

To detect the signaling network mediated by Rho-GDIγ, according to the network, randomly selected VCP (valosin-containing protein) gene was assayed 3 days after treating cells with overexpression plasmid pEGFP-C1-Rho-GDIγ , which can overexpressed Rho-GDIγ protein in vivo. VCP is an abundant and ubiquitously expressed multifunctional protein that is a member of the AAA+ (ATPase associated with various activities) protein family. It has been reported to be similarly expressed in most mammalian tissues [26] and targeted deletion of VCP in mice is embryonic lethal [27]. VCP interacts with at least 30 different cellular proteins, some of which may differentially mediate its functions [28] ranging from organelle biogenesis to protein degradation [29]. Our results indicated that the expression of VCP was induced in response to Rho-GDIγ, implied that there is a relationship between VCP and Rho-GDIγ. The fold changes measured using quantitative real-time PCR, which revealed that VCP gene was changed when Rho-GDIγ was overexpressed.

3 Materials and Methods

3.1 Cell culture

Neural stem cell line C17.2 were grown in high-glucose DMEM (Invitrogen) supplemented with 10% fetal calf serum (Hyclone, USA) and 5% horse serum on 6cm Corning

Table 1: Function enrichment analysis for genes in the functional network

Function	P-value
regulation of neurogenesis	6.6E-4
system development	6.9E-4
anatomical structure morphogenesis	9.7E-4
cellular component organization and biogenesis	9.9E-4
nervous system development	1.0E-3

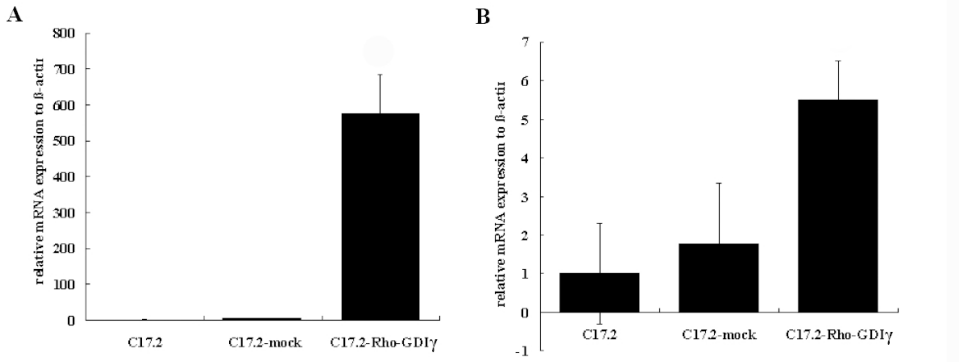

Figure 2: Overexpression of Rho-GDIγ in C17.2 cells upregulates VCP. The expression of VCP gene was evaluated by real-time PCR. After cells were transfected with pEGFP-C1-Rho-GDIγ vector for 3days, Total RNA was extracted as described in Methods. when Rho-GDIγ was upregulated by pEGFP-C1-Rho-GDIγ (A), VCP (B) was upregulated. The average $\pm$ SD from two independent experiments performed in triplicate is shown.

culture dishes in standard humidified 5% CO_2 at 37°C (Snyder).

3.2 Gene transfection

Lipofectamine 2000 reagent (Invitrogen) was used for gene transfection according to the protocol provided. The cells were seeded in 96-well plates at the density of 1×10^4 cells/100μl each well. Tweenty-four hours later, the pEGFP-C1-Rho-GDIγ vectors 1μg in the Lipofectamine 2000 / DMEM (W/V, 1:1) 50μl was added. Five hours later, the cells were changed with fresh medium, and then cultured for use.

3.3 Total RNA extraction, cDNA synthesis, and real-time RT-PCR analysis

To validate the effects when Rho-Rho-GDIγ was overexpressed, real-time PCR analysis were conducted .After transfected for 3days the cells were harvested by trypsinization and washed twice by PBS. Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, USA) following the supplier's instructions. The RNA samples were reverse transcribed into cDNA according to the manufacturer's protocol (TaKaRa RNA PCR Kit (AMV) Ver. 3.0). Real-time PCR was performed in a real-time RT-PCR ma-

chine (Bio-Rad mini opticon Real-time PCR system) using the Takara's Real-Time PCR Kits (Takara). At least three replicates were performed on each sample and each experimental gene was tested by three PCR runs. In each run of PCR, mouse housekeeping gene GAPDH was used as the reference transcript. All cDNA samples were analyzed for the transcript of interest and the housekeeping gene in independent reactions. Data were analyzed by Opticon Monitor software. Each sample was analyzed in triplicate.

3.4 Statistical analysis

All expression values are expressed as means$\pm$ SD. Analysis of variance with the student's paired *t*-test was used to determine the significance of differences in multiple comparisons.

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