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Time-course network analysis reveals TNF-α can promote G_1/S transition of cell cycle in vascular endothelial cells

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ABSTRACT

Motivation: Tumor necrosis factor-alpha (TNF-α), a major inflammatory cytokine, is closely related to several cardiovascular pathological processes. However, its effects on the cell cycle of vascular endothelial cells (VECs) have been the subject of some controversy. To investigate the molecular mechanism underlying this process, we constructed time-course protein–protein interaction (PPI) networks of TNF-α induced regulation of cell cycle in VECs using microarray datasets and genome-wide PPI datasets. Then, we analyzed the topological properties of the responsive PPI networks and calculated the node degree and node betweenness centralization of each gene in the networks. We found that p21, p27 and cyclinD1, key genes of the G1/S checkpoint, are in the center of responsive PPI networks and their roles in PPI networks are significantly altered with induction of TNF-α. According to the following biological experiments, we proved that TNF-α can promote G_1/S transition of cell cycle in VECs and facilitate the cell cycle activation induced by vascular endothelial growth factor.

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1 INTRODUCTION

Tumor necrosis factor-α (TNF-α), a major inflammatory cytokine, plays a pivotal role in cardiovascular disease and induces multiple changes in vascular endothelial cells (VECs), which affect several pathological processes including tumor angiogenesis (Bradley, 2008; Zhang *et al.*, 2009). In addition to its well-characterized properties such as altered cell adhesion and increased apoptosis, TNF-α also modulates the cell cycle in VECs (Fajardo *et al.*, 1992; Lindner *et al.*, 1997; Wojciak-Stothard *et al.*, 1998). However, its effects on the cell cycle processes of VECs have been the subject of some controversy. Previous studies indicate that TNF-α induces cell cycle arrest and blocks proliferation of VECs (Dormond *et al.*, 2002). Inhibition of TNF-α accelerates endothelial recovery after angioplasty (Krasinski *et al.*, 2001). Frater-Schroder *et al.* (1987) found that TNF-α can enhance VECs proliferation and enhance angiogenesis in rabbit cornea assays. Fajardo *et al.* (1992) revealed that low doses of TNF-α can increase DNA synthesis and decrease the proportion of G_1 phase cells. Moreover, our recent studies showed that TNF-α-induced cell cycle may be regulated by dynamic pathway interactions in VECs (Huang and Li, 2010) and that there is a complex gene network behind TNF-α-induced cell cycle processes in VECs (Gu *et al.*, 2010). We also demonstrate the advantages of protein–protein interaction (PPI) networks in studies of disease mechanisms and drug actions (Li *et al.*, 2011; Wu *et al.*, 2008; Zhao and Li, 2010). In this study, we constructed time-course PPI networks of TNF-α-induced regulation of cell cycle in VECs and analyzed their topological properties to reveal the molecular mechanisms underlying TNF-α-induced cell cycle processes.

2 METHODS

2.1 Time-course microarray dataset

A time-course microarray dataset of TNF-α-stimulated human umbilical vein endothelial cells (HUVECs) was downloaded from NCBI gene expression omnibus (GEO) database: GSE9055, Affymetrix Human Genome U133 Plus 2.0 Array, HUVECs stimulated with 10 ng/ml TNF-α, 0–8 h (Wada *et al.*, 2009). Original CEL format files were processed and normalized by dChip (Li and Wong, 2001). Differentially expressed genes (DEGs) were selected as those with base-2 log-transformed fold changes >1.0 (fold change >2.0) by comparing the gene expression value at the corresponding time point with the value at time zero. Then all the probe sets were mapped to known Entrez genes. To avoid noise caused by lowly expressed genes, we filtered out genes which were lowly expressed in HUVECs across 100 arrays collected from GEO.

2.2 Gene Ontology assay

DEGs from each of 16 time points were used to enrich to Gene Ontology (GO) terms, 'regulation of apoptosis' (GO:0042981), 'regulation of cell adhesion' (GO: 0030155), 'regulation of metabolic process' (GO:0019222) and 'regulation of cell cycle' (GO:0051726) by DAVID Bioinformatics Resources 6.7 (Dennis *et al.*, 2003) (Supplementary Fig. S1). Those DEGs related to term 'regulation of cell cycle' in each time point were defined as 'seed' genes to construct the time-course responsive PPI networks (Supplementary Table S1).

2.3 Construction of PPI networks

ClustEx (v0.31) (Gu *et al.*, 2010) was used to construct PPI networks from two inputs: one is the global background PPI network, which was gathered by merging the Human Protein Reference database (HPRD, v8.0) (Keshava Prasad *et al.*, 2009; Peri *et al.*, 2003) and the BioGRID database (v2.0.58) (Stark *et al.*, 2006). The background PPI network remained the same in the whole study. The other input was the seed genes at each time point as described in Section 2.2, which were used as starting nodes to identify

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the corresponding gene module (the 'responsive PPI network') from the background PPI network.

For each of the 16 time points, we used those DEGs which were also annotated as 'regulation of cell cycle' in the GO database as the seed genes. We constructed one responsive PPI network for each time point using ClustEx (Supplementary Table S2, Supplementary Fig. S2–S4). Meanwhile, genes in responsive PPI networks were enriched to KEGG pathway (Supplementary Table S3) by DAVID Bioinformatics Resources 6.7 (Dennis *et al.*, 2003)

2.4 Topological property analysis of PPI networks

Protein–protein networks were considered to be a type of undirected network. The clustering coefficient $C$of a network is defined as

$$C = \sum_i \frac{C_i}{N},$$

where N is the total number of nodes in the connected network. C_i is the clustering coefficient of node i which is defined as

$$C_i = \frac{2e_i}{k_i(k_i-1)},$$

where k_i is the number of neighbors of node i and e_i is the number of connected pairs between all neighbors (Assenov *et al.*, 2008). Node degree of node i is the number of edges linked to the node (Assenov *et al.*, 2008). Betweenness centrality of a node i in an undirected network is defined as

$$b_i = \left(\sum_{s\neq i\neq t} \frac{\varphi_{st}(i)}{\varphi_{st}}\right) \Big/ \left(\frac{(N-1)(N-2)}{2}\right),$$

where s and t are nodes in the network different from n, ϕ_{st} is the number of shortest paths from s to t, $\phi_{st}(i)$ is the number of shortest paths from s to t that n lies on and N is the total number of nodes in the network (Assenov *et al.*, 2008)

Each gene's node degree and node betweenness centrality in each PPI network at each time point were normalized. The normalized node degree at time j, was defined as

$$D_{ij} = \frac{(d_{ij}-\mu_j)}{\sigma_j},$$

where μ_j is the average node degree of the network at time point j, $\mu_j = \sum_i d_{ij}/N_j$ and $\sigma_j = \sqrt{\sum_i (d_{ij}-\mu_j)^2/(N-1)}$. Node betweenness centralization was normalized using the same method. Total node degree is defined as $D_i = \sum_j D_{ij}$ and total node betweenness centrality is defined as $B_i = \sum_j B_{ij}$.

2.5 Cell culture

HUVECs were isolated from freshly obtained human umbilical cords by established methods (Yan *et al.*, 2010). Cells were grown on gelatin-coated dishes in standard endothelial cell medium (ECM, ScienCell Research Laboratories). ECM consists of 500 ml of basal medium, 25 ml of fetal bovine serum, 5 ml of endothelial cell growth supplements and 5 ml of penicillin/streptomycin solution. Cells used for this study were from passages 4–6 and maintained at 37˚C in a humidified atmosphere of 5% CO_2 in air.

2.6 Cell cycle assay

HUVECs were plated in 60 mm dishes (50 000 cell/cm^2) and cultured for 48 h. Cells were confluent before stimulation (~120 000 cell/cm^2). Cells were harvested and fixed in 70% ethanol and stored at -20˚C overnight. Cells were washed twice with ice-cold phosphate buffer saline (PBS) and incubated with RNase and propidium iodide for 30 min and then cell cycle analysis was performed by a Flow CytoMeter (FCM).

2.7 Western blot

Proteins from cells were extracted with RIPA lysis buffer [150 mM NaCl, 10 mM Tris, pH 7.5, 1% NP40, 1% deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche)]. Proteins from total cell lysates were resolved by 10% SDS–PAGE gel, transferred to the nitrocellulose membrane, blocked in 5% non-fat milk in PBS/Tween-20, blotted with the antibodies with recommend dilutions (Cell Signalling) and blotted with Goat anti Rabbit IgG (1:3000, Santa Cruz). Visualization of the second antibody was performed using a chemiluminescence detection procedure according to the manufacturer's protocol (Amersham Pharmacia Biotech). GAPDH was used as loading control.

2.8 Experimental statistics

All experimental results were expressed as means ± SD unless indicated otherwise. Differences between two groups were assessed by two-tailed Student's t-test, and $P < 0.05$ was considered significant.

3 RESULTS

3.1 Responsive PPI networks of TNF-α-induced cell cycle process in HUVECs

DEGs at each time point were enriched to biological processes term of GO. Result showed that the term 'regulation of cell cycle' (GO: 0051726) was significantly associated with stimulation by TNF-α in VECs (Supplementary Fig. S1). Thirty-five DEGs were associated with the term 'regulation of cell cycle' during induction (Supplementary Table S1), and their gene expressions were clustered as shown in Figure 1A. These genes were defined as 'seed' genes to construct time-course responsive PPI networks by ClustEx. Results showed that the number of nodes (gene) and number of edges (gene–gene interaction) in the responsive PPI networks increased with stimulation of TNF-α (Fig. 1B). The average number of neighbors and clustering coefficient also increased with stimulation of TNF-α (Fig. 1C). These results suggest that TNF-α might activate a signaling transduction cascade to regulate cell cycle processes (Supplementary Table S3).

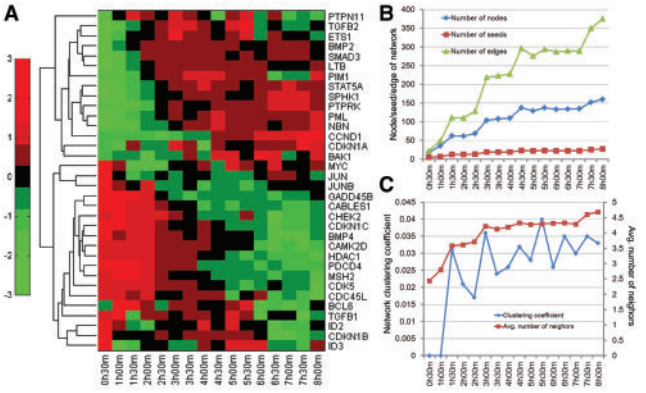

Fig. 1. Network attributes of time-course PPI networks of TNF-α-induced regulation of cell cycle. (**A**) Heat map of time-course microarray results of the DEGs annotated with GO term 'regulation of cell cycle'. Red indicates upregulated gene expression and green indicates downregulated gene expression. (**B**) Dynamical changes of PPI networks of TNF-α-induced regulation of cell cycle. (**C**) Dynamical changes of average number of neighbors and network clustering coefficient.

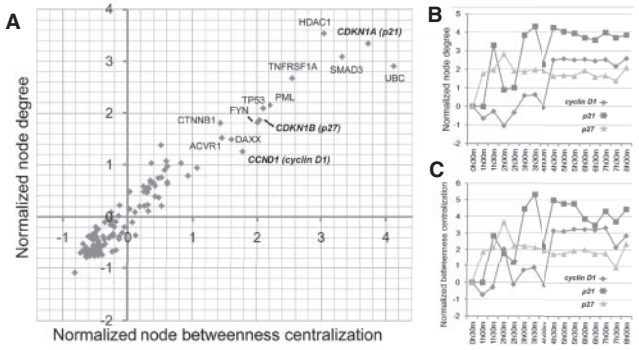

Fig. 2. Network centrality of time-course PPI networks of TNF-α-induced regulation of cell cycle. (**A**) Node degree and betweenness centralization of each gene in the time-course PPI networks were analyzed as described in Section 2. Those nodes which had both normalized node degree and normalized betweenness centralization >1 are marked with their gene symbol and their detailed information are listed in Supplementary Table S4. (**B** and **C**) Normalized node degree and normalized betweenness centralization of CDKN1A (p21), CDKN1B (p27) and CCND1 (cyclinD1) in the time-course PPI network show the dynamic relationship between these core genes and the whole networks.

3.2 TNF-α altered topological properties of p21, p27 and cyclinD1 in the responsive PPI networks

To determine the key genes of TNF-α-induced regulation of cell cycle, we analyzed each gene's node degree and node betweenness centrality in each responsive PPI network. We added up the normalized node degree and the normalized betweenness centrality of each gene to calculate the total node degree and total betweenness centrality and determine the importance of each gene in the whole process. We found that CDKN1A(p21), CDKN1B(p27) and CCND1(cyclinD1), the key genes of the G1/S checkpoint, were also the most important key genes in the responsive PPI networks of TNF-α-induced regulation of cell cycle (Fig. 2A). Node degree and node betweenness of p21 and cyclinD1 were significantly changed with induction of TNF-α and p27 was significantly changed at the first 2 h, but it remained stable in the following time points (Fig. 2B and C). These results suggested that the G_1/S checkpoint might be one of the most important targets of TNF-α-induced regulation of cell cycle.

3.3 TNF-α promotes G_1/S transition of cell cycle in VECs and facilitates the cell cycle activation induced by VEGF

To determine if TNF-α could alter the G_1/S checkpoint of VECs, we first got quiescent HUVECs (Supplementary Fig. S2). Then, we treated quiescent HUVECs as shown in Figure 3A. After inductions, the cells were harvested for cell cycle assay. Results showed that 8 h after induction with TNF-α, the percentage of VECs in S phase was not significantly different from the ECM-treated control (Fig. 3B), but after 24 h the percentage of VECs in S phase was significantly increased in both groups of pulse induction of TNF-α (for 8 h) and continuous induction of TNF-α (for 24 h) (Fig. 3C). At the same time, 8 h of induction by TNF-α also significantly promoted VEGF-induced cell cycle (Fig. 3C). In western blot, the expression of p21, p27 and cyclinD1 were sensitive to stimulation of TNF-α at the

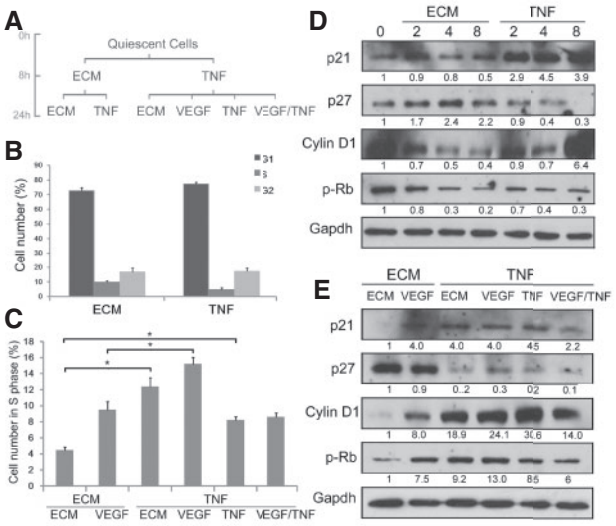

Fig. 3. TNF-α promotes G_1/S transition of cell cycle in VECs which facilitate the cell cycle activation induced by VEGF. (**A**) As shown in the flow chart, quiescent VECs were treated with TNF-α (10 ng/ml) or media ECM alone for 8 h and then cells were induced with different cytokines for the following 16 h. During this process, the concentration of TNF-α and VEGF were both 10 ng/ml. 'VEGF/TNF' shows cells that were treated with TNF-α (10 ng/ml) and VEGF (10 ng/ml) together. (**B**) Quiescent VECs were treated with TNF-α (10 ng/ml) or ECM for 8 h and then cells were harvested for FCM. Result shows mean ± SD for triplicate independent experiments ($*P < 0.05$). (**C**) Quiescent ECs were treated with different cytokines as indicated and then cells were harvested for FCM. Results show mean ± SE for triplicate independent experiments ($*P < 0.05$). (**D**) Quiescent ECs were treated with TNF-α (10 ng/ml) or media control for 2, 4 and 8 h and then G_1/S checkpoint gene expressions were analyzed with western blot. Results are shown from one of three similar experiments. The numbers show each gene's relative expression by gray level. (**E**) Quiescent VECs were treated with different cytokines as indicated and then G_1/S checkpoint gene expressions were analyzed with western blot. Results are shown from one of three similar experiments. The numbers show each gene's relative expression by gray analysis.

protein level. Four hours induction of TNF-α was enough to change the expression of p21 and p27, and 8 h induction was enough to increase the expression of cyclinD1. We used phosphorylated Rb as marker of G_1/S transition. Results showed that the expression of phosphorylated Rb is consistent with cell cycle assay (Fig. 3D and E). All these results showed that TNF-α can promote G_1/S transition of cell cycle in VECs and facilitate the cell cycle activation-induced VEGF.

4 DISCUSSION

Quiescent VECs can rapidly reverse in response to signaling from the microenvironment; however, tight regulation of the cell cycle is necessary for VECs, for one of the most important properties of VECs is to separate blood from underlying tissues (Augustin *et al.*, 2009; Dejana, 2004). In the present study, VEGF treatment alone doubled the number of cells in S phase after 24 h (Fig. 3C, ECM/VEGF). An 8 h pulse of TNF-α followed by removal of TNF-α (TNF/ECM) nearly tripled the amount of cells in S phase and,

strikingly, an 8 h pulse of TNF-α followed by treatment with VEGF alone (TNF/VEGF) caused the highest amount of S phase induction. Conversely, continuous treatment with TNF-α for 24 h (TNF/TNF) or addition of VEGF after 8 h to cells continuously treated with TNF-α (TNF/VEGF + TNF) caused fewer cells to enter S phase than the 8 h TNF-α pulse. The pulse of TNF-α facilitated the cells for entry into S phase, but continuous TNF-α treatment also acted to repress entry into S phase in the absence of VEGF induction. These results suggest that TNF-α has a double role in regulation of the cell cycle in VECs and that the cell cycle of VECs is controlled by multiple cytokines in a balanced way (Mantovani and Sica, 2010).

In this study, time-course microarray datasets and functional relationships of PPIs were used to construct responsive PPI networks. PPI networks are a type of scale-free network (Han, 2008) in which node degree and node betweenness centrality can be used to determine the network center (Barabasi *et al.*, 2011). Liu *et al.* (2007) compared a metabolic network with the KEGG (Kyoto Encyclopedia of Genes and Genomes) database and found that enzyme phylogenetic profile correlated best with betweenness centrality and also quite closely with degree. Based on these studies, time-course microarray datasets and the functional relationship of PPIs were used to construct responsive PPI networks in the present study. By employing node degree and node betweenness centrality, we quantified the role of genes in the time-course PPI network which made it easier to identify the key genes in the network. Combined with laboratory experiments, we found that TNF-α could affect the G_1/S cell cycle transition in VECs. These results show that time-course PPI network analysis could also be a useful way to study other time-course biological processes.

In summary, combined with computational and experimental studies, we found that TNF-α can promote G_1/S transition of cell cycle in VECs and facilitate the cell cycle activation induced by VEGF. All these results suggest that TNF-α can break the resting cell cycle and wake up quiescent VECs.

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Conflict of Interest: none declared.

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