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Original Article

Effect of Stat1 and Stat3 on the progression of viral myocarditis in mice by regulating the development of the vascular system

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Abstract: Myocarditis is an inflammatory heart disease mainly caused by viral infection. Viral myocarditis can be divided into the acute viral phase, subacute immune phase, and chronic cardiac remodeling phase. Although myocarditis can be diagnosed clinically by histology and cytology, its biomarkers are not clear enough and can cause misdiagnosis. Thale's mouse encephalomyelitis virus (TMEV) belongs to the genus *Myocardia* and can cause myocarditis in susceptible mice. Using transcriptome microarray data, this study used infected mice as the research subjects and performed bioinformatics analysis, including differential analysis, co-expression analysis, enrichment analysis, and predictors of pivot regulators. Through WGCNA analysis, we obtained nine related dysfunction modules. Reviewing the functions and pathways of interest , we investigated the regulation of vasculature development, which were associated with the other nine dysfunction modules. Finally, the transcription factors and ncRNA of the regulatory dysfunction module were analyzed to obtain key regulators such as Stat1, Stat3, and G730013B05Rik. Among them, Stat1 and Stat3 have significant regulatory effects on vasculature development. In summary, by controlling vascular system development, we believe that Stat1 and Stat3 affect the pathogenesis of viral myocarditis. Stat1 and Stat3 may be used as therapeutic targets for the treatment of viral myocarditis induced by TMEV infection, and may also serve as biomarkers for the diagnosis of myocarditis.

Keywords: Myocarditis, vascular system, Stat1, Stat3, transcription factors

Introduction

In 1995, the World Health Organization defined myocarditis as an inflammatory disease of the myocardium through histological, immunological and immunohistological criteria [1]. At present, there are about 1.5 million cases of myocarditis found worldwide each year, with an estimated incidence of 10-20 cases per 100,000 people [2]. According to statistics, more than half of the cases of myocarditis occur in patients under 40 years of age, and after autopsy analysis, the prevalence rate is 3.5%-5% [3]. Dilated cardiomyopathy is a common indication for heart transplantation and is believed to be the cause of accidental death in young adults [4]. Despite the best medical management, the overall mortality has not changed over the past 30 years [5]. A study of sudden death syndrome has linked viruses (enterovirus, adenovirus, parvovirus B19, and Epstein-Barr virus infections) to patients with sudden death syndrome [6]. Epidemiological studies have shown that most Coxsackie B virus infections are an important causes of myocarditis. Although most of them are benign, the effects cannot be underestimated. In order to treat myocarditis better and faster, it is necessary to diagnose myocarditis accurately. Currently, many studies have validated the diagnosis by classical methods of endomyocardial biopsy (EMB) through establishing histological, immunological and immunohistochemical criteria [7-9]. Some studies have used the new T2*w MRI techniques to rapidly and sensitively determine the pattern and severity of acute and chronic enteroviral myocarditis [10]. The pathophysiology of viral myocarditis can be broken down into direct viral-mediated damage, which can also trigger acute and chronic autoimmune responses and subsequent adverse remodeling [11]. The pathogenesis

summary analysis shows the cause of myocarditis. These include infectious diseases (Echovirus, spore virus), autoimmune diseases (dermatomyositis, giant cell myocarditis), allergic reactions (cephalosporins, tetracycline), toxicity of drugs (amphetamine, phenolamine in children), and radiation therapy [12]. After infection with viral myocarditis, patients develop natural and adaptive immune responses that gradually evolve into severe injury, persistent inflammation and persistent viral infections and dilate the cardiomyopathy [3]. As a result, the vital organs and tissues of the body do not have enough oxygen and nutrients to get rid of the waste, eventually leading to congestive heart failure [13, 14]. Clinically, typical manifestations of myocarditis are often accompanied by other viral diseases (fever, myalgia, respiratory or gastrointestinal symptoms). It is associated with symptoms of heart failure with a dilated cardiomyopathy phenotype [15, 16]. Among them, sudden death is a characteristic of myocarditis, which is usually diagnosed histologically [17]. Arrhythmia patients can be treated with heart rhythm adjustments, but most patients with arrhythmia myocarditis need a permanent pacemaker after restoring atrioventricular conduction [18]. Studies have shown that thyroid hormone and antibody levels contribute to chronic viral myocarditis (VMC) in children and diseases caused by complications of arrhythmia [19]. In the case of normal coronary angiography, both adults and children with simulated myocardial infarction had severe chest pain. At this time, the results of electrocardiogram examination and serum creatinine kinase were increased [20]. However, from a diagnostic point of view, cardiomyopathy is easily identified by some clinical features. So we need to better understand its molecular levels, gene levels, and pathogenesis. Through specific biomarkers, we found key indicators of myocarditis and made further systematic and accurate diagnosis. After comparing the analytical data, we know that RBP4 can be used for early screening as a specific biomarker [21]. Serum galectin-3 may be a useful biomarker for viral infections of myocarditis caused by acute myocardial fibrosis [22]. Studies have shown that miR-20b may be a potential therapeutic target for the treatment of viral myocarditis and a useful marker for the diagnosis of viral myocarditis [23]. In this study, the expression of genes related to myocarditis in mice infected with encephalomyelitis virus was analyzed to further understand the pathogenesis of myocarditis.

Materials and methods

Data resources

For high-throughput gene expression and other functional genomic data sets, the Gene Expressions Comprehensive Database is a public knowledge base. With the rapid development of technology, high-throughput data are now used for many other data applications, including detection of genomic methylation, chromatin structure, and genomic-protein interaction data. In the GEO database, we downloaded gene expression data from mice infected with TMEV and compared them to uninfected mice and performed a differential analysis [24].

Difference analysis

The differential expression analysis of the gene expression profile data of this study was performed using the R language limma package [25-27]. First, the background correction function is used and normalization of the data, setting the threshold as $P<0.05$.

Co-expression analysis

In order to explore the molecular progression of viral myocarditis in mice, we conducted differential analysis in infected mouse samples (4, 7, 60 days) and normal mouse samples, and finally obtained differential gene expression profiles [28]. Based on the regulatory capacity of each gene in the dysfunctional module, we explored the key factors leading to dysfunctional modules, which are considered to be the critical genes responsible for the development of viral myocarditis in mice.

Enrichment analysis

The exploration of functional and signaling pathways contributes to the study of molecular mechanisms of disease. For the genes of the dysfunctional modules, we performed an enrichment analysis of functions and pathways. It explores gene expression of mouse viral myocarditis and is an effective means of treating myocarditis. Therefore, we obtained nine dysfunctional gene modules in both infected C3H

mice and uninfected mice. The R language Clusterprofiler package [29] was used for enrichment analysis of Go function and KEGG pathway. Cluster profile is a Bioconductor software package for functional clustering analysis. Besides, for the integrated module network, Cytoscape's BinGO [30] application was also used for path analysis. The operational mechanism of the relevant modules was identified by further enriching the function and pathway of the module genes.

Transcription factors and non-coding RNAs that regulate dysfunctional modules

The transcription and post-transcriptional regulation of genes are often driven by non-coding RNA (ncRNA) and transcription factors (TF). Therefore, we scientifically tested the expressed genes of viral myocarditis in mice. A pivot regulator is defined as a modulator that has a significant regulatory role in the development of mouse viral myocarditis, including non-coding RNA and TF. We require that the control connection between each regulator and each module be greater than or equal to two. At the same time, according to the calculation of hypergeometric test, the target significance of enrichment in each module is *P*-value <0.01.

Statistical analyses

The results of the transcriptome microarray data test were analyzed with bioinformatics analysis, including differential analysis, co-expression analysis, enrichment analysis, and prediction of pivot regulators. Through WGCNA analysis, we obtained nine related dysfunction modules.

Results

Determining the disordered molecules of viral myocarditis in mice

Biologists have conducted experiments and studies on the pathogenesis of viral myocarditis in mice, and have identified underlying genes for the development of viral myocarditis in mice. However, the links between the complex molecules of these likely genes and their overall impact are unclear. In order to identify the role of differential gene expression (DEG) in mouse viral myocarditis, this study first integrated differential genes to obtain genes that may lead to the development of viral myocarditis in mice. We believe that there is an expression disorder of viral myocarditis in these differential genes.

Mouse viral myocarditis functional disorder module

Biological networks characterize global underlying pathogenic mechanisms, with each module marking potential mechanisms. First, we constructed an expression profile matrix in the samples based on the 448 differentially expressed genes and their interaction genes that are dysregulated in mouse viral myocarditis. Gene expression behaviors in mice infected with viral myocarditis (4, 7, 60 days) and uninfected mice were clustered into individual modules. By identifying the co-expression panel as a module, we obtained nine functional barrier modules (**Figure 1A**, **1B**). Based on the functional disorder module, we identified the essential genes and acquired core genes, including Vav1, Hif1an, and Phf11d (Table S1). By correlating the module with phenotypic data (**Figure 1C**), we can find that the MEpink module is associated with the gene expression of mouse viral myocarditis at the beginning of infection (4 days of disease). At seven days after infection, the MEyellow module was associated with gene expression in mouse viral myocarditis. At 30 days after infection, the MEred and MEbrown modules were associated with gene expression in mouse viral myocarditis.

Functions and pathways involved in the gene of interest

We performed enrichment analysis on GO function and KEGG pathway of 9 module genes and obtained 33,457 biological processes, 3,676 cells, 6,311 molecular functions, and 1,699 KEGG pathways. Then we used Cytoscape to visualize the results of GO enrichment analysis, and we can see that these functions are mainly focused on biological processes such as biological metabolic processes and gene recombination.

On the other hand, we visualized the enrichment results of the KEGG pathway (**Figure 2**), reflecting that the differentially expressed genes in infected mice with viral myocarditis and uninfected mice are mainly related to different viral infections, including influenza A

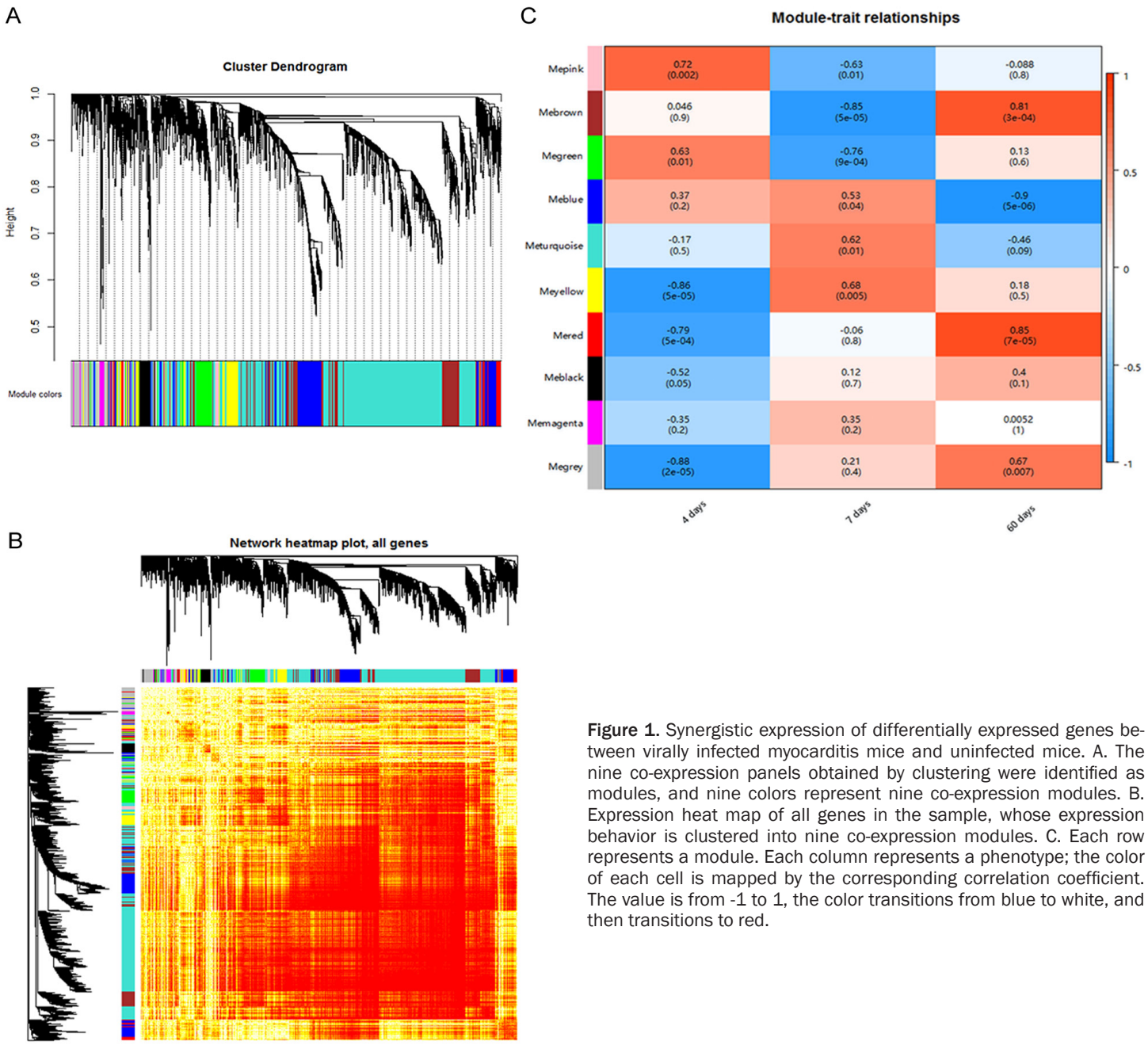

Figure 1. Synergistic expression of differentially expressed genes between virally infected myocarditis mice and uninfected mice. A. The nine co-expression panels obtained by clustering were identified as modules, and nine colors represent nine co-expression modules. B. Expression heat map of all genes in the sample, whose expression behavior is clustered into nine co-expression modules. C. Each row represents a module. Each column represents a phenotype; the color of each cell is mapped by the corresponding correlation coefficient. The value is from -1 to 1, the color transitions from blue to white, and then transitions to red.

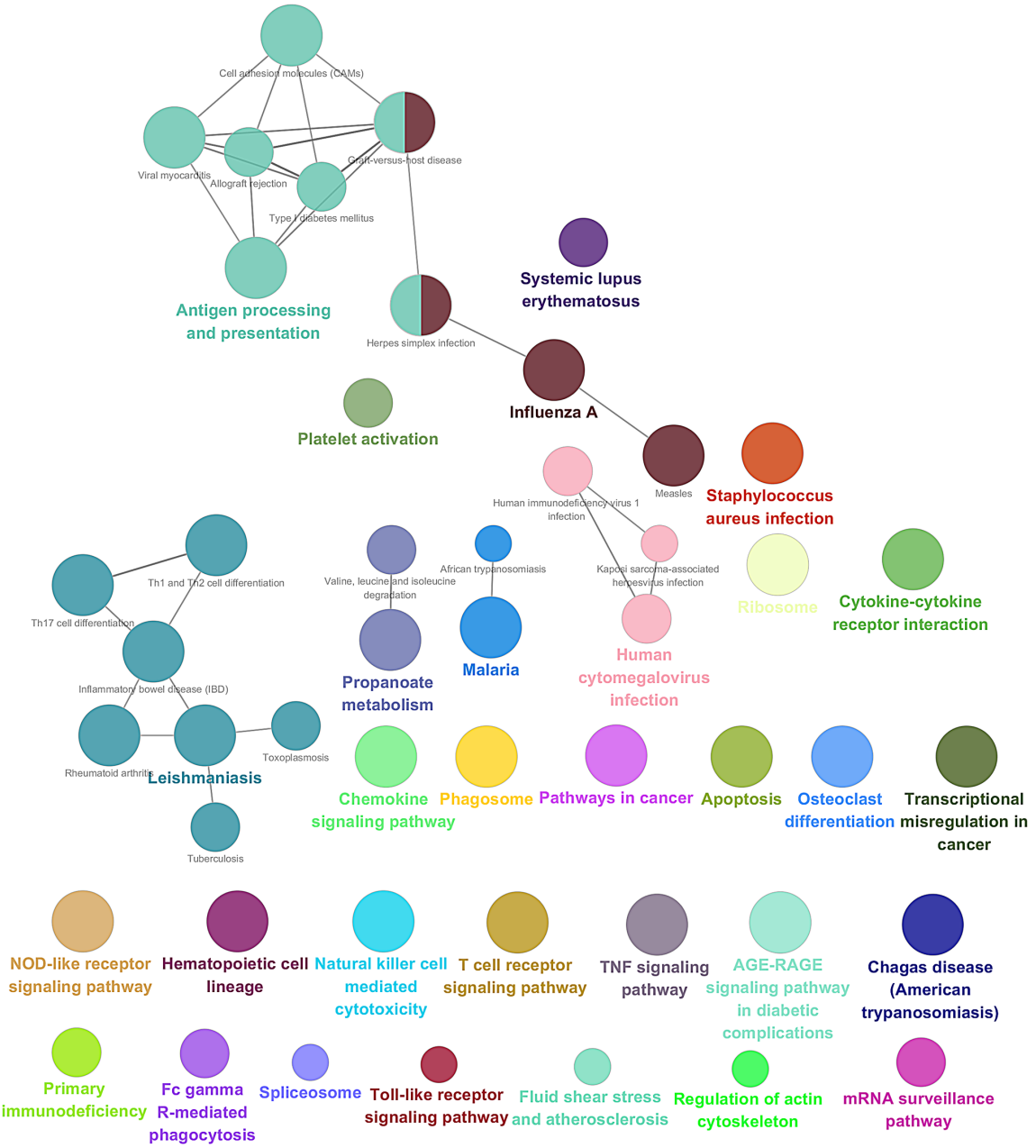

Figure 2. GO enrichment analysis and KEGG enrichment analysis. KEGG enrichment analysis, each color represents a different pathway process.

virus and Shiman disease. For the functions and pathways that regulate the most genes in the dysfunctional module, they can be considered to play the most critical role in the dysfunctional module. In this study, the obtained dysfunctionality modules were analyzed, and since the biological functions associated with the nine modules were relatively large, screening was required. The regulation of vasculature development can be a crucial feature. Its main function is to regulate vascular system development, including the genes IDs Stat1, Stat3.

TF and ncRNA that drive the development of viral myocarditis in mice

In this study, we performed a critical analysis of the co-expression module genes and explored key transcriptional regulators that regulate the development of viral myocarditis in mice. The

results (**Figure 3A**, **3B**) showed that a total of 151 ncRNAs involved 178 ncRNA-module regulatory pairs and 55 transcription factors affected 65 TF-module target pairs. Besides, in the ncRNA pivot analysis, we obtained three dysfunctional module genes involved in regulation (**Figure 3A**). In the TF pivot analysis, we selected transcription factors with higher associations, including Stat1, Stat3, Rb1, Trp53, Cebpb, Spi1 and Rela (**Figure 3B**). Among them, Stat1 and Stat3 are connected to the most modules, and the expression of 3 modules is connected. Therefore, we can judge the potential role of Stat1 and Stat3 in the development of mouse viral myocarditis. These transcription factors and ncRNAs may regulate the progression of viral myocarditis in mice by mediating dysfunctional modules.

Discussion

Viral myocarditis is a kind of cardiomyopathy caused by viral infection, which is one of the high risk diseases threatens human health. The odds ratio in the untreated group was 7.39 (95% CI 0.91 to 59.86), the survival ratio in the treatment group was 49.5%, and the survival rate in the placebo group was 35.9% (risk difference was 13.6%, 95% CI was 5.1 to 22.1%, *P* value =0.001) [31]. Common symptoms of viral myocarditis include fever, discomfort, myalgia, vomiting, and diarrhea. Adults may experience difficulty breathing, chest pain and arrhythmia. Children have a snoring breath and intercostal retraction. Infants have outbreak syndrome, including fever, hypoxia with purpura, respiratory distress, and even cardiac arrest.

Patients with severe myocarditis require circulatory support in the form of extracorporeal membrane oxygenation and ventricular assist devices. Clinically, we can use some methods to diagnose myocarditis, which monitor cardiac function and morphological changes. Moreover, myocardial edema imaging is the most effective parameter for predicting left ventricular ejection fraction [32]. We need to further study its genetic level and explore the pathogenesis of its genetic level. After data analysis, the pathogenic genes were further analyzed, and nine functional disorder modules were obtained. Based on the functional barrier module to identify the critical genes of each module, we acquired core genes based on Vav1, Hif1an, Phf11d, *etc*. Studies have shown that the heart contains two significant subsets of conventional dendritic cells (CDC), namely CD103+ and CD11b+. Among them, antigen-specific T cells play an essential role in the clinical period of viral myocarditis, and IL-35, CD4+, and EBI3+ T have been found to play an indispensable role in the progression of VMC [33-35]. In this study, after obtaining the dysfunctional module and the core genes, the functions and modules of the gene of interest were analyzed. In the pathway analysis of the involved genes, we obtained a 9-module regulation of vasculature development, including in the management of vascular system development. This indicates that the involvement of Stat1 and Stat3 changed the development of the cardiovascular system and ultimately affected the development of myocarditis.

Some studies report that interleukin-17A (IL-17A) and interleukin-13 (IL-13) have been linked the pathogenesis of viral myocarditis. Among them, SNP rs2275913 in IL-17A gene may affect susceptibility, while IL-13 can reduce cardiac damage and protect heart function by enhancing M2 macrophage polarization [36, 37]. Studies have shown that the expression level of TNF-α, IL-18 and cTnI and the expression level of miR-1 and miR-146b can be used to predict viral myocarditis in children [38]. By studying these regulatory factors, the diagnostic level of VMC can be effectively enhanced, and the accuracy of its determination can be further guaranteed.

Finally, we performed a pivotal analysis of co-expression modules. We explored the vital transcriptional regulators that regulate the progression of viral myocarditis. The results showed that there were a total of 151 ncRNAs and 55 transcription factors. Besides, in ncRNA and TF pivot assays, we obtained potential regulatory factors including G730013B05Rik, Rb1, Stat1, Stat3, Trp53, Cebpb, Spi1, and Rela. We initially identified dysfunctional molecules involved in the pathogenesis of viral myocarditis. Through the above analysis, regarding the prevention and treatment of myocarditis, we can start from the potential mechanism of action for prevention and treatment.

In contrast, the regulation of TGF-β1/Smad7 signaling pathway promotes myocardial fibro-

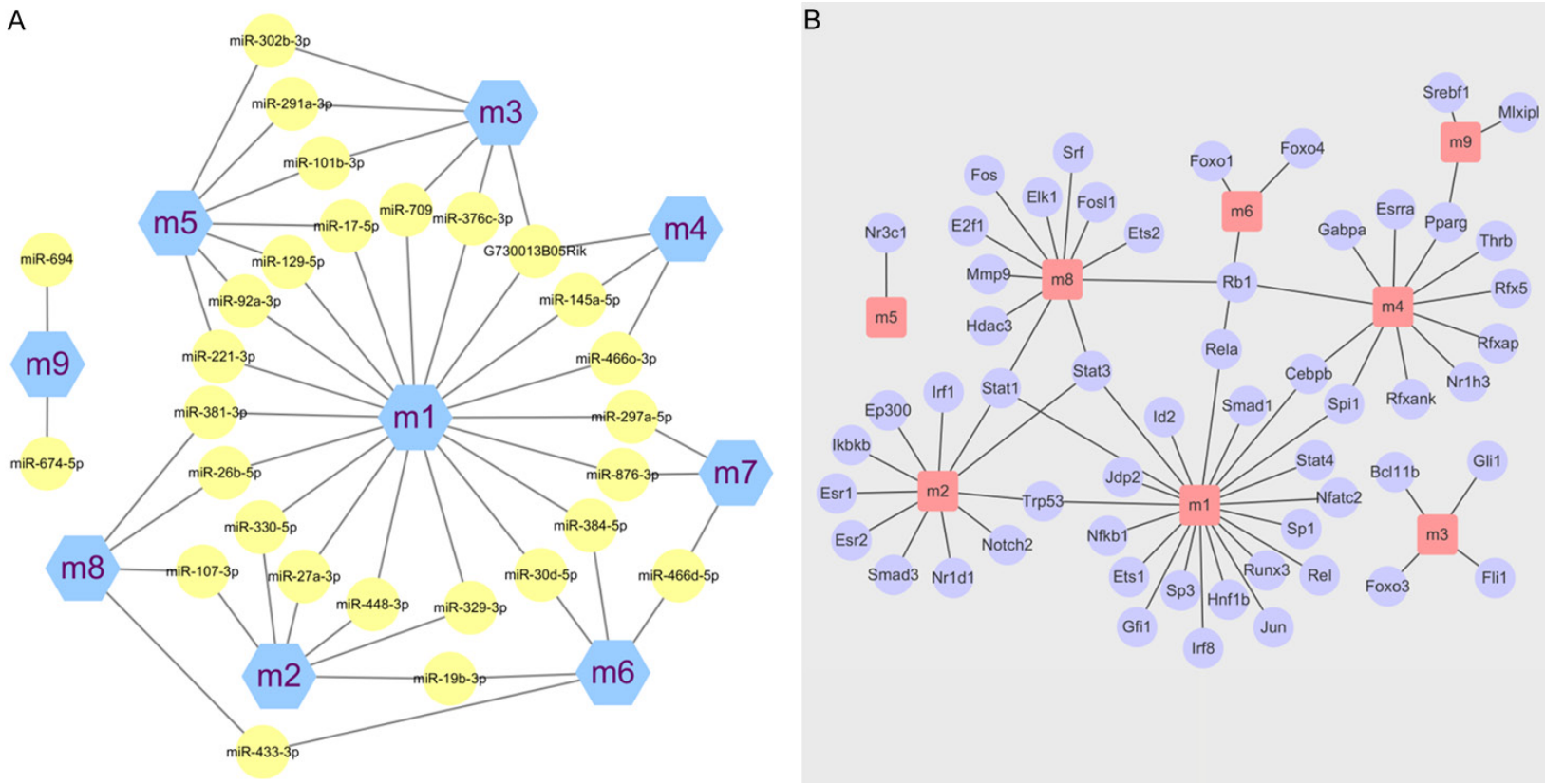

Figure 3. Modulatory effects of regulators on dysfunction modules. A. The blue hexagon represents the module, and the yellow circle represents the ncRNA. B. The red square represents the module, and the purple circle represents the TF.

sis in VMC mice [39]. In the acute and resolution/chronic phase of Biya, cardiac-related miR-208a levels were significantly increased in the acute phase. Therefore, the determination of miR-208b level has prognostic significance for the functional recovery of the left ventricle [40]. Based on the study of gene expression specificity in mice with viral myocarditis, we found that Stat1 and Stat3 may regulate the development the of vascular system to guide the development of viral myocarditis. This study not only helps us understand the potential role of gene regulation in viral myocarditis, but also provides a reference for clinical treatment. Many drugs have been put into use, but the study of their active ingredients requires further research. At present, total flavonoids of astragalus (TFA) and dried root extract (ARDE) are used in the treatment of viral myocarditis. Its mechanism of action is to reduce the levels of miR-146b and miR-155 to improve cardiac function in patients with VMC. TFA prevents loss of mRNA and protein levels of calmodulin. ARDE regulates miR-1 levels to rescue CVB3-induced endogenous Cx43 expression [41-43]. Chinese patented drug Shenfu injection (SFI), andrographolide, tanshinone IIA (TSA), Shenqi Fuzheng Injection (SFI) and cinnamaldehyde have been shown to have the function of protecting myocardium and effectively relieving myocarditis [43-47]. Ulinastatin (UTI), CQ10 and trimetazidine, ivabradine are widely used in clinic [24, 48-51]. In summary, all the mechanisms of action are based on the molecular regulatory mechanisms at the gene level to inhibit the development of viral myocarditis. Therefore, through the analysis of biological information data, this study further confirmed that Stat1 and Stat3 affect the development of viral myocarditis in mice by regulating the development of the vascular system.

Disclosure of conflict of interest

None.

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Table S1. Hub gene of modules

color	HubGenes	Module
black	Podn	m7
blue	Phf11d	m2
brown	Smim20	m3
green	Hif1an	m5
magenta	Cidec	m9
pink	Fam107a	m8
red	Gpnmb	m6
turquoise	Vav1	m1
yellow	Gusb	m4