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Original Article

Silent gene Pitx1 reveals the potential pathogenesis of clubfoot

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Abstract: Background: As one of the most common congenital defects of the musculoskeletal system, clubfoot affects about one in every 1,000 live births. Studies have shown that mutations in the PITX1-TBX4-HOXC transcription pathway may contribute to familial clubfoot and vertical talus. Despite extensive research, the etiology and pathogenesis of clubfoot is still unclear. Therefore, the aim of this study is to identify genes regulated by Pitx1 that lead to the onset of congenital valgus, using a modular analysis method. Methods: Data collection, weighted gene correlation network analysis, enrichment analysis, and data perspective analysis were performed. Results: Three co-expression modules are enriched in clubfoot. Among them, Mynn, Snca, and Actn2 genes are differently and significantly expressed, and they also play an active supervisory role in the dysfunction module, and are considered to be the driving genes for clubfoot. Connectivity analysis revealed that Ubc and Actn2 regulate multiple functional pathways. The enrichment results indicate that the modular genes are significantly involved in striated muscle tissue development, striated muscle contraction, muscle system process, muscle contraction. Furthermore, they are also involved in ubiquinone and other terpenoid-quinone biosynthesis, transcriptional misregulation in cancer, lysosome and other signaling pathways. Finally, pivot ncRNA (including let-7d-5p and Terc) and pivot TF (including Myod1 and Myog) were identified as meaningful regulatory dysfunction modules. Conclusion: The results of this study identified a differentially regulated protein interaction network involved in clubfoot, and revealed its core dysfunction module and potential regulatory factors.

Keywords: Clubfoot, differential analysis, protein interaction network, enrichment analysis, regulatory factor, Pitx1

Introduction

Clubfoot is one of the most common musculoskeletal deformities found at birth. It is characterized by talus (ankle) and calcaneal (heel) deformation, which gives the foot a characteristic "spherical" appearance [1]. If not treated, it can lead to long-term dysfunction, deformity and discomfort [2, 3]. In addition to these core symptoms, there are some complications, such as edema, which is the most common complication of clubfoot [4]. Furthermore, the known complications from surgical treatment of clubfoot is ankle or subtalar valgus deformity, which causes the heel fibula and forearm to strike, or flat foot deformity [5]. Clubfoot is related to many high-risk factors, such as smoking in men, primiparas and mothers [1]. In genetic studies, a genome-wide association analysis of chromosome 12q24.31 between NCOR2 and ZNF664 (rs7969148) found the strongest evidence related to clubfoot, that is, it has significant differences in replication with healthy people [6]. In addition, several chromosomal deletion regions, including 2q31-33, are associated with clubfoot and may contribute to the phenotype that creates to idiopathic clubfoot [7]. At present, researchers have made progress in exploring the pathogenesis of congenital clubfoot from medical and biological perspectives. The abnormal expression of HOX is closely linked to the occurrence and development of congenital clubfoot (CCF) in children, which mainly plays a role through oxidative damage, inflammation and apoptosis pathways [8]. Collagen IX type alpha 1 (COL9A1) has been designated as a candidate pathogenic gene for idiopathic congenital clubfoot (ICTEV) [9]. It has been shown that exposure to all-trans retinol in the placenta of pregnant rats can promote

apoptosis, suggesting a possible correlation between placental apoptosis and congenital clubfoot [10]. In addition, the deletion of 5'HOXC gene is associated with lower limb deformities including clubfoot and vertical talus [11]. On the other hand, the abnormal expression of HOXD13 and FHL1 is related to the occurrence of clubfoot deformities [12]. These findings have deepened our understanding of the pathogenesis of congenital clubfoot and are a major consideration for our further research.

Although previous studies on congenital clubfoot have been reported, the overall effect of these results remains elusive. To further explore the role of genes in the pathogenesis of congenital clubfoot, we performed a systematic segmentation analysis to identify disease-related dysfunction modules and core molecules. In conclusion, the protein-protein interaction network we made for differentially expressed genes not only reveals the relationship between modules and differentially expressed genes, but also provides guidance for further research and treatment of congenital clubfoot by biologists.

Materials and methods

Data collection

The NCBI Gene Expression Omnibus database (GEO Datasets) [13] includes a broad classification of high-throughput experimental data, including single-channel and dual-channel microarray-based gene abundance measurements, genomic DNA and protein molecule experimental data. In addition, it includes data from non-array-based high-throughput functional genomics and proteomics techniques. First, we collected a set of gene expression profiles of hindlimb buds from mice with and without the Pitx1 gene knockout. Its number in the GEO datasets is GSE27363 [14]. We then screened the ncRNA-RNA (protein) interaction pairs with a score $\geq$ 0.5 from the RAID v2.0 database [15]. At the same time, all human transcription factor target data was downloaded and used in the TRRUST V2 database [16] to predict TF.

Differentially expressed genes

In order to consider the driver of congenital clubfoot caused by Pitx1 gene, we analyzed the differences between Pitx1 knockout mice and wild-type mice. Differential expression analysis of gene expression profile data in this study was implemented by R language limma package [17-19]. First, background Correct function was used for background correction and standardization. Second, the normal array function quantile normalization method was used to filter out control probes and low expression probes. Then, the default parameters of the lmFit and eBayes functions were used to identify differentially expressed genes in the datasets.

Co-expression analysis

In order to study the co-expression of congenital clubfoot differentially expressed genes, we used weighted gene co-expression network analysis (WGCNA) [20] to analyze the matrix of congenital clubfoot differentially expressed genes to find co-expressed gene modules. First, the weighted value of correlation coefficient, that is, the N power of the gene correlation coefficient, was used to calculate the correlation coefficient (Person Coefficient) between any two genes. The connections between genes in the network obey scale-free network, making the algorithm more biologically meaningful. Then, a hierarchical clustering tree is built using the correlation coefficients between genes. Different branches of the clustering tree represent different gene modules, and different colors represent different modules. According to the regulation of genes, in each functional module, we excavated the key genes that lead to the module to malfunction, and they were considered to be the key genes that cause congenital clubfoot.

Function and pathway enrichment analysis

Exploring the function of genes and its signal pathways is helpful for studying the molecular mechanism of diseases. Enrichment of genes in the dysfunction modules is an effective way to explore the underlying mechanism of congenital clubfoot. Therefore, we used the R language Cluster profiler package [21] to analyze the enrichment of Go function and KEGG pathway of the congenital clubfoot module genes. Cluster Profiler is a software package of Bioconductor, which can perform statistical analysis and visualization on functional clustering of gene sets or gene clusters. In addition, we used

BinGO [22] application of Cytoscape to analyze the integrated modules network.

Transcription factors and ncRNA analysis of regulatory dysfunction modules

Gene transcription and post-transcriptional regulation are usually driven by non-coding genes (ncRNA) and transcription factors (TF). Therefore, we scientifically predicted and tested its role in the congenital clubfoot dysfunction module. Pivot regulators, including ncRNA and TF, are defined as regulators that have important regulatory effects on modules in the pathogenesis of congenital clubfoot. We required that there were more than two regulatory links between each regulator and each module, and *p*-values less than 0.01 calculated in each module based on the hypergeometric test were taken to be significant differences.

Blood samples from patients with congenital clubfoot

Blood samples were obtained from congenital clubfoot patients that were diagnosed in the Zhangjiakou First Hospital. This research was conducted with the approval of the Ethical and Scientific Committees of Zhangjiakou First Hospital and informed consent was obtained from each patient recruited.

Verify the expression level of key genes by qPCR

Real-time PCR was performed to verify the results, and β-actin was used as the internal control. Total RNA was isolated from the blood using TRIzol reagent (Invitrogen, Thermo Fisher Scientific). The RNA samples were reverse transcribed into cDNA using PrimeScript™ RT reagent Kit (Takara Bio, Otsu, Japan). Then, qPCR reaction was carried out using SYBR® Premix Ex Taq II (Takara Bio, Otsu, Japan). Real-time PCR was performed with the following protocol: 30 s at 95°C, followed by 45 cycles of 5 s at 95°C, 30 s at 60°C, and 15 s at 95°C, 30 s at 60°C and 15 s at 95°C. Relative expression of mRNA was determined by the $2^{-\Delta\Delta CT}$ method.

Statisticasl analysis

Data were shown as *mean* ± *SD*, Statistical analysis were carried out by GraphPad Prism 8 (GraphPad Software, La Jolla, CA, United States). A probability value of $P < 0.05$ was considered to be statistically significant.

Results

Identification of abnormally expressed molecules in congenital clubfoot

Many researchers have carried out several experiments on the pathogenesis of congenital clubfoot, and identified some possible pathogenic genes of congenital clubfoot. However, the complex molecular connections and overall effect of these genes remain unclear. In order to study the role of Pitx1 gene in the pathogenesis of congenital clubfoot, we analyzed the differential expression of Pitx1 gene based on microarray data. We then analyzed the differential genes between the knockout Pitx1 gene samples and the wild-type samples, and obtained 1101 differential genes (Table S1) that may be related to congenital clubfoot. We believe that some of these differentially expressed genes caused congenital clubfoot.

Recognize the dysfunction modules related to congenital clubfoot

In order to observe the complex synergistic relationship between these genes from the perspective of expression behavior, we clustered the expression behavior of congenital clubfoot samples into modules. First, the expression profile matrix was constructed based on 1011 congenital clubfoot differentially expressed genes and their interacting genes. Then, we obtained three dysfunction modules for congenital clubfoot based on weighted gene co-expression network analysis (WGCNA) (**Figure 1A**, **1B**). We further identified key genes for each dysfunction module. They are Mynn, Snca and Actn2 (Table S2). Based on the association between the module and phenotypic data, we found that MEblue and MEbrown are related to the knockout of Pitx1 gene, while MEturquoise is related to the non-knockout of Pitx1 gene (**Figure 1C**).

Function and pathway enrichment amalysis of genes of interest

Function and pathway are crucial mediators of the physiological response the disease. Exploring the functions and pathways of genes involved in dysfunctional modules will help build a molecular bridge between modules and diseases in systems biology and deepen the understanding of the underlying molecular mechanisms of diseases. By performing GO

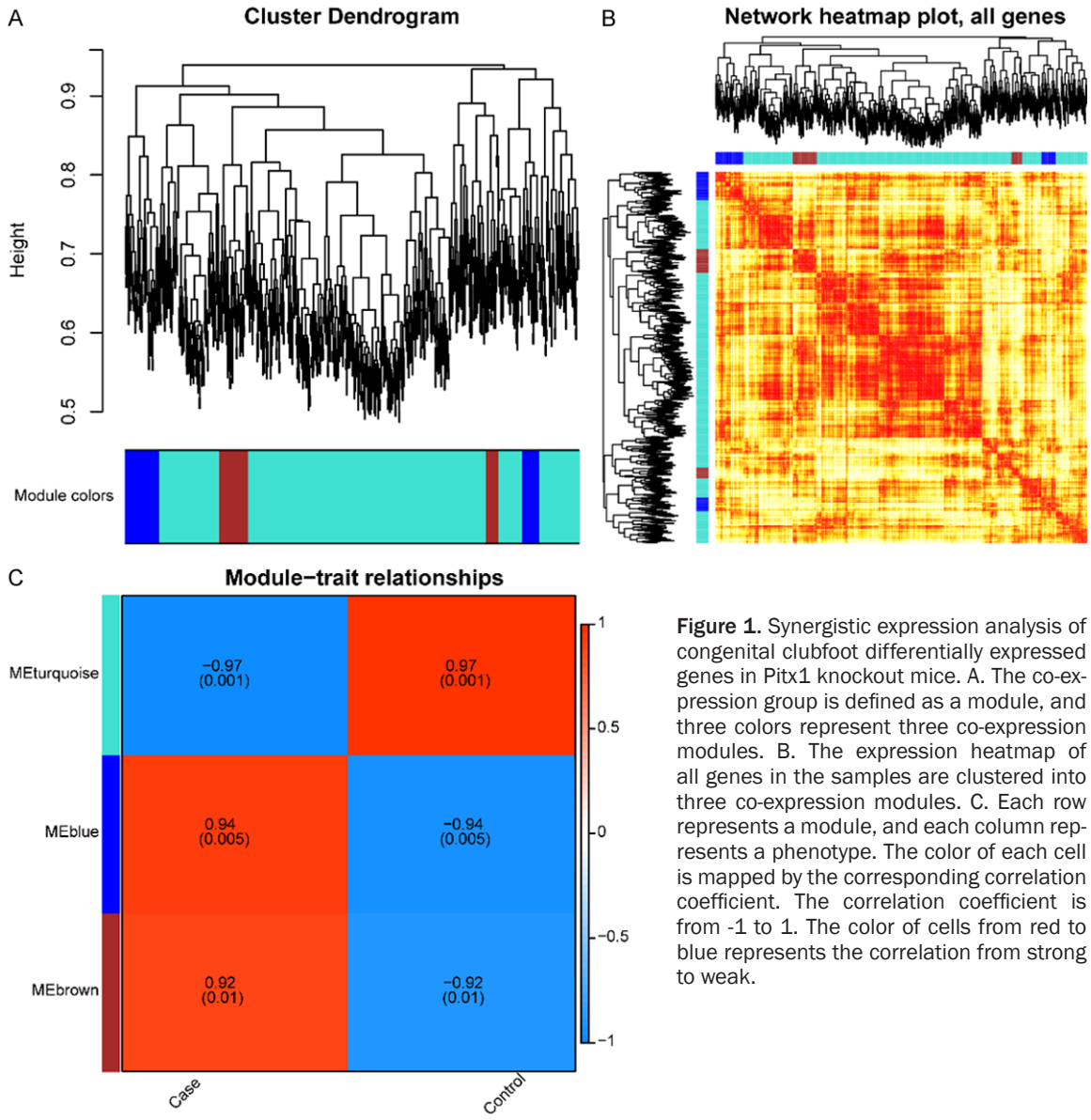

Figure 1. Synergistic expression analysis of congenital clubfoot differentially expressed genes in Pitx1 knockout mice. A. The co-expression group is defined as a module, and three colors represent three co-expression modules. B. The expression heatmap of all genes in the samples are clustered into three co-expression modules. C. Each row represents a module, and each column represents a phenotype. The color of each cell is mapped by the corresponding correlation coefficient. The correlation coefficient is from -1 to 1. The color of cells from red to blue represents the correlation from strong to weak.

function and KEGG pathway enrichment analysis on the three modules, we found that differential genes were enriched in 473 biological processes (BP), 113 cell components (CC), 134 molecular functions (MF) and 11 KEGG pathways (**Figure 2**; Table S3). The main focused items of GO function analysis are: striated muscle tissue development, striated muscle contraction, muscle system process, muscle contraction and other biological processes. The enrichment results of KEGG pathway reflect that these differentially expressed genes are mainly involved in ubiquinone and other terpenoid-quinone biosynthesis, transcriptional misregulation in cancer and lysosomes. As for the most regulated functions and pathways of genes in the dysfunction module, we considered that genes play a decisive role in the dysfunction module. According to the connectivity, we leveraged Ubc and Actn2, the univarsal and pathway-regulating driving genes. Considering the overall situation, we integrated these three modules and use BinGO for path analysis.

TF and ncRNA driving congenital clubfoot

Although many researchers have paid attention to the regulation of single or multiple TFs and ncRNAs on clubfoot, few studies have focused on their overall effects on dysfunction mecha-

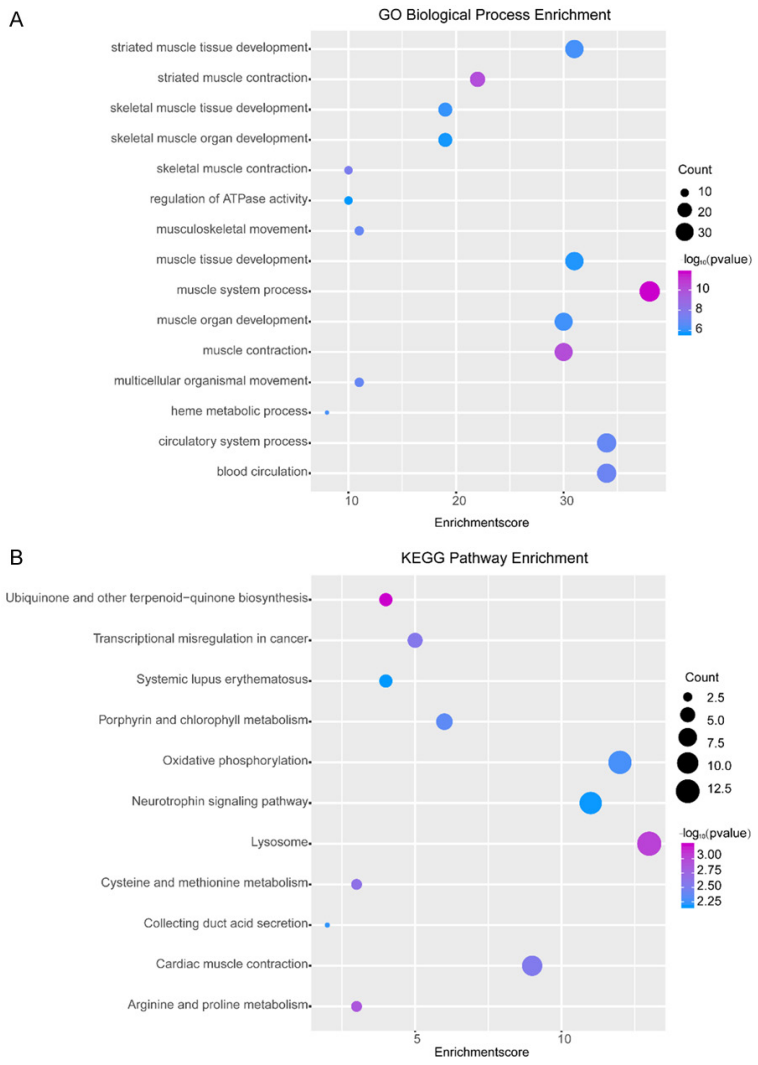

Figure 2. Function and pathway enrichment analysis of modular genes (excerpts). A. Terms for GO function enrichment analysis of module genes. B. KEGG pathway enrichment analysis of modular genes. The color of terms from blue to purple indicates its richness from low to high. The circle of entries from small to large indicates that the proportion of module genes involved in GO function or KEGG pathway from small to large.

nisms and their bridge role in the development of clubfoot. Therefore, based on the targeted regulatory relationship between TF and ncRNA, in this study, we performed a pivotal analysis of co-expressed module genes to explore key regulatory factors affecting the molecular pathogenesis of clubfoot. The prediction results showed that 121 ncRNAs are involved in 121 ncRNA-module regulatory pairs, and 7 transcription factors are involved in 7 TF-module target pairs (Tables S4, S5). By introducing the above results into cytoscape, we observed the regulation of regulators in the dysfunction modules (**Figure 3A**, **3B**). In addition, we also analyzed the number of pivot regulatory modules and found ncRNAs (let-7d-5p and Terc, etc.) and TFs (Myod1 and Myog, etc.) that regulate multiple dysfunctional modules. These transcription factors and ncRNAs may regulate the process of congenital clubfoot by mediating dysfunction modules. In summary, we identified these potential regulators as dysfunctional molecules of congenital clubfoot.

qPCR verification of the differentially expressed genes

As shown in **Figure 4**, the expression level of key genes was verified by qPCR. Pitx1 and Actn2 expression levels were down-regulated in Pitx1 knockout mice and patients with congenital clubfoot. However, Mynn showed high expression in Pitx1 knockout mice and patients with congenital clubfoot. We can see that the datasets analysis results were consistent with qPCR results.

Discussion

Congenital clubfoot is a common congenital malformation characterized by adduction of the hindfoot, midfoot and forefoot, as well as varus and cavity deformities of the subtalar joint complex [23]. Some studies have found that the prevalence of congenital clubfoot is higher in Chinese people, than in people from other contries [24]. Currently, the treatment of congenital clubfoot is aimed at correcting functional foot pain [25]. Despite research having studied congenital clubfoot in many ways, its pathogenesis remains unclear. In this study, we collected gene expression profile data from hindlimb shoots of Pitx1 knockout and wild-type mice from the NCBI GEO DataSets. To understand the molecular mechanism by which Pitx1 regulates congenital clubfoot, we analyzed the differentially expressed genes of con-

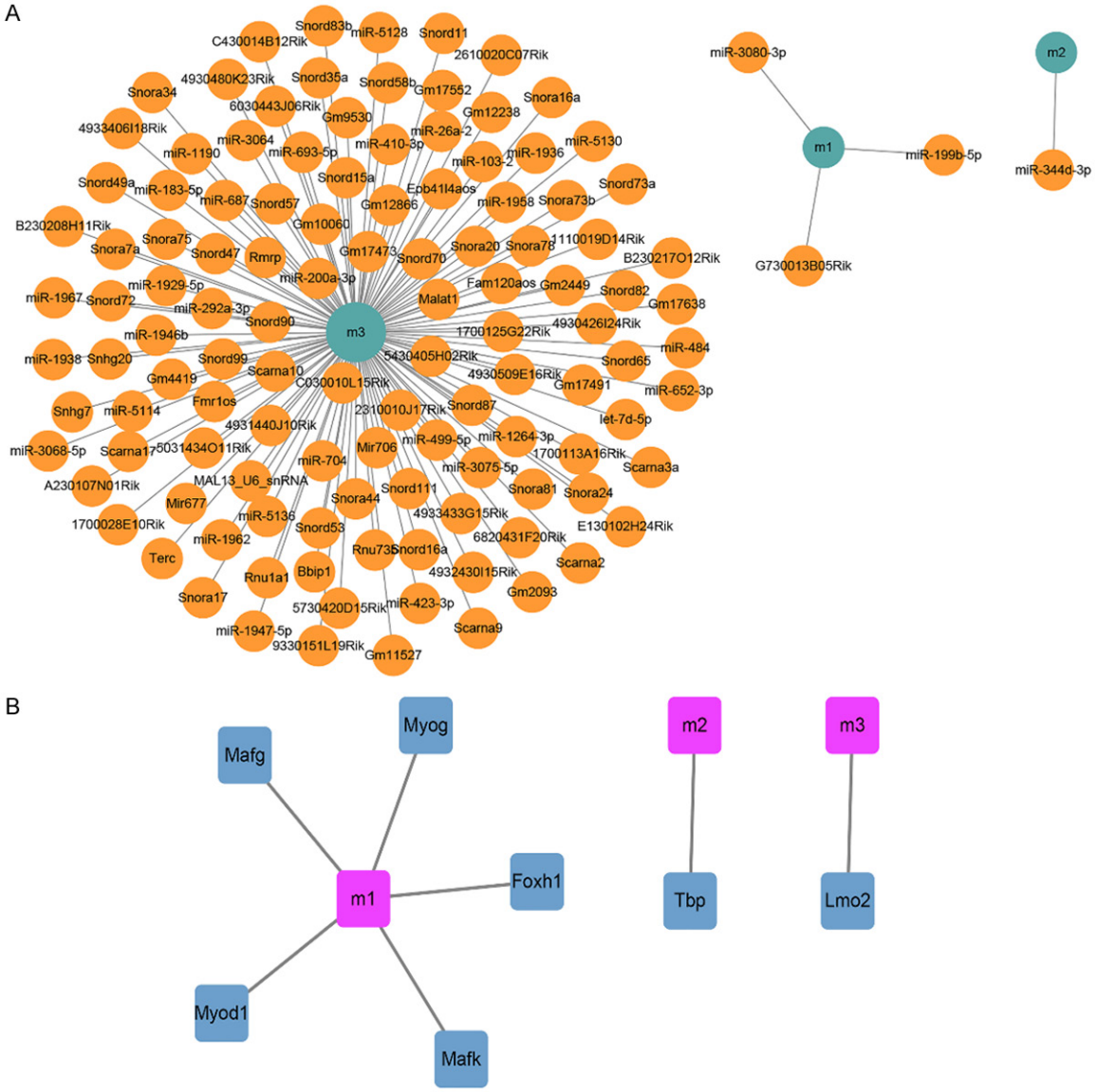

Figure 3. Regulators to regulate the dysfunction module. A. Blue circles represent modules and orange circles represent ncRNA. B. The purple square represents the module and the blue square represents the TF.

genital clubfoot, and the functional modules of congenital clubfoot gene driven by transcription factors and ncRNA regulatory factors. At the module level, module genes are significantly involved in the active regulation of innate immune responses, muscle organ development, ATPase activity, muscle contraction and regulation of muscle system processes. In addition, they also participate in signaling pathways such as in lysosomes, myocardial contraction, porphyrin and chlorophyll metabolism, oxidative phosphorylation and neurotrophic factors. Currently, there is compelling evidence that lysosomes have a wide range of functions and are involved in many basic processes. For example, secretion, plasma membrane repair, signal transduction and energy metabolism. The important role of lysosomes in autophagy puts these organelles at the intersection of several cellular processes, which has important significance in physiological and pathological activities [26]. Recent evidence suggests that activation of myocardial contraction may depend in part on a voltage-sensitive release mechanism similar to skeletal muscle (VSRM) [27]. Oxidative phosphorylation provides most of the ATP for life activities in advanced animals and is responsible for setting and maintaining

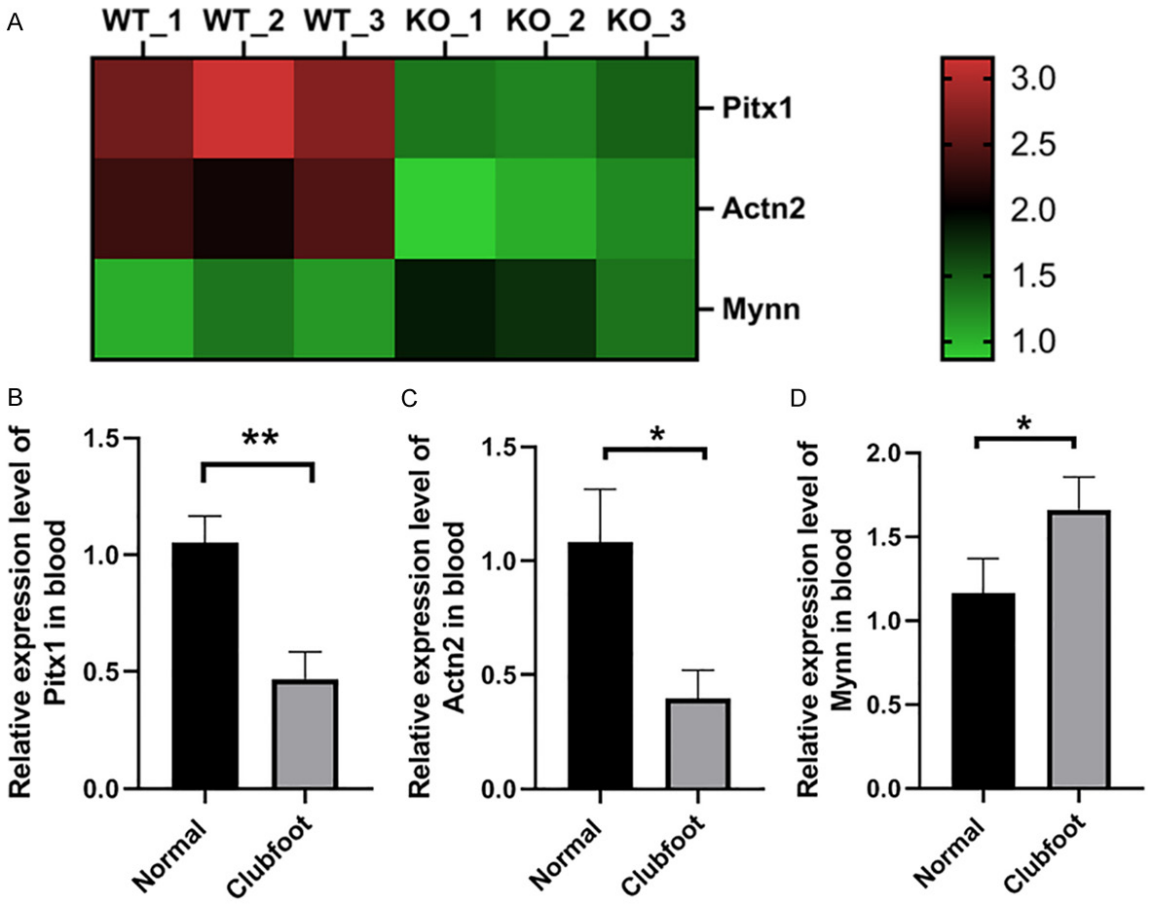

Figure 4. The relative expression level of Pitx1, Mynn and Actn2 in Pitx1 knockout mice and patients with congenital clubfoot. A. Datasets analysis results. B-D. qPCR results. *$P < 0.05$, **$P < 0.01$.

metabolic homeostasis [28]. In addition, the fundamental mechanisms of target-derived neurotrophic proteins include the cellular biological processes of endocytosis and the reverse transport of Trk receptors from the growth cone to the cell body [29]. In existing studies, the effects of these pathways on congenital clubfoot have not been found, but they can be used as the direction for further research.

On the other hand, at the molecular level, we identified three core genes by co-expression analysis. They are Mynn, Snca and Actn2. These core genes are not only differentially expressed in the congenital clubfoot, but also play important regulatory roles in the dysfunction module. In addition, based on connectivity, we take full advantage of the universal and regulatable driver genes Ubc and Actn2. It should be noted that MYNN rs10936599 has a strong cumulative association with bladder cancer risk, and TERC can inhibit bladder cancer cell growth [30]. The autophagy-lysosome pathway (ALP) regulates the homeostasis of SNCA/α-synuclein and is impaired in synaptic nucleoprotein diseases, including Parkinson's disease and Lewy body dementia (DLB) [31]. ACTN2 is a human sarcomere homologous gene encoding α-actin-2, which is mainly distributed in skeletal muscle and myocardial [32]. ACTN2 mutations may be the source of familial cardiac phenotypic heterogeneity [33]. The absence of Ubc-13 destroys the transport of MIG-14/Wntless from the body to the Golgi apparatus, resulting in the incorrect separation of MIG-14 from lysosomes and the damage of Wnt-dependent processes [34]. In the study of the above genes, they have not found their effect on congenital clubfoot. However, they may still play a huge potential role in the pathogenesis and regulation of congenital clubfoot, and they can be used as candidate molecules for further molecular experimental verification.

In addition, we predicted 121 ncRNAs that participated in the pathogenesis of congenital clubfoot via mediating modules. Based on statistical analysis, we found ncRNAs that regulate only one dysfunctional module, which are let-7d-5p and Terc. The differential expression of microRNAs in plasma between AE-IPF and S-IPF may be a potential AE-IPF biomarker for IPF [35]. Meanwhile, the mammalian TERC RNA component is introduced into the mitochondria, processed into shorter TERC-53, and then transported back to the cytoplasm. Cytosolic TERC-53 levels will affect mitochondrial function. Mitochondrial dysfunction plays a major role in many diseases [36]. The effects of ncRNA on congenital clubfoot were not found in the above studies. However, other ncRNAs that significantly regulate the dysfunction modules of congenital clubfoot may also participate in its elementary process. They can be used as candidates for further molecular experimental verification.

Finally, we identified seven differentially expressed transcription factors involved in the congenital clubfoot dysfunction module. According to statistical analysis, we found that Myod1, Myog, Foxh1, Mafg, Mafk, Tbp and Lmo2 regulate one dysfunction module each. The expression levels of Fhl1, Hgf, MyoD1, Myogenin and Myh4 were different in hind limbs of mice at different gestational ages. With the exception of Fhl1, all genes that control skeletal muscle development peak in the lower limbs at E17 [37]. The transcription factor EGR1 promotes the differentiation of bovine skeletal muscle satellite cells by regulating the expression of MyoG [38]. Meanwhile, Foxh1 is a Smad DNA-binding partner. During early development, Foxh1 mediates TGF β-dependent gene expression [39]. In addition, MAFG is a bZIP transcription regulator and belongs to the small MAF (sMAFs) protein family. MiR-128 participates in the stress response of skeletal muscle cells by targeting MAFG [40]. Overexpression of the transcription factor MafK inhibits T cell proliferation and *in vivo* function [41]. Some studies have found that TBP inactivation is one of the causes of spinocerebellar ataxia type 17 (SCA17) [42]. On the other hand, Lmo2 transcription factors participate in hematopoiesis and vascular remodeling and promote endothelial cell migration by regulating Sphk1 (sphingosine kinase) [43]. Of these seven differentially expressed transcription factors, only Myod1 is associated with the pathogenesis of congenital clubfoot. Other transcription factors are candidates for further study.

Disclosure of conflict of interest

None.

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Table S2. Key genes of dysfunction module

colour	HubGenes	Module
blue	Mynn	m2
brown	Snca	m3
turquoise	Actn2	m1

Table S4. ncRNA pivot that regulates module genes

module	pivot	module Links	*P* value
m1	G730013B05Rik	30	0.00251
m1	miR-3080-3p	2	0.000736
m1	miR-199b-5p	3	0.005787
m2	miR-344d-3p	4	0.001538
m3	miR-200a-3p	5	0.001531
m3	miR-410-3p	5	0.005477
m3	miR-292a-3p	2	0.004481
m3	miR-499-5p	4	0.002623
m3	miR-183-5p	4	0.002848
m3	let-7d-5p	5	0.006776
m3	miR-1264-3p	2	0.009881
m3	miR-1936	2	0.00144
m3	MAL13_U6_snRNA	2	0.006408
m3	Terc	2	0.00097
m3	Mir677	2	0.000833
m3	miR-3064	2	0.001999
m3	miR-1962	2	0.007121
m3	miR-1967	2	0.00097
m3	miR-484	2	0.001616
m3	miR-5114	2	0.007867
m3	miR-1929-5p	2	0.002205
m3	miR-1947-5p	2	0.000706
m3	miR-26a-2	2	0.00059
m3	miR-3075-5p	2	0.00144
m3	miR-5128	2	0.001116
m3	miR-423-3p	2	0.000388
m3	miR-5130	2	0.001116
m3	miR-1946b	2	0.001999
m3	miR-1190	2	0.000706
m3	miR-1938	2	0.001803
m3	miR-103-2	2	0.000706
m3	miR-3068-5p	2	0.000302
m3	miR-704	2	0.00097
m3	miR-687	2	0.00097
m3	miR-693-5p	2	0.00242
m3	miR-652-3p	2	0.000163
m3	miR-1958	2	0.00391
m3	miR-5136	2	0.00059
m3	Bbip1	2	0.000706
m3	Snord70	2	0.000388

m3	C030010L15Rik	2	0.000483
m3	Mir706	2	0.00059
m3	4930480K23Rik	2	0.00097
m3	Malat1	2	0.005088
m3	2610020C07Rik	2	0.000833
m3	5031434011Rik	2	0.000388
m3	Scarna3a	2	0.00059
m3	1110019D14Rik	2	0.000706
m3	Snord90	2	0.000302
m3	Snora75	2	0.001116
m3	Snora44	2	0.001616
m3	1700113A16Rik	2	0.001273
m3	Gm12238	2	0.001116
m3	Gm17638	2	0.00059
m3	Snord65	2	0.000706
m3	Snora81	2	0.002205
m3	Gm11527	2	0.000483
m3	Snora17	2	0.005088
m3	Gm17491	2	0.001116
m3	6820431F20Rik	2	0.001273
m3	Snord15a	2	0.000388
m3	Fam120aos	2	0.000483
m3	4933406I18Rik	2	0.000483
m3	Snhg7	2	0.000706
m3	5730420D15Rik	2	0.00097
m3	5430405H02Rik	2	0.001803
m3	B230217O12Rik	2	0.001116
m3	Snora16a	2	0.000706
m3	Snord73a	2	0.000388
m3	Snora34	2	0.000302
m3	Snord11	2	0.000388
m3	Snora20	2	0.00059
m3	Gm2449	2	0.000483
m3	Gm12866	2	0.000302
m3	4930509E16Rik	2	0.000483
m3	Snord16a	2	0.000483
m3	Snhg20	2	0.000833
m3	Snora78	2	0.001116
m3	Snora24	2	0.002205
m3	Gm17473	2	0.000302
m3	Snord83b	2	0.001616
m3	Snord99	2	0.000833
m3	6030443J06Rik	2	0.00059
m3	Scarna2	2	0.00097
m3	Scarna17	2	0.001616
m3	Snord111	2	0.00097
m3	Scarna9	2	0.00097
m3	C430014B12Rik	2	0.00059
m3	Gm9530	2	0.000833

m3	Gm10060	2	0.000388
m3	Fmr1os	2	6.53E-05
m3	A230107N01Rik	2	0.000227
m3	4933433G15Rik	2	0.00059
m3	2310010J17Rik	2	0.001803
m3	Rnu1a1	2	0.000388
m3	Snord35a	2	0.001273
m3	B230208H11Rik	2	0.000706
m3	E130102H24Rik	2	0.001273
m3	Snord53	2	0.00097
m3	Rmrp	2	0.00242
m3	9330151L19Rik	2	0.000302
m3	1700125G22Rik	2	0.000833
m3	Gm4419	2	0.000388
m3	Snord47	2	0.001273
m3	Snord82	2	0.000388
m3	Snora7a	2	0.000302
m3	Snora73b	2	0.000706
m3	Epb41l4aos	2	0.000388
m3	Rnu73b	2	0.000706
m3	Scarna10	2	0.00144
m3	4931440J10Rik	2	0.000483
m3	4930426I24Rik	2	0.000483
m3	Snord57	2	0.00059
m3	1700028E10Rik	2	0.00097
m3	Snord49a	2	0.001116
m3	Gm2093	2	0.00144
m3	4932430I15Rik	2	0.000302
m3	Snord87	2	0.000388
m3	Snord72	2	0.000483
m3	Gm17552	2	0.000833
m3	Snord58b	2	0.000227

Table S5. TF pivot that regulates module genes

module	pivot	module Links	Pvalue
m1	Myod1	6	0.00142
m1	Myog	4	0.000314
m1	Foxh1	2	0.006035
m1	Mafg	2	0.000634
m1	Mafk	2	0.001871
m2	Tbp	2	0.000322
m3	Lmo2	2	9.26E-05