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Full Length Research Paper

Study on transcription regulation network in rheumatoid arthritis via bioinformatics analysis

Jie Chen, Jun Xia*,Siqun Wang, Yibing Wei, Jianguo Wu, Gangyong Huang, Feiyan Chen and Jingcheng Shi

Department of Orthopedics, Huashan Hospital affiliated to Shanghai Fudan University, No.12, Middle Urumqi Road, Shanghai, 200040, China.

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Rheumatoid arthritis (RA) is a systemic, inflammatory autoimmune disease with irreversible joint destruction. It is a form of autoimmunity, however, its cause is incompletely known. The objective of this study was to identify potential transcription regulation between transcription factors and differentially expressed genes in RA by using the microarray data and transcriptional network analysis. In addition, their underlying molecular mechanisms were also explored by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. Our results showed that JUN, ETS2, CREB1, PPARG, and SPI1 were crucial transcription factors in our transcriptome networks and these transcription factors could regulate the DEGs expression to involve in RA by promoting or inhibiting effect. For example, JUN could promote FN1 expression; ETS2 promoted FLT1 expression; CREB1 promoted CD4 expression and inhibited F3 expression; PPARG could also inhibit MMP9 expression; SPI1 promoted CSF3R expression. In addition, four significant pathways were identified associated with RA development, including hematopoietic cell lineage, pathways in cancer, MAPK signaling pathway, antigen processing and presentation. ETS2 and PPARG could inhibit hematopoietic cell lineage pathway; ETS2, Jun, and SPI1 promoted the pathway in cancer; CREB1 suppressed MAKP signaling pathway, but promoted antigen processing and presentation. However, further experiments are still needed to confirm the conclusion.

Key words: Rheumatoid arthritis, bioinformatics, network.

INTRODUCTION

Rheumatoid arthritis (RA) is prevalent in 0.31 to 0.85% of adult people, with female predominance (Biver et al., 2009; Nakasa et al., 2011). Although, it principally attacks synovial joints, many tissues and organs are affected, thus resulting in lung disease (Remy-Jardin et al., 1994), cardiovascular (Avouac and Allanore, 2008), and renal amyloidosis (de Groot, 2007). RA is characterized by polyarticular inflammation of synovial tissue, which causes pain, swelling, and stiffness of the joints of the hands, wrists, and feet in particular (van der Helm-van Mil et al., 2005).

RA is a form of autoimmunity, the causes of which are still incompletely known. Previous study suggests that the disease involves abnormal B cell-T cell interaction, with presentation of antigens by B cells to T cells and consequent production of rheumatoid factor (RF) and anticitrullinated peptide antibodies (ACPA). Inflammation is then driven either by B cell or T cell products stimulating release of tumor necrosis factor (TNF) and other cytokines. If TNF release is stimulated by B cell products in the form of RF or ACPA-containing immune complexes, through activation of immunoglobulin Fc receptors, then the sign of RA can be seen (O'Neill et al., 2007). Some infectious organisms are suspected to trigger RA because epidemiological studies have confirmed a potential association between RA and Epstein-Barr virus. Individuals with RA are more likely to exhibit an abnormal immune response to the Epstein-Barr virus (Ferrell et al., 1981).

Efforts of intensive research to increase our understanding of the molecular basis of RA have been undertaken and there are growing evidences showing

*Corresponding author. E-mail: jun_xiajx@hotmail.com

that transcription factors (TFs) play a critical role in disease by regulating transcription and expression of many genes. For example, c-Fos/AP-1 controls the expression of inflammatory cytokines and matrix-degrading matrix metalloproteinases (MMPs) important in arthritis via promoter AP-1 binding motif (Shiozawa and Tsumiyama, 2009). Further, an understanding of these new developments in TFs and pathways may pave the way for innovative combinatorial approaches for treatment of RA and possibly chemoprevention. Several TFs have been demonstrated to be related with MAPK signaling pathway. In response to inflammatory cytokines, the MAPK family of serine/threonine kinases [the c-Jun N-terminal kinases (JNKs) and the extracellular signal-regulated kinases (ERKs)] phosphorylates and activates the activating protein-1 (AP-1) family member c-Jun, which dimerizes with c-Fos to drive transcription of multiple MMP genes. In addition to c-jun, the ERK pathway regulates the activity of erythroblastosis twenty-six (Ets) transcription factors, which cooperate with AP-1 proteins in multiple MMP promoters (Vincenti and Brinckerhoff, 2002). However, high-throughput functional analysis of multiple transcription factors and their target genes in RA is still rare. Therefore, the objective of this study was to identify potential transcription regulation relationships between transcription factors and differentially expressed genes in RA, using the microarray data and transcrip-tional network analysis. In addition, their underlying molecular mechanisms were also explored by KEGG pathway enrichment.

MATERIALS AND METHODS

Data source

Affymetrix microarray data

One transcription profile of RA GSE10500 was obtained from a public functional genomics data repository GEO (http://www.ncbi.nlm.nih.gov/geo/) which is based on the Affymetrix platform data (Wachi et al., 2005).

Pathway data

KEGG (Kyoto Encyclopedia of Genes and Genomes) is a collection of online databases dealing with genomes, enzymatic pathways, and biological chemicals (Kanehisa, 2002). The pathway database records networks of molecular interactions in the cells, and variants of them specific to particular organisms (http://www.genome.jp/kegg/). A total of 130 pathways, involving 2287 genes, were collected from KEGG.

Regulation data

There are approximately 2600 proteins in the human genome that contain DNA-binding domains, and most of them are presumed to function as TFs (Wachi et al., 2005). The combinatorial use of a subset of the approximately 2000 human TFs easily accounts for the unique regulation of each gene in the human genome during development (Brivanlou and Darnell, 2002).

These TFs are grouped into 5 super class families, based on the presence of conserved DNA-binding domains. TRANSFAC database contains data on TFs, their experimentally-proven binding sites, and regulated genes (Wingender, 2008).

Transcriptional Regulatory Element Database (TRED) was built in response to increasing needs of an integrated repository for both cis- and trans- regulatory elements in mammals (Jiang et al., 2007). TRED was used for the curation for transcriptional regulation information, including TF binding motifs and experimental evidence. The curation is currently focusing on target genes of 36 cancer-related TF families.

A total of 774 pairs of regulatory relationship between 219 TFs and 265 target genes were collected from TRANSFAC (http://www.gene-regulation.com/pub/databases.html). A total of 5722 pairs of regulatory relationship between 102 TFs and 2920 target genes were collected from TRED (http://rulai.cshl.edu/TRED/). By combining the two regulation datasets, a total of 6328 regulatory relationships between 276 TFs and 3002 target genes were obtained (Table 1).

Methods

Differentially expressed genes (DEGs) analysis

The limma method (Smyth, 2004) was used to identify DEGs. The original expression datasets from all conditions were processed into expression estimates using the Robust Multiarray Average (RMA) method with the default settings implemented in Bioconductor, and then the linear model was constructed. The fold change value larger than 2 and p-value less than 0.05 were considered as the threshold of DEGs.

Co-expression analysis

For demonstrating the potential regulatory relationship, the Pearson correlation coefficient (PCC) was calculated for all pair-wise comparisons of gene-expression values between TFs and the DEGs. The regulatory relationships whose absolute PCC are larger than 0.6 were considered as significant.

Regulation network construction

Using the regulation data that have been collected from TRANSFAC database and TRED database, we matched the relationships between differentially expressed TFs and its differentially expressed target genes.

Based on the aforementioned two regulation datasets and the pathway relationships of the target genes, we built the regulation networks by Cytoscape (Shannon et al., 2003). Based on the significant relationships (PCC > 0.6 or PCC < -0.6) between TFs and its target genes, 77 putative regulatory relationships were built. The result of regulation network is shown in Figure 1. In order to visualize the relationship between KEGG pathway and TFs, KEGG pathways were enriched among the DEG genes in the regulatory network (Figure 1).

Significance analysis of pathway

We adopted an impact analysis that includes the statistical significance of the set of pathway genes but also considers other crucial factors such as the magnitude of each gene's expression change, the topology of the signaling pathway, and their interactions (Draghici et al., 2007). In this model, the Impact Factor (IF) of a pathway Pi is calculated as the sum of two terms:

Table 1. Regulation datasets.

Source	Regulation	TFs	Targets	Link
TRANSFAC	774	219	265	http://www.gene-regulation.com/pub/databases.html
TRED	5722	102	2920	http://rulai.cshl.edu/TRED/
Total	6328	276	3002	

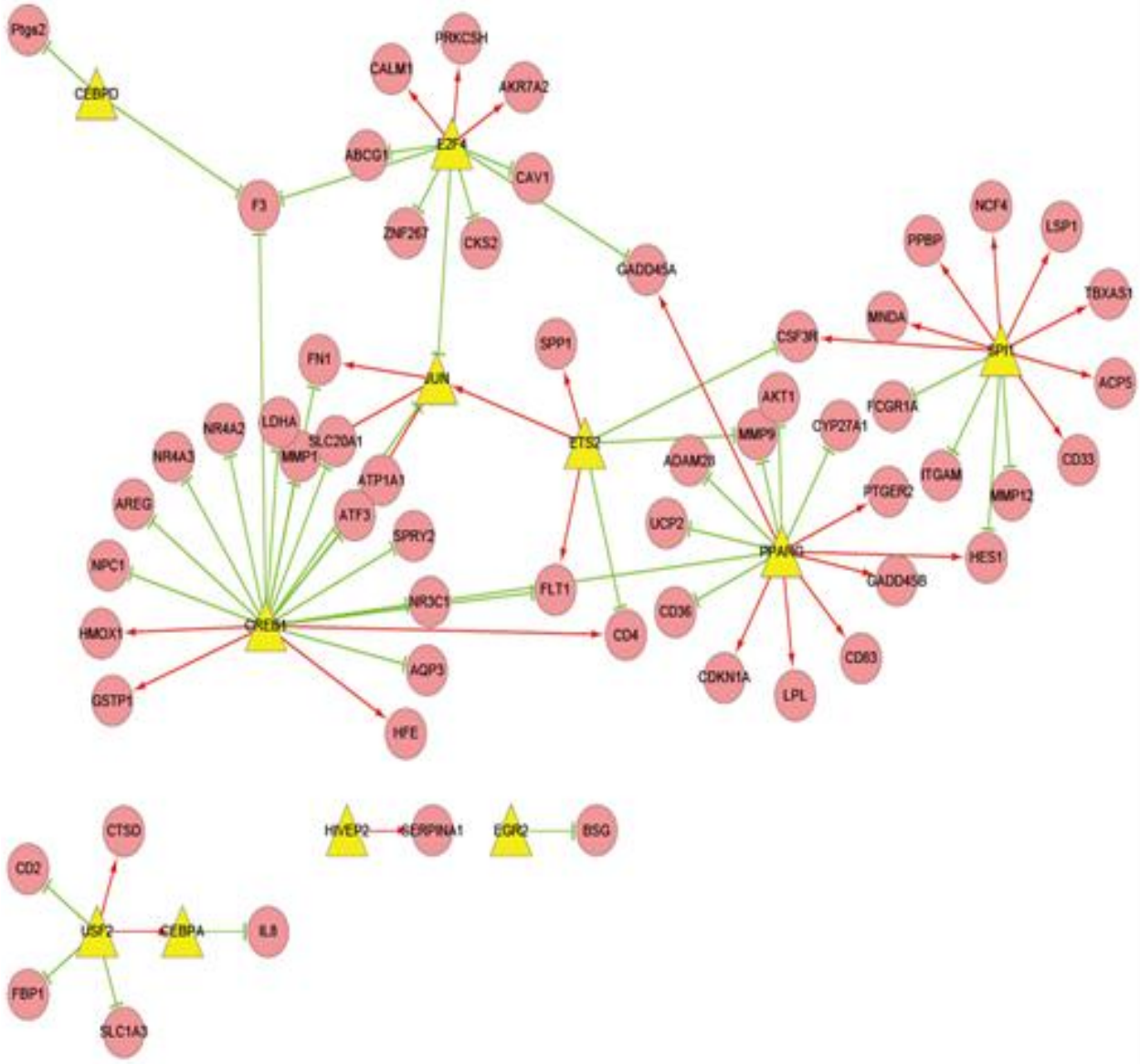

Figure 1. Regulation network construction in RA. Yellow triangle indicates the transcription factor; red circle indicates the differentially expressed genes; red line indicates that the transcription factors could promote the expression of differentially expressed genes; the green line indicates that the transcription factors could inhibit the expression of differentially expressed genes.

$$IF(Pi) = \log(\frac{1}{pi}) + \frac{\sum_{g \in Pi} |PF(g)|}{|\square E| \cdot N_{de}(Pi)} \quad (1)$$

The first term is a probabilistic term that captures the significance of the given pathway P_i from the perspective of the set of genes contained in it.

It is obtained by using the hyper geometric model in which p_i is the probability of obtaining at least the observed number of

Table 2. Significant pathways in RA.

Pathway name	Impact factor	p-value
Hematopoietic cell lineage	8.364	9.90E-04
MAPK signaling pathway	10.335	0.001092989
Pathways in cancer	7.85	0.002351094
Antigen processing and presentation	163.55	0.052055907

differentially expressed gene, N_{de}, just by chance (Tavazoie et al., 1999; Draghici et al., 2003).

The second term is a functional term that depends on the identity of the specific genes that are differentially expressed, as well as on the interactions described by the pathway (that is, its topology).

The second term sums up the absolute values of the perturbation factors (PFs) for all genes g on the given pathway P_i. The PF of a gene g is calculated as follows:

$$PF(g) = \Delta E(g) + \sum_{u \in USg} \beta_{ug} \cdot \frac{PF(u)}{N_{ds}(u)} \quad (2)$$

In this equation, the first term $\Delta E\ (g)$ captures the quantitative information measured in the gene expression experiment. The factor $\Delta E\ (g)$ represents the normalized measured expression change of the gene g. The first term $\Delta E\ (g)$ in the above equation is a sum of all PFs of the genes u directly upstream of the target gene g, normalized by the number of downstream genes of each such gene $N_{ds}(u)$, and weighted by a factor β_{ug}, which reflects the type of interaction: $\beta_{ug} = 1$ for induction, $\beta_{ug} = -1$ for repression (KEGG supply this information about the type of interaction of two genes in the description of the pathway topology). US_g is the set of all such genes upstream of g. We need to normalize with respect to the size of the pathway by dividing the total perturbation by the number of differentially expressed genes on the given pathway, N_{de} (P_i). In order to make the *IF*s as independent as possible from the technology, and also comparable between problems, we also divide the second term in Equation 1 by the mean absolute fold change ΔE, calculated across all differentially expressed genes. The result of the significance analysis of pathway is shown in Table 3.

RESULTS

Regulation network construction in RA

Publicly available microarray data sets GSE10500 were obtained from GEO. A total of 888 genes with the fold change > 2 and p-value < 0.05 were collected as DEGs using the limma method.

To get the regulatory relationships, the co-expressed value (PCC ≥ 0.6) was chosen as the threshold. Finally, we got 77 regulatory relationships between 11 different expressed TFs and their 62 differently expressed target genes. By integrating the regulatory relationships mentioned earlier, a regulation network of RA was built between TFs and their target genes (Figure 1). In this network, CREB1, E2F4, PPARG, SPI1, JUN, and ETS2 had higher degrees from a local network, suggesting that these genes may play an important role in RA. Importantly, we found JUN could promote FN1 expression; ETS2 promoted FLT1 expression; CREB1 promoted CD4 expression and inhibited F3 expression; PPARG could also inhibit MMP9 expression; and SPI1 promoted CSF3R expression.

Significant pathway in RA

To identify the relevant pathways changed in RA, we used a statistical approach on pathway level. Significance analysis at single gene level may suffer from the limited number of samples and experimental noise that can severely limit the power of the chosen statistical test. Pathway can provide an alternative way to relax the significance threshold applied to single genes and may lead to a better biological interpretation. So, we adopted a pathway based impact analysis method that contained many factors, such as the statistical significance of the set of DEGs in the pathway, the magnitude of each gene's expression change, the topology of the signaling pathway, and their interactions. The impact analysis method yielded many significant pathways, such as hematopoietic cell lineage, MAPK signaling pathway, pathways in cancer, antigen processing and presentation (Table 2).

Regulation network between TFs and pathways in RA

To further investigate the regulatory relationships between TFs and pathways, we mapped DEGs to pathways and got a regulation network between TFs and pathways (Figure 2). In the network, CEBPA, CEBPD, E2F4, ETS2, JUN, SPI1 and PPARG were shown as hub nods linked to RA related pathways. ETS2 and PPARG could inhibit hematopoietic cell lineage pathway; ETS2, Jun, and SPI1 promoted the pathway in cancer; CREB1 suppressed MAKP signaling pathway, but promoted antigen processing and presentation.

DISCUSSION

From the result of regulation network construction in RA, we could find that many TFs and DEGs closely related with RA have been linked by our method. The gene, JUN,

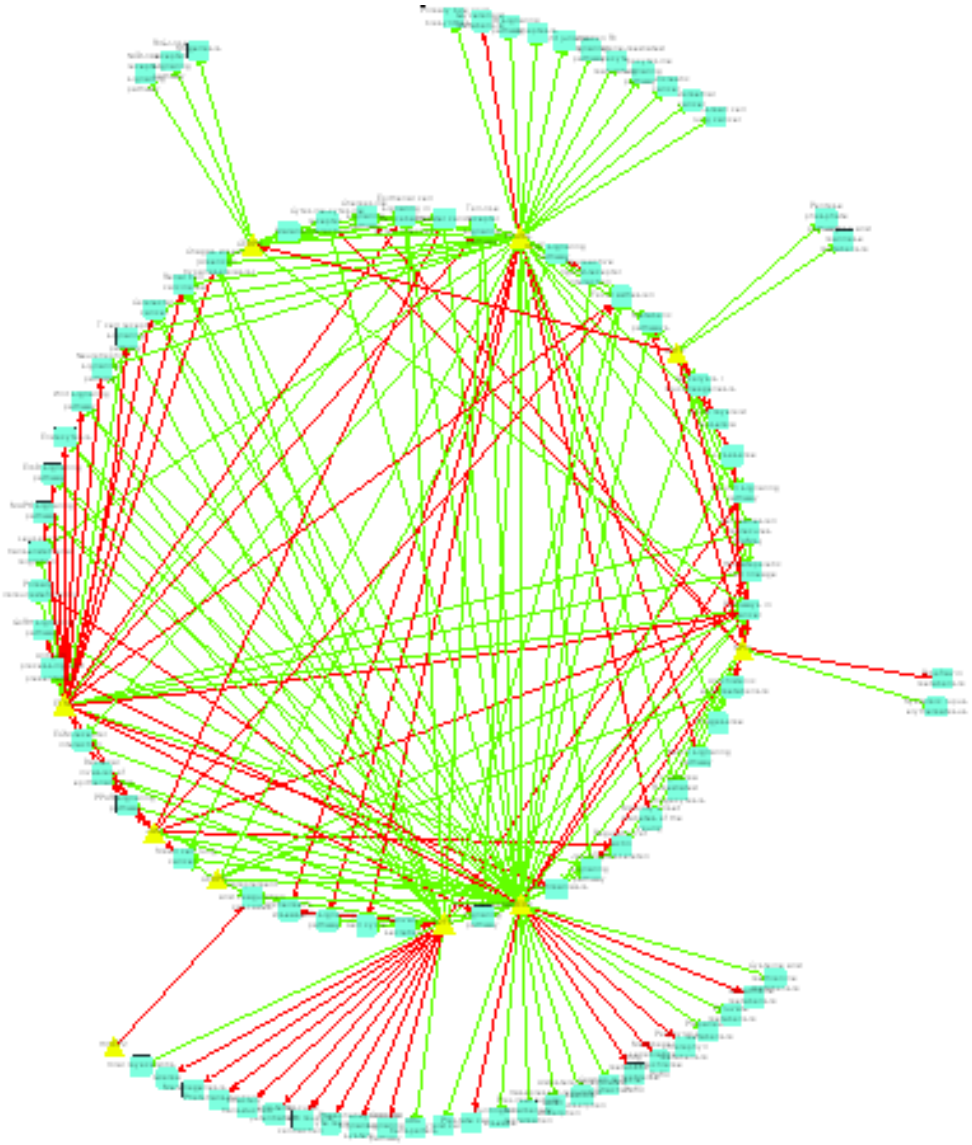

Figure 2. Regulation network between TFs and pathways in RA. Red line indicates that a promoting effect between transcription factors, and between transcription factors and pathway. The green line indicates an inhibiting effect between transcription factors, and between transcription factors and pathway.

ETS2, CREB1, PPARG, and SPI1 were crucial TFs in our transcriptome networks and these TFs could regulate the DEGs expression by promoting or inhibiting effect. Therefore, we would discuss these interaction relationships based on previous studies.

JUN, a signal-transducing transcription factor of the AP-1 family, is normally implicated in cell cycle progression, differentiation and cell transformation. C-jun has been demonstrated as constitutive signal transmitters in solid RA tissues, which may probably result from a continuing inflammatory stimulus (Dooley et al., 1996). Further study reveals that the expression of c-Jun is significantly down-regulated when normal cells are transfected with miRNA-146 which is negative regulator in immune and inflammatory response and strongly expresses in RA (Nakasa et al., 2011). FN1 encodes fibronectin, a glycoprotein present in a soluble dimeric form in plasma, and in a dimeric or multimeric form at the cell surface and in extracellular matrix. Fibronectin is expressed at high levels in patients with RA (Fyrand et al., 1978) and it mediates various physiological processes through interactions with cell-surface integrin receptors and growth factors. Citrullination of Fn can alter interactions between Fn and its receptors and growth factors, consequently contributing to mechanisms of RA pathogenesis such as perturbed angiogenesis and

apoptosis (Chang et al., 2005). Ap-1 is significantly elevated to involve in cell migration and invasion, and high AP-1 activity, as an effector of both c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) pathways, induces the up-regulated expression of fibronectin (Wang et al., 2010).

ETS2 is a member of the ETS (E-twenty six) family of transcription factors and it is implicated in regulating numerous genes involved in proliferation, differentiation, apoptosis, and senescence processes. One group used synovial tissues from 22 patients with RA to test the genes expression, and they found a strong activation of the ETS-2 nuclear oncogene in about one third of RA tissues, which may also be part of a pathway leading to advanced disease stages (Dooley et al., 1996). FLT1 encodes a member of the vascular endothelial growth factor receptor (VEGFR) family. This protein binds to VEGFR-A, VEGFR-B and placental growth factor and plays an important role in angiogenesis and vasculo-genesis. FLT-1 is increased expression associated with placenta growth factor (PlGF), the enhanced expression of PlGF and FLT-1 may contribute to rheumatoid inflammation by triggering production of pro-inflammatory cytokines (Yoo et al., 2009). The expression of VEGFR-1 has been found to be dependent on ETS transcription factors with ETS-1 and ETS-2 demonstrating the strongest activation *in vitro* (Singh et al., 2002).

CREB1 gene encodes a transcription factor that is a member of the leucine zipper family of DNA binding proteins. This protein involves in cAMP pathway and binds as a homodimer to the cAMP-responsive element. CREB is found to up-regulate in the peripheral blood mononuclear cells from patients with RA to involve in the over-expression of Gi protein associated A (3) adenosine receptor (Ochaion et al., 2009). Further, Perez-Garcia et al. (2011) showed that the ratio between phospho-CREB (p-CREB) and CREB is higher in osteoarthritis and significantly lower in RA fibroblast-like synoviocytes after vasoactive intestinal peptide treatment.

CD4 is a surface monomeric glycoprotein present on the helper/inducer subset of T lymphocytes and macro-phages. CD4 binds as a T-cell co-receptor to conserved areas of the major histocompatibility complex II on antigen-presenting cells, and thereby participates in the formation of the immunological synapse and the provision of the so-called "second signal" required for full activation of T-helper cells (Kinne et al., 2010). Therefore, a specific diagnosis of CD4 may be effective for immunological diseases, such as RA. Patients with RA have raised levels of soluble CD4 in both their sera and synovial fluid compared to age-matched healthy controls (Symons et al., 1991). The genetic polymorphisms at the CD4 enhan-cer gene are associated with the risk of development of RA through analysis the RA patients by polymerase chain reaction-restriction fragment length polymorphism method (Lo et al., 2008). CREB- 1 has been demon-strated to specifically bind to the -79 to -52 region of the CD4 promoter, promoting CD4 transcription (Flamand et al., 1998).

F3 gene encodes coagulation factor III which is a cell surface glycoprotein. This factor enables cells to initiate the blood coagulation cascades, and it functions as the high-affinity receptor for the coagulation factor VII. F3 is found as up-regulated expression in osteoarthritis (Appleton et al., 2007). In addition, TNFα and IL-17 are classical and key cytokines involved in RA pathogenesis, and IL-17 and TNFα induced synergistically the expression of tissue factor (F3, 151 fold) to initiate the coagulation cascade in RA (Hot et al., 2012). F3 contains an AP-1 binding site in its regulatory region. F3 is significantly up-regulated as JUN approaches statistical significance in lung injury model, which activates a pro-inflammatory transcriptional program (Gharib et al., 2009). JUN is inhibited by CREB1 and E2F4 in our study, thus leading to the suppression of F3 subsequently.

Peroxisome proliferator-activated receptor gamma (PPARγ) is ligand-activated transcription factors, whose activation has been linked to several physiologic pathways including those related to the regulation of RA. PPAR-γ can be expressed in RA synovial cells, and the PPAR-γ activation inhibits expression of inflammatory cytokines, such as TNF-α and IL-1β (Ji et al., 2001) and IL-1β-induced matrix metalloproteinase (MMP)-1 synthesis which can degrade the components of the extracellular matrix and leads to joint destruction (Fahmi et al., 2002). MMP-9 is also an inducible MMP, but its role in connective tissue destruction in arthritis appears to be secondary, since it contributes to the degradation of collagen only after the chains of the triple helix have been cleaved by the interstitial collagenases (Vincenti and Brinckerhoff, 2002). Recent studies indicate that MMP-9 is profoundly down-regulated by PPARγ agonists (Shu et al., 2000). These results suggest that PPAR-γ agonists may provide a new therapeutic approach for RA.

SPI1 gene (also known as PU.1) encodes an ETS-domain transcription factor that activates gene expres-sion during myeloid and B-lymphoid cell development. The nuclear protein binds to a purine-rich sequence known as the PU-box, found near the promoters of target genes, and regulates their expression in coordination with other transcription factors and cofactors. Migration inhibitory factor (MIF)-deficient macrophages are shown to have impaired activity of the PU.1 transcription factor, which is known to regulate the mouse Tlr4 gene, resulting in reduced cell surface expression of the lipopoly-saccharide receptor TLR-4 (Roger et al., 2001). However, the over-expression of MIF is reported in serum, synovial fluid, and cultured synovial fibroblasts from patients with RA (Leech et al., 1999), thus, leading to activation of PU.1 transcription factor and high expression of TLR-4, which is detected at an early stage of RA (Ospelt et al., 2008). CSF3R encodes a receptor for colony stimulating factor 3 who controls the production, differentiation, and function of granulocytes. CSF3R is shown up-regulated

more than threefold in early RA (Olsen et al., 2004). Expression of CSF3R is controlled by the myeloid transcription factors PU.1 in the 5′ untranslated region of G-CSF receptor promoter, at bp +36 and +43. Mutation of these sites prevents PU.1 binding and reduces promoter activity by 75% (Smith et al., 1996).

In addition, our findings indicated that four significant pathways were associated with RA development, including hematopoietic cell lineage, pathways in cancer, MAPK signaling pathway, and antigen processing and presentation. These four pathways were also modulated by corresponding TFs.

Hematopoietic cell lineage can differentiate into various immune cells, such as lymphocytes or macrophage and therefore it may be associated with RA, a quintessential autoimmune syndrome. In RA, circulating bone marrow–derived hematopoietic progenitor cells are diminished, and concentrations stagnates at levels typical of those in old control subjects. HPCs from RA patients display growth factor non-responsiveness and sluggish cell cycle progression (Colmegna et al., 2008). CSF3R is also shown to be up-regulated more than threefold in early RA (Olsen et al., 2004). In the TF-DEGs regulation network, we found that ETS2 could inhibit CSF3R expression, thus inhibiting hematopoietic cell lineage pathway. CD36 provides a signal that sufficiently lowers the activation threshold of T cells from patients with RA such that the cells can be activated by synovial self-antigens (Goronzy et al., 2004). Phosphorylation of PPAR-γ results in decreased CD36 gene transcription (Han et al., 2000), alleviating RA development.

There is evidence that pathway in cancer is closely related with RA development. However, this relation between cancer and RA is complex. A number of previous investigations proved that methotrexate-treated RA patients have an increased incidence of melanoma, non-Hodgkin's lymphoma, lung cancer, hematologic and kidney cancer (Buchbinder et al., 2008; Chen et al., 2011). While other publications found the reduced risk for colorectal cancer, large bowel cancer and stomach cancer in patients with RA, at the same time they also found the excess of lymphomas of RA patients (Gridley et al., 1993; Thomas et al., 2000). Overall, we can see that the susceptibility to cancer of RA patients is tissues specific, and the mechanisms of this phenomenon need further investigations. In our study, we found that Jun, ETS2, and SPI1 all promoted the pathway in cancer, which seemed to be in accordance with our TFs-DEGs interaction relationship. JUN could promote FN1 expression, which is a high expression in cancer (Nam et al., 2010) and plays a key role in the regulation of adhesion, migration and metastasis of tumors (Li et al., 2011). ETS2 promoted FLT1 expression, which has been proved to be up-regulated in the majority of human squamous cell carcinomas (Lichtenberger et al., 2010); SPI1 promoted CSF3R expression. Aberrant expression of G-CSF and its receptor have been observed in solid tumors like ovarian cancer, bladder cancer and squamous cell carcinoma (Beel and Vandenberghe, 2009).

Mitogen-activated protein kinases (MAPKs) have been implicated as playing key regulatory roles in the production of these pro-inflammatory cytokines and downstream signaling events, leading to joint inflammation and destruction (Thalhamer et al., 2008). For example, IL-23p19 is over-expressed in RA synovial fibroblasts and IL-17 appears to up-regulate the expression of IL-23p19 in RA synovial fibroblasts via PI3-kinase/Akt, NF-κB- and p38-MAPK-mediated pathways (Kim et al., 2007). Our results indicated that CREB inhibited MAPK signaling pathway. Phosphorylated CREB has been proposed to directly inhibit NF-κB activation by blocking the binding of CREB binding protein to the NF-κB complex, thereby limiting pro-inflammatory responses (Wen et al., 2010).

Emerging evidences have demonstrated antigen processing and presentation pathway is involved in RA progression. The proteoglycan aggrecan, which is a major structural component of cartilage, has been identified as a candidate auto-antigen in rheumatoid arthritis (RA). Studies have also defined an essential requirement for auto-antigen-specific B cells as antigen presenting cells (APC) in RA. These B cells specifically bind aggrecan leading to efficient processing and the generation of the immunogenic T cell epitope 84-103 that is recognized by both aggrecan-specific T cell hybridomas (Wilson et al., 2011). Some lipid antigens can be recognized and presented by CD1 glycoproteins, whose expression is under the control of transcription factors, CREB1. Therefore, we suggest that CREB1 may be involved in RA by promoting antigen processing and presentation pathway.

In conclusion, our present findings shed new light on the biology of RA progression and have implications for future research. We showed that JUN, ETS2, CREB1, PPARG, and SPI1 were crucial TFs in our transcriptome networks and these TFs could regulate the DEGs expression to involve in RA by promoting or inhibiting effect. Four significant pathways were identified associated with RA development, including hematopoietic cell lineage, pathways in cancer, MAPK signaling pathway, antigen processing and presentation. These four pathways were also modulated by corresponding TFs. However, further experiments are still needed to confirm the conclusion.

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