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Original Article

Analysis for time series gene expression profiles during recovery phase of severed rat medial collateral ligament

Chunhui Ma[1], Qi Lv[2], Songsong Teng[3], Yinxian Yu[3], Kerun Niu[3], Chengqin Yi[3], Biao Cheng[4]

[1]Shanghai General Hospital of Nanjing Medical University, Shanghai 200080, China; [2]Department of Radiology, Tong Ji Hospital, Tong Ji University, Shanghai 200065, China; [3]Department of Orthopedic Surgery, Shanghai General Hospital, Shanghai Jiao Tong University, Shanghai 200080, China; [4]Shanghai Tenth People's Hospital of Nanjing Medical University, Shanghai 200072, China

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Abstract: Here we intended to depict the dynamic changes of gene expression profiles during the recovery phase of severed rat medial collateral ligament (MCL). The GSE10720 gene expression profiles of severed rat MCL after 1, 2, 4, 7, 10 and 14 day(s) of injury were downloaded from Gene Expression Omnibus (GEO) database. STEM (Short Time-series Expression Miner) software was conducted to cluster genes with similar expression patterns, followed by identification of differentially expressed genes (DEGs) between injured group and sham control group, Gene Ontology (GO) annotation and pathway enrichment analysis. Subsequently, gene functional interaction (FI) network and protein-protein interaction (PPI) network were respectively constructed using Reactome FI and STRING. Transcription factors (TFs) were predicted by Animal TFDB. Three significant temporal expression patterns were uncovered after MCL injury, among which the most significant cluster consisted of 3854 DEGs. Of these DEGs, 102 constructed the gene FI network consisting of 9 significant modules. GO analysis revealed that cell differentiation was the most significant GO term enriched by 240 DEGs, and among these DEGs, 33 genes, such as *EGFR, VEGFC, ITGAV, ITGA1*, significantly enriched in both focal adhesion and ECM-receptor interaction pathways. PPI network demonstrated 10 genes with degree > 20. Totally 22 TFs were predicted to target to these 240 DEGs. The expression profiles of severed rat MCL significantly changed during the recovery phase. DEGs in the early 14 days of MCL healing, such as *VEGFC, EGFR, ITGAV and LAMB2*, are more likely to play essential roles during this process.

Keywords: Medial collateral ligament, time-series expression analysis, function enrichment analysis

Introduction

Medial collateral ligament (MCL) is composed of two portions, i.e., the superficial MCL (sMCL) and deep MCL (dMCL) [1]. sMCL is the prime limitation for abduction rotation [2], while dMCL acts as the anchor to fix the medial meniscus to the tibia and femur [3]. As the major static stabilizer against valgus rotation of knee joint, MCL injury can affect the function of medial meniscus and then lead to the occurrence of osteoarthritis [4]. Fortunately, even the severed MCL, as a kind of soft tissue injury, can be healed without surgical intervention, although its recovery is very difficult and requires a very long period [5, 6]. For instance, gene transfer strategy has been used to induce DNA fragments into injured ligaments to promote or depress the expression of certain genes and accelerate the healing process [7, 8]. The repairing mechanism of injured MCL, however, has not been extensively studied, which results in the limited the understanding of key factors involving in the recovery phase.

Microarray of time series gene expression is widely used in the research of connotatively dynamic changes of certain biological processes, and is therefore considered as one of the optimal approaches to identify genes/processes/pathways involved in repairing injured MCL [9, 10]. Owens *et al.* (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE10720) submitted expression profiling of severed rat MCL after 1, 2, 4, 7, 10 and 14 days of injury to the Gene Expression Omnibus (GEO)

database (http://www.ncbi.nlm.nih.gov/geo/). Nevertheless, this vast quantities of valuable data has not been deeply mined, and litter information has been derived from it.

The present study analyzed this set of expression profiles for severed rat MCL by utilizing the software of Short Time-series Expression Miner (STEM) designed specifically for the analysis of short time series microarray gene expression data. Accompanied with the high-throughput bioinformatics approaches such as Gene Ontology (GO) annotation, pathway enrichment, prediction of transcription factor (TF), as well as protein-protein interaction (PPI) network and gene functional interaction (FI) network, we aimed to uncover crucial genes for better understanding the recovery mechanisms of MCL injury and provide insight for improvement of MCL repair.

Materials and methods

Agilent microarray data

Gene expression profiles of severed rat MCL were downloaded with the accession number of GSE10720 from GEO database. This dataset was submitted at Mar 04, 2008 and updated at Dec 06, 2012 based on the platform of Agilent-013162 Whole Rat Genome Microarray G4131A. In the original study, rats in the injured group underwent ligament severing after general anesthesia. At a series of time points following injury (1, 2, 4, 7, 10 and 14 days after injury), the sham control groups were exposed to the same anesthesia regime and a bilateral sham surgery was performed but the ligament was not severed. Totally 72 samples were available, including 5 replicates from the injured group and sham group at each time point (3 replicates at the 7^{th} day), 5 replicates from no-manipulated ligament, 5 replicates from fibroblasts ligament and 3 replicates for universal reference RNA labelled by Cy3 and Cy5, respectively.

Cluster analysis of genes by short time series analysis

STEM was the first software program specifically designed for analysis of short time series microarray gene expression data (3-8 time points), and permits efficient biological interpretations of the data through integrating with GO [11]. The same gene sets have most closely gene expression pattern as determined by the correlation coefficient. The algorithm employed in STEM can then determine which gene set has a statistically significant higher number of genes using a permutation test.

Herein, the raw data downloaded from GEO database in TXT format was firstly normalized based on average normalization algorithms. The tool of "Short Time-series Expression Miner" in STEM v1.3.6 software, was then performed to cluster genes and identify gene sets co-regulated time dependently with correlation coefficients > 0.09 and all other settings as the defaults [10]. Student's *t*-test was used to identify differentially expressed genes (DEGs) between sham group and injured group and false discovery rate (FDR) < 0.05 for one or more time points was considered as the threshold.

Gene distribution analysis

To detect the major chromosome related with MCL healing process, all DEGs in the most significantly expression pattern selected by STEM were analyzed for their distribution in rat chromosomes.

Construction of gene FI network

Reactome FI Viz, as a Cytoscape app, was recently developed by Guanming Wu *et al.* for [12] assisting biologists to analyze network- and pathway-based data in a biologically intuitive and user-friendly way. It is described that this app could be used to unravel pathway and network patterns, reveal pathways significantly enriched by genes, as well as search for gene signatures from gene expression data sets. We thus performed Reactome FI Viz to construct the gene FI network for network-based analysis *via* merging interactions extracted from Reactome and other pathway databases, and simultaneously generate a sub-network for module analysis based on the module size and average correlation. All parameters were set as the defaults. For better understanding the potential role of these network modules, genes in each module were further analyzed by pathway enrichment with FDR < 0.05.

GO analysis

STEM supports efficient and statistically rigorous biological interpretations for short time series data through its integration with the GO.

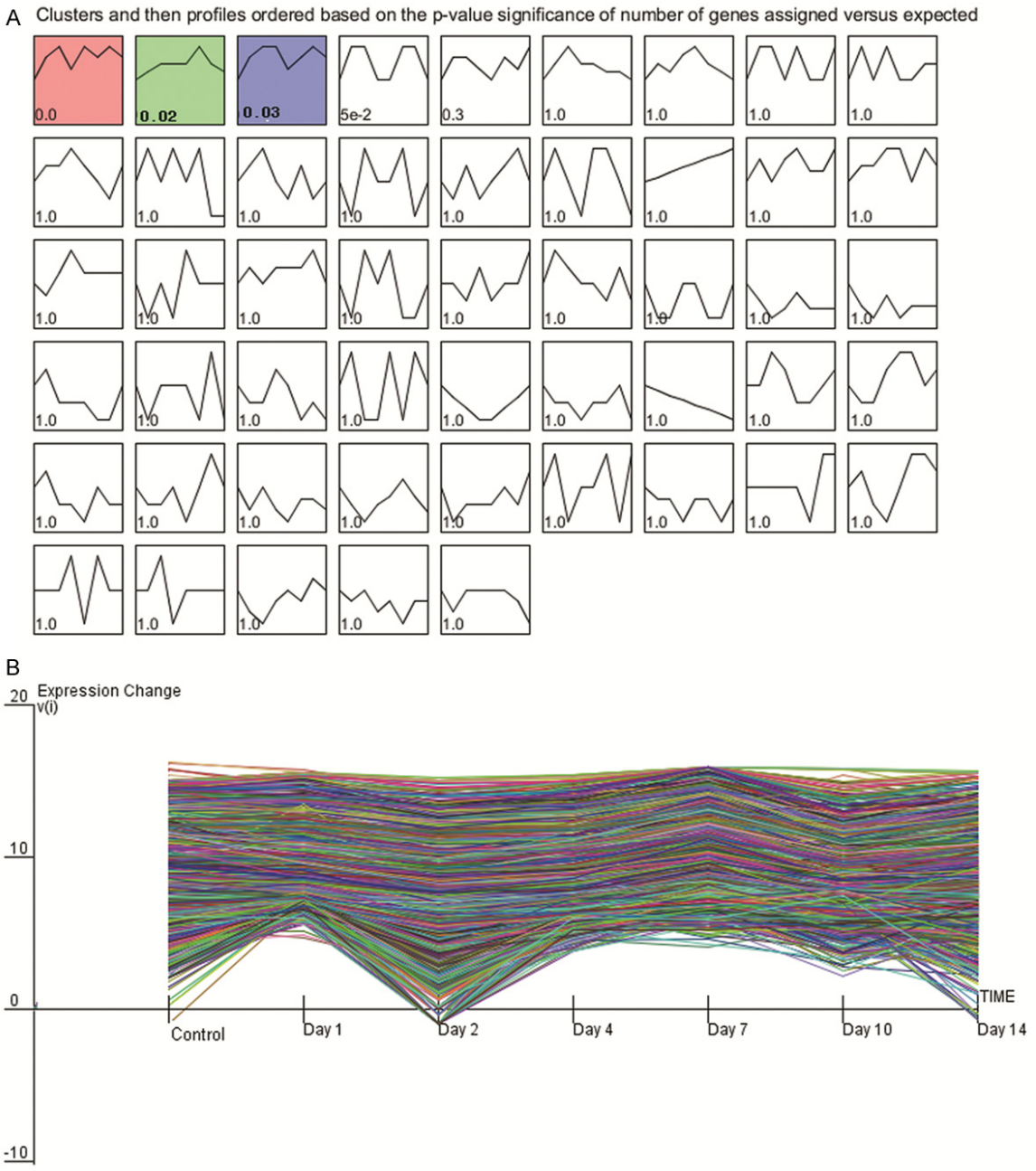

Figure 1. STEM clustering analysis of significant differentially expressed genes in MCL after injury. A. Three profiles (respectively labeled as profile 1, profile 2 and profile 3) with a statistically significant number of genes assigned. Each box represents one expression profile/pattern. The number in the bottom left corner represents *p*-value. B. One model profile detailed in expression graphs. The curves in different color show individual gene expression profiles. The distinct and representative temporal expression profiles sampled at 7 time points, control, 1 d, 2 d, 4 d, 7 d, 10 d, 14 d.

Hence STEM itself can determine GO term enrichments for a set of genes avoiding exporting into external GO software. The STEM integration with GO is bidirectional as it can also determine the behavior of genes in a given GO category and identify temporal expression profiles enriched by genes in that category. In our analysis, the DEGs in the most significant gene clusters were mapped to GO terms by STEM with a threshold of FDR < 0.05.

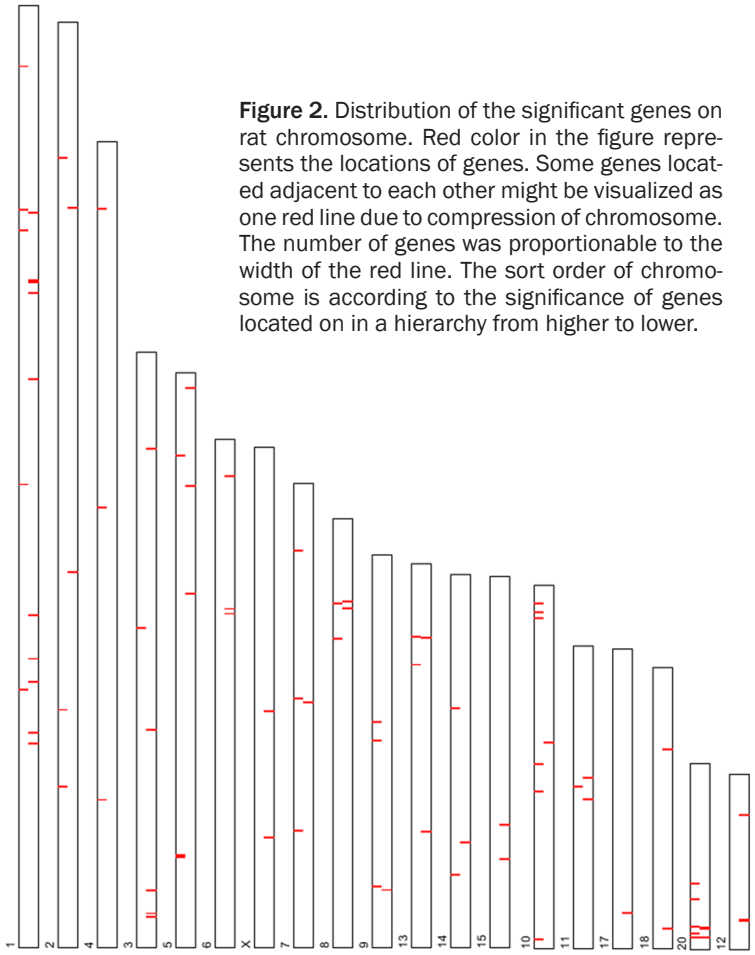

Figure 2. Distribution of the significant genes on rat chromosome. Red color in the figure represents the locations of genes. Some genes located adjacent to each other might be visualized as one red line due to compression of chromosome. The number of genes was proportionable to the width of the red line. The sort order of chromosome is according to the significance of genes located on in a hierarchy from higher to lower.

Pathway enrichment analysis

Traditionally, the individual gene analysis (IGA) was used to evaluate the significance of individual genes between two compared groups. Due to its defect in threshold value selection, gene set analysis (GSA), free from the problems of the 'cutoff-based' methods, has developed [13]. GSA directly scores pre-defined gene sets for differential expression and especially aims to identify gene sets with 'subtle but coordinated' expression changes that cannot be detected by IGA methods [14]. In this study, genes enriched in the most significant GO term were respectively analyzed for pathway enrichment by Functional Classification Tool with a threshold of FDR < 0.05 [15].

Construction of PPI network

The STRING database (http://string-db.org) is developed with the aim to provide a critical assessment and integration of physical as well as functional PPI based on resource from neighborhood, gene fusion, co-occurrence, co-expression experiments, databases and text mining [16]. Herein, PPI pairs of DEGs enriched in the most significant GO term were predicted by using STRING, and those with PPI score > 0.04 were retrieved for construction of PPI network. Cytoscape 2.8 (http://cytoscape.org/) was finally conducted to visualize the PPI network.

TF prediction

TFs are regarded as key regulators for gene expression in all organisms. In this study, TFs targeting to the DEGs enriched in the most significant GO term were predicted by using AnimalTFDB [17]. Information on the TF families was also obtained.

Results

Time series gene expression profile analysis

Time-series analysis was conducted to assess the modulation of gene expression profiles from severed rat MCL collected in the time-course experiment by using the STEM software. The expression profiles totally mapped to 50 model temporal expression patterns among which 3 were statistically significant (**Figure 1A**). Briefly, gene expression levels in these clusters were evaluated on the first day (day 1) after injury, decreased to the lowest on day 2, and then gradually increased until day 4, followed by relatively stable expression levels no the 4-10th day and decreasing on the 12-14th day (**Figure 1B**). Totally 3854 DEGs (Supplementary Table 1) with FDR < 0.05 constituted the most significant cluster, which were further analyzed for chromosome distribution and GO annotation.

Distribution of DEGs on chromosomes and their GO annotation

The distribution of these 3854 DEGs on chromosomes was examined to determine the pref-

Table 1. Gene ontology (GO) terms of genes in the significant expression profile (FDR < 0.05)

Category ID	Category name	#Genes	FDR	Category ID	Category Name	#Genes	FDR
GO:0030154	Cell differentiation	240	0.002	GO:0010941	Regulation of cell death	143	0.008
GO:0051649	Establishment of localization in cell	162	0.002	GO:0006446	Regulation of translational initiation	11	0.01
GO:0009653	Anatomical structure morphogenesis	194	0.002	GO:0048513	Organ development	246	0.014
GO:0008219	Cell death	164	0.002	GO:0044271	Cellular nitrogen compound biosynthetic process	195	0.016
GO:0051171	Regulation of nitrogen compound metabolic process	197	0.002	GO:0018130	Heterocycle biosynthetic process	189	0.024
GO:0016265	Death	164	0.002	GO:0048518	Positive regulation of biological process	292	0.024
GO:0016482	Cytoplasmic transport	66	0.002	GO:0006793	Phosphorus metabolic process	208	0.024
GO:0015031	Protein transport	98	0.002	GO:0034654	Nucleobase-containing compound biosynthetic process	184	0.028
GO:0045184	Establishment of protein localization	102	0.002	GO:0050793	Regulation of developmental process	159	0.03
GO:0048646	Anatomical structure formation involved in morphogenesis	93	0.002	GO:0033554	Cellular response to stress	102	0.036
GO:0042981	Regulation of apoptotic process	137	0.002	GO:0070887	Cellular response to chemical stimulus	178	0.036
GO:0043067	Regulation of programmed cell death	138	0.002	GO:0007399	Nervous system development	172	0.038
GO:0019219	Regulation of nucleobase-containing compound metabolic process	190	0.002	GO:0006796	Phosphate-containing compound metabolic process	203	0.038
GO:0010033	Response to organic substance	245	0.002	GO:0048193	Golgi vesicle transport	15	0.04
GO:0070647	Protein modification by small protein conjugation or removal	35	0.008	GO:0006355	Regulation of transcription, DNA-dependent	150	0.044

#FDR represents the *p*-value corrected by false discovery rate.

Table 2. The summary of critical functional categories in significant expression patterns

Functional categories	Profile 1	Profile 2	Profile 3
Response to organic substance	√	√	√
Translational elongation	√		√
Protein targeting to mitochondrion	√		
Regulation of cell death		√	√

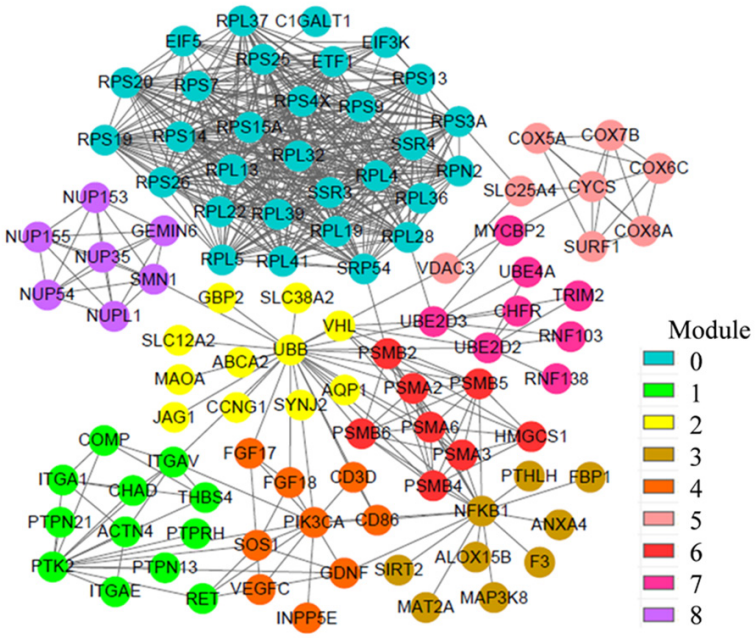

Figure 3. Gene functional interaction network and module analysis. Circle represents the differentially expressed genes between injured group and sham control group; edge between two nodes represents functional interaction; each color represents one functional module.

erential chromosomes related to severed rat MCL recovery, which was with great biological significance. As shown in **Figure 2**, relatively more DEGs located on chromosome 1, compared with those on the other chromosomes.

To investigate the main biological processes involving in severed rat MCL repair, the 3854 identified DEGs were mapped to GO terms in STEM. After filtering by the criterion of FDR < 0.05, 30 significant GO terms were enriched, including cell differentiation (FDR = 0.002), cell death (FDR = 0.002), regulation of nitrogen compound metabolic process (FDR = 0.002) (**Table 1**). Among these enriched GO terms, cell differentiation was enriched with the largest number of DEGs (240/3854, 6.23%). Besides, GO analysis was also conducted for gene sets in each temporal expression cluster. The high-enriched GO terms for each temporal expression cluster, such as response to organic substance, translational elongation and regulation of cell death, were found to enrich in more than one expression patterns (**Table 2**). Besides, a few of genes were annotated with the process of angiogenesis and inflammation, such as *ITGA1, ITGAV* and *VEGFC* (Data not shown).

Gene FI network and module analysis

Gene FI network was constructed by totally 102 in 3854 DEGs, which consisted of 9 significant modules (**Figure 3**). Pathway enrichment analysis showed that most genes in Module 0 enriched in pathways like SRP-dependent cotranslational protein targeting to membrane, Eukaryotic Translation Elongation/Termination and Ribosome; while genes in Modules 1 were mainly related to focal adhesion. Significantly enriched pathways by all gene sets from each module were shown in **Figure 4**. No significant pathways were found to be enriched by gene sets from Module 2 and 3.

Pathway enrichment analysis for genes mapping to the most significant GO terms

To further explore genes that play important roles in MCL repair, the 240 genes significantly enriched in the GO term of cell differentiation were further analyzed by pathway enrichment with the software of Functional Classification Tool. As shown in **Table 3**, these genes mainly participated in two pathways including focal adhesion and extracellular matrix (ECM)-receptor interaction. In addition, focal adhesion was the most significant pathway and enriched by 24 DEGs.

Analysis for PPI network for genes mapping to the most significant GO terms

By using STRING, PPI network was constructed with 581 edges and 180 nodes from 240

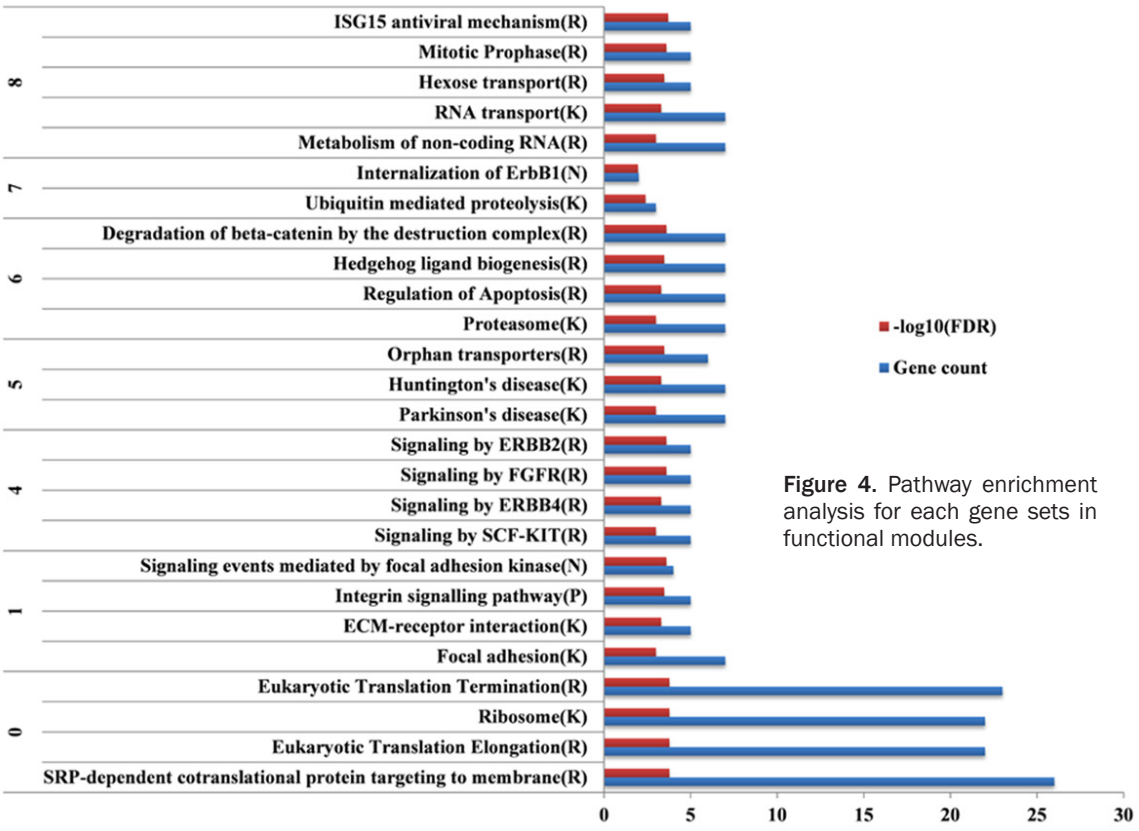

Figure 4. Pathway enrichment analysis for each gene sets in functional modules.

Table 3. Pathway enrichment analysis of genes enriched in GO term of cell differentiation

Term	Count	FDR#	Genes
rno04510:Focal adhesion	22	2.42E-07	CAV3, EGFR, CAV2, ROCK1, PGF, ITGA1, MYLK2, COL2A1, VEGFC, PTK2, LAMB2, RASGRF1, LAMA5, CCND2, ITGAV, BCL2, MAPK9, RELN, COL11A2, COL11A1, FN1, THBS4
rno04512:ECM-receptor interaction	11	0.00526	LAMB2, CD36, LAMA5, ITGAV, ITGA1, RELN, COL2A1, COL11A2, COL11A1, FN1, THBS4

#FDR represents the *p* value corrected by false discovery rate.

DEGs significantly enriched in GO terms of cell differentiation (**Figure 5**). Among all these nodes, 10 were characterized with relatively high level degrees (> 20), including BCL2 (degree = 42), JUP (degree = 33), JUND (degree = 32), MAPK9 (degree = 32), EGFR (degree = 31), PTK2 (degree = 28), DHH (degree = 23), HIF1A (degree = 22), RET (degree = 20) and AGT (degree = 20).

TFs targeting to DEGs mapping to the most significant GO terms

Based on Animal TFDB, a total of 22 TFs were predicted to be targeted by the 240 DEGs which enriched in the most significantly GO terms of cell differentiation (**Table 4**). These 22 TFs belonged to 13 TF families, including BHLH, HOMEOBOX, etc.

Discussion

MCL of the knee is one of the most commonly injured ligaments [18]. A more in-depth analysis of the molecular mechanism involved in MCL healing may offer novel opportunities for the development of new therapies for MCL injury. In our research, time series expression profiles of severed rat MCL during recovery phase were systematically analyzed by high-throughput bioinformatic approaches including STEM analysis, GO annotation, pathway enrichment, TF prediction, and construction of gene FI network and PPI network. Results showed that expression profiles were significantly clustered into three temporal expression patterns, and DEGs in the most significant cluster were mainly correlated with cell differentiation. Several

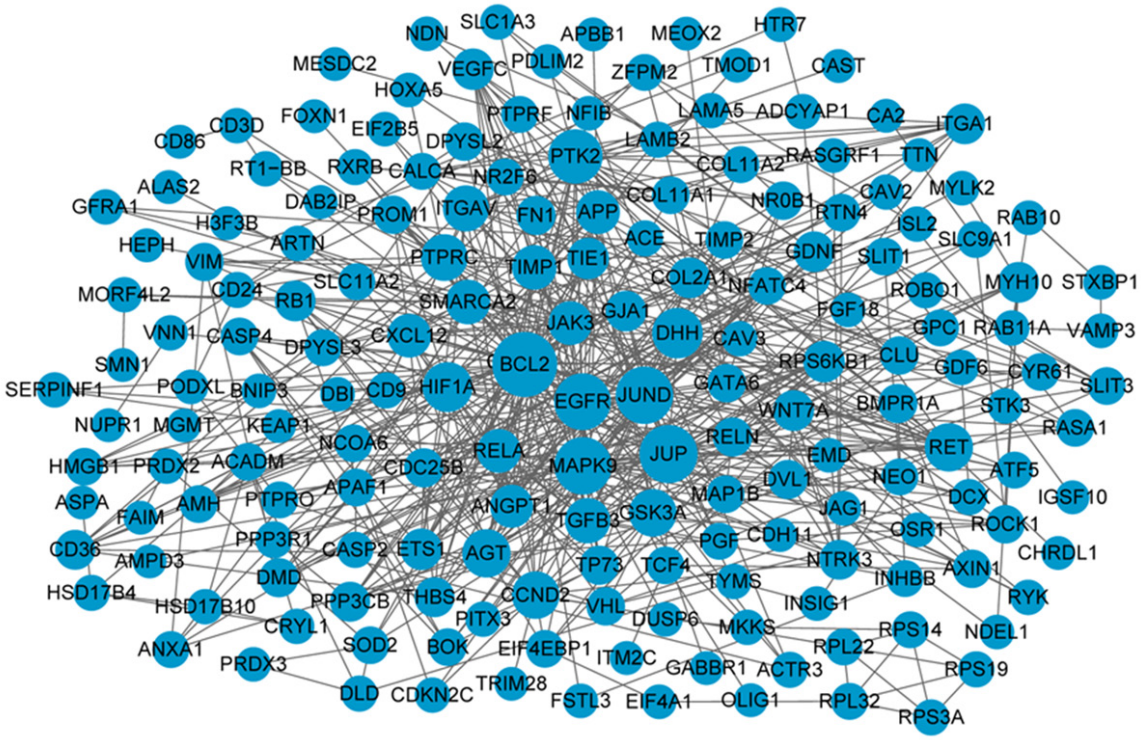

Figure 5. Protein-protein interaction network. Circle represents the differentially expressed genes between injured group and sham control group; edge between two nodes represents functional interaction; the size of node is positively correlated with its degree.

genes, such as *ITGA1, ITGAV* and *VEGFC* were annotated with both processes of angiogenesis and inflammation. Gene FI network construction and sub-network analysis further demonstrated the crucial role of these genes in cell proliferation and focal adhesions. PPI network revealed several key genes, such as *EGFR*, with relatively high level of degrees.

Integrated results of GO analysis and cluster analysis suggest that cell differentiation is the most significant GO terms related with MCL healing. The subsequent pathway analysis for 240 genes participating in cell differentiation revealed that DEGs were significantly enriched in two pathways including focal adhesion and ECM-receptor interaction. Focal adhesions mainly serve as sub-cellular macromolecules that mediate the regulatory effects of ECM adhesion on cell behavior [19]. Therefore, these genes, such as *EGFR*, *VEGFC*, *ITGAV, ITGA1*, might control tissue and organ morphogenesis and maintain tissue function by regulating ECM-receptor interaction.

A recent study showed that angiogenesis plays an important role in MCL healing process, and VEGF has been recognized as a supporting factor for stem cell therapy for MCL healing [20]. Corral *et al.* [21] reported that VEGF could improve granulation tissue formation in both normal and ischemic wounds. Moreover, VEGF is proved as an important factor modulating MCL repair through a paracrine effect [22]. VEGFC, as a member of VEGF family, is active in angiogenesis and endothelial cell growth, and can also affect the permeability of blood vessels [23]. In addition, ITGA1 and ITGAV, the members of integrin superfamily, could interact with several ECM proteins to mediate cell adhesion and may play roles in cell migration, inflammation and angiogenesis [24-25]. Consistent with previous studies, genes *ITGA1, ITGAV* and *VEGFC* were predicted to involve in the process of angiogenesis in the present study. The expression of these gene was adjusted during the recovery of MCL injury. Therefore, we suggest that these genes might play crutcial roles in the early phase of MCL healing through modulating interaction with ECM proteins.

For the cell proliferation stage, there is ample evidence that basic fibroblast growth factor (bFGF), growth and differentiation factor (GDF-

Table 4. Result for prediction of transcription factors

Gene id	Gene symbol	Family
29560	HIF1A	BHLH
84382	TCF4	BHLH
60394	OLIG1	BHLH
361391	CBFB	CBF
245980	NR2F6	COUP
81524	NFIX	CTF/NFI
29227	NFIB	CTF/NFI
24356	ETS1	ETS
287469	FOXN1	FORK HEAD
252886	FOXD4	FORK HEAD
24413	NR3C1	GCR
29279	MEOX2	HOMEOBOX
57233	ISL2	HOMEOBOX
29609	PITX3	HOMEOBOX
311604	ZHX3	HOMEOBOX
58850	NR0B1	OTHER NUCLEAR RECEPTOR
362675	TP73	P53
305897	NFATC4	RHD
309165	RELA	RHD
361801	RXRB	THYROID HORMONE RECEPTOR
314930	ZFPM2	ZF-C2H2
298878	OSR1	ZF-C2H2

5) [26], macrophage migration inhibitory factor (MIF) [27] and lysyl oxidase (LOX) family [28, 29] could stimulate the healing of MCL injury by promoting proliferation and migration of MCL fibroblasts. Although these genes were not screened out by our research, GO analysis in our results supports the importance of cell proliferation and differentiation in repair of injured MCL. In addition, *EGF* was predicted as a potential repair gene to promote MCL healing. EGF has been reported to stimulate cell proliferation by activating members of the EGFR family [30]. Experimental evidence suggest cell proliferation might be due to EGFR dimerization and tyrosine autophosphorylation [31]. In addition, EGF has been reported to promote the cell proliferation of rabbit MCL [32]. Two other ECM-related genes, laminin, beta 2 (*LAMB2*) and laminin, alpha 5 *(LAMA5*), both of which are the major noncollagenous constituent of basement membranes, were also involved in pathway of ECM-receptor interaction. They have also been further proved to be implicated in a wide variety of biological processes including cell adhesion, differentiation, migration [33]. Neither *LAMB2* nor *LAMA5* have been studied in MCL healing, and further studies will be necessary to better define their role in early phase of MCL recovery. Since the raw microarray data only covered the 14 days following the injury, it was difficult to detect the genes activity in the third phase of repair, which occurred in the following weeks to months after injury.

Taken together, we systematically analyzed the time series gene expression profiles of rat MCL at the recovery phase by STEM integrated with high-throughput bioinformatics approaches. Our results revealed that the gene expression profiles of injured MCL significantly changed during the recovery phase. DEGs in the early 14 days of MCL healing, such as *VEGFC, EGFR, ITGAV and LAMB2,* are more likely to play essential roles in cell differentiation and proliferation, immune response and focal adhesion. However, our results are still need to be validated by experiments.

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Disclosure of conflict of interest

None.

Address correspondence to: Biao Cheng, Department of Orthopedic Surgery, Shanghai Tenth People's Hospital, Shanghai Tong Ji University, No.301 Yanchang Middle Road, Zhabei District, Shanghai, 200072, China. Tel: +86-21-6630-0588; Fax: +86-86-21-56771300; E-mail: drchengbiao123@163.com; Chengqin Yi, Department of Orthopedic Surgery, Shanghai General Hospital, Shanghai Jiao Tong University, Shanghai 200080, China. E-mail: dryichengqing@163.com

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