Molecular Neurobiology (2018) 55:8251–8262
https://doi.org/10.1007/s12035-018-0963-6

# A Novel Five-Node Feed-Forward Loop Unravels miRNA-Gene-TF Regulatory Relationships in Ischemic Stroke

Sreekala S. Nampoothiri[1] · S. M. Fayaz[1] · G. K. Rajanikant[1]

Received: 14 July 2017 / Accepted: 14 February 2018 / Published online: 9 March 2018


## Abstract

The complex and interlinked cascade of events regulated by microRNAs (miRNAs), transcription factors (TF), and target genes highlight the multifactorial nature of ischemic stroke pathology. The complexity of ischemic stroke requires a wider assessment than the existing experimental research that deals with only a few regulatory components. Here, we assessed a massive set of genes, miRNAs, and transcription factors to build a miRNA-gene-transcription factor regulatory network to elucidate the underlying post-transcriptional mechanisms in ischemic stroke. Feed-forward loops (three-node, four-node, and novel five-node) were converged to establish regulatory relationships between miRNAs, TFs, and genes. The synergistic function of miRNAs in ischemic stroke was predicted and incorporated into a novel five-node feed-forward loop. Significant miRNA-TF pairs were identified using cumulative hypergeometric distribution. Two subnetworks were derived from the extensive miRNA-TF regulatory network and analyzed to predict the molecular mechanism relating the regulatory components. NFKB and STAT were identified to be the chief regulators of innate inflammatory and neuronal survival mechanisms, respectively. Exclusive novel interactions between miR-9 and miR-124 with TLX, BCL2, and HDAC4 were identified to explain the post-stroke induced neurogenesis mechanism. Therefore, this network-based approach to delineate miRNA, TF, and gene interactions might promote the development of effective therapeutics against ischemic stroke.

**Keywords** Ischemic stroke · Network biology · miRNA · Genes · Transcription factor · Feed-forward loop · Neurogenesis

## Introduction

microRNAs (miRNAs) are differentially expressed following neuronal injury and critically regulate ischemic stroke pathophysiological processes [1–3]. The introduction of miRNA mimic or antagomir promotes neuroprotection depending on the functional role of miRNAs in ischemia with specific target genes and transcription factors [4]. With the increasing number of miRNAs and the associated regulatory components being explored, it has become indispensable to uncover novel strategies defining the underlying mechanisms associated with cerebral ischemia.

Systems biology provides a holistic platform to comprehend and envisage pathological processes [5, 6]. However, the systems biology approach to decipher ischemic stroke regulatory components is still in its infancy. Biological networks are formed from network motifs and demonstrated using feed-forward loops (FFLs), the primary type being a three-node FFL, comprising a miRNA, a TF, and a common gene target [7, 8]. A three-node FFL could be extended to generate a four-node FFL consisting of coexpressed genes as joint targets between miRNA and TF [8].

This study brings together a massive set of ischemic stroke genes, important miRNAs, and transcription factors to build a novel five-node feed-forward loop by introducing an additional miRNA-miRNA interaction to the existing four-node FFL. The synergistic activity of miRNA is considered to be of utmost importance owing to the differential expression of multiple miRNAs in ischemic stroke [4]. The five-node FFL renders all possible regulatory relationships between miRNAs, genes, and TFs and plays a promising role in elucidating the complexity of any disease, particularly ischemic stroke.

All FFLs are classified into three main types, miRNA FFL, TF FFL, and composite FFL, according to the regulatory

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s12035-018-0963-6) contains supplementary material, which is available to authorized users.

✉ G. K. Rajanikant
rajanikant@nitc.ac.in

[1] School of Biotechnology, DBT - Centre for Bioinformatics, National Institute of Technology Calicut, Calicut 673601, India

module between miRNA and TF [8]. The FFLs are schematically illustrated in Fig. 1. In the present study, composite FFLs were integrated together to form a major miRNA-TF regulatory network for ischemic stroke. Two subnetworks were generated and analyzed to determine the crucial miRNAs, genes, and transcription factors in cerebral ischemia. Furthermore, the proposed five-node FFL was used to determine core miRNAs and interactions associated with post-stroke induced neurogenesis (Fig. 2). There has been a limited insight on ischemic stroke at the systems level, and hence, the integration of disease regulatory components in a network might help in delineating intricate molecular mechanisms and developing therapeutic interventions against ischemic stroke.

## Materials and Methods

### Acquisition of Ischemic Stroke-Related Genes, miRNAs, and TFs

Ischemic stroke-related genes were retrieved from GeneCards [9], an integrated database that mines a broad range of gene-related information. The keyword search of "brain ischemia" or "cerebral ischemia" enlisted genes ($n = 831$) that were sorted according to the gene description. The genes were subjected to an enrichment analysis using Gene Ontology Consortium [10, 11]. microRNAs involved in the ischemic stroke were obtained through a comprehensive literature search as well as through the databases such as miR2Disease [12], PhenomiR [13], and Human microRNA Disease Database (HMDD) [14]. The selected miRNAs were either directly related to cerebral ischemia or involved in the ischemic stroke pathology through other etiological disorders. The transcription factors (TF) were retrieved from the ChIPBase [15] and transcriptional regulatory element database (TRED) [16]. In addition, we also selected the transcription factors present in the collected ischemic stroke gene list.

### Generation of Regulatory Interaction Pairs Between miRNAs, Genes, and TFs

miRNA-gene pairs were extracted from miRTarbase [17], a database of experimentally validated miRNA-target interaction. miRNA-gene pairs were filtered such that they showed strong evidence of interaction in humans and were in compliance with the ischemic stroke gene list. miRNA and transcription factor (miRNA-TF) pairs were also obtained using miRTarbase [17]. The regulatory relationship between TF and miRNA was retrieved from chIPBase [15], an online repository, to obtain information on the transcriptional regulation of miRNAs. Furthermore, TF-gene pairs were acquired from TRED [16]. The gene-gene coexpression data was retrieved using a GeneMANIA [18] plugin for Cytoscape v3.5.0. The ischemic stroke gene list was given as an input for which a gene-gene coexpression network was obtained. miRNA-miRNA interaction was established based on the common gene targets between two miRNAs [19], and the pairs were enriched using the Kyoto Encyclopedia of Genes and Genomes (KEGG) [20] database as well as literature search.

### Significant miRNA-TF Pairs in an Ischemic Stroke Regulatory Network

The significant miRNA and TF pairs that coregulate the common target genes were identified by performing a cumulative hypergeometric test [8]. miRNA-gene-TF units were produced by combining the miRNA-gene and TF-gene pairs. Considering the common ischemic stroke-related genes between a pair of miRNA and TF, the $p$ value was computed using the following formula:

$$p = \sum_{i=|N_{(\mathrm{miR})}|\cap|N_{(\mathrm{tf})}|}^{\min\left(|N_{(\mathrm{miR})}|,|N_{(\mathrm{tf})}|\right)} \left\{ \binom{|N_{(\mathrm{miR})}|}{i} \binom{\mathrm{total}-|N_{(\mathrm{miR})}|}{|N_{(\mathrm{tf})}|-i} \right\} \Bigg/ \binom{\mathrm{total}}{|N_{(\mathrm{tf})}|}$$

where $N_{(\mathrm{miR})}$ denotes the number of target genes for a given ischemic stroke miRNA, $N_{(\mathrm{tf})}$ represents the number of ischemic stroke target genes for the corresponding TF, total is the number of common genes between all the ischemic stroke-related genes regulated by TFs and repressed by miRNAs, and $i$ denotes the number of common genes between a given miRNA and a TF. Further, the false discovery rate (FDR) was used to perform multiple test corrections and the TF-miRNA pairs with the corrected $p$ value $< 0.05$ were considered to be significant.

### Construction of Three-, Four-, and Five-Node FFLs

For a three-node FFL, the significant TF-miRNA pairs were filtered by identifying the type of interaction between them (miRNA-TF, TF-miRNA, or both) to construct miRNA, TF, and composite FFLs, respectively. Similarly, in a four-node FFL, significant TF and miRNA pairs formed miRNA, TF, and composite FFLs. The gene-gene coexpression data retrieved from GeneMANIA was incorporated to complete the four-node FFL interaction.

A novel five-node FFL was introduced in this study, an extension to the four-node FFL. The existing miRNA in a four-node FFL was extended to pair with another miRNA such that it satisfied the miRNA-miRNA interaction pair. It was ensured if the included miRNA forms a significant pair with TF.

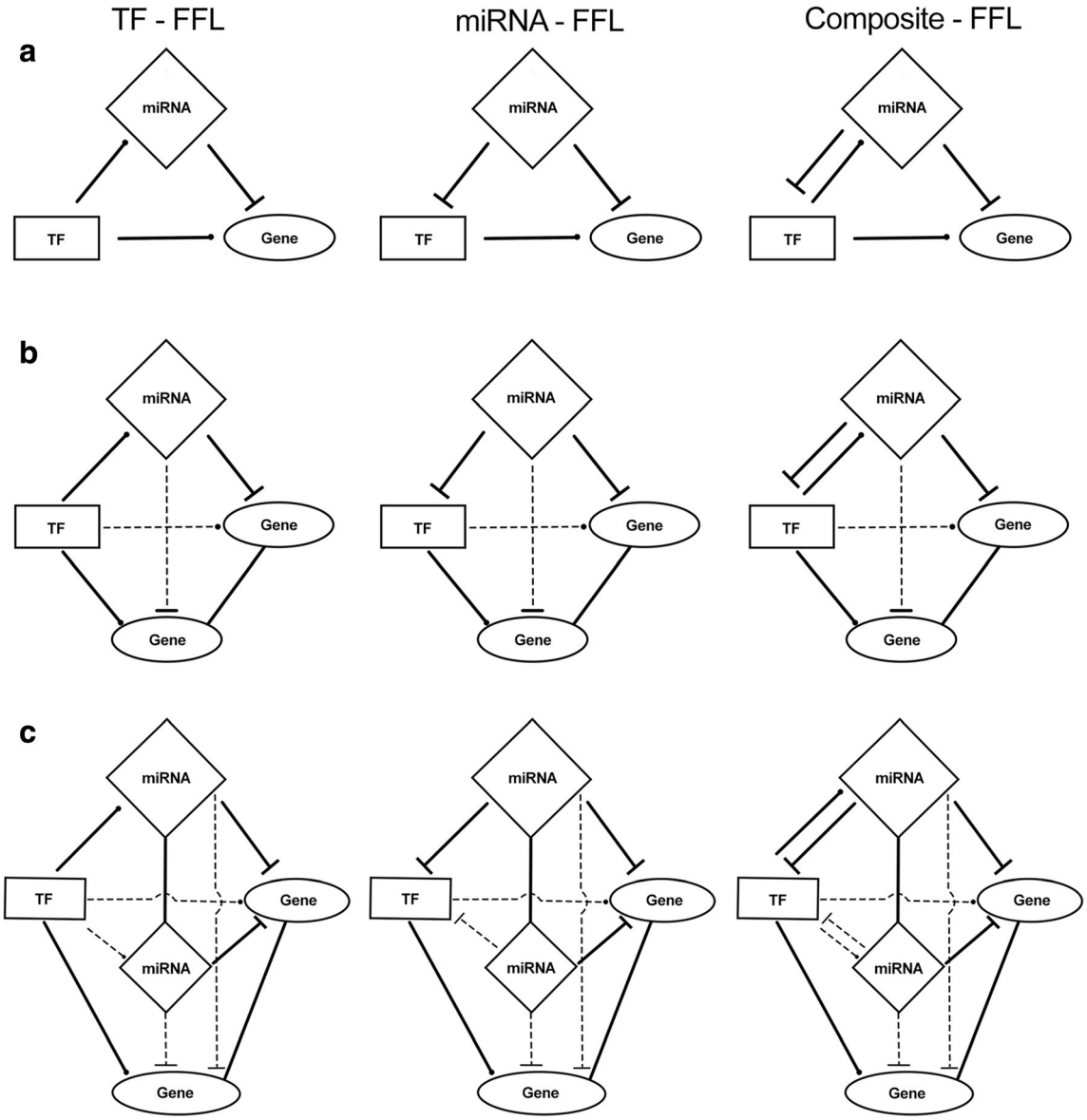



**Fig. 1** Schematic representation of the feed-forward loops. **a** Three-node TF FFL (TF-miRNA, TF-gene, miRNA-gene), three-node miRNA FFL (miRNA-TF, miRNA-gene, and TF-gene), and three-node composite FFL (miRNA-TF, miRNA-gene, TF-miRNA, and TF-gene). **b** Four-node TF FFL (TF-miRNA, TF-gene, miRNA-gene, and gene-gene), four-node miRNA FFL (miRNA-TF, miRNA-gene, TF-gene, and gene-gene), and four-node composite FFL (miRNA-TF, miRNA-gene, TF-miRNA, TF-gene, and gene-gene). **c** Five-node TF FFL (TF-miRNA, TF-gene, miRNA-gene, gene-gene, and miRNA-miRNA), five-node miRNA FFL (miRNA-TF, miRNA-gene and TF-gene, gene-gene, and miRNA-miRNA), and five-node composite FFL (miRNA-TF, miRNA-gene, TF-miRNA, TF-gene, gene-gene, and miRNA-miRNA)

## Development of miRNA-Gene-TF Regulatory Network and Generation of Subnetworks

The composite FFLs (three-, four-, and five-node) were converged together to form a miRNA-gene-TF regulatory network using Cytoscape v3.5.0. The resultant regulatory network was analyzed using the network analyzer tool of Cytoscape v3.5.0. The network was treated as undirected, and important parameters such as average short path length, average clustering coefficient, betweenness centrality, closeness centrality, average neighborhood connectivity, degree distribution of nodes, the frequency of number of shared neighbors, topological coefficients, and edge betweenness were calculated. Subnetworks were generated based on the distribution of nodes and links to reduce the complexity of the network by identifying network hubs. The target genes in the identified subnetworks were subjected to an enrichment analysis using Enrichr [21] to establish the integrity of the subnetwork. It was then closely analyzed to study and predict the ischemic stroke processes associated with the specific transcription factor, connected gene targets, and miRNAs.

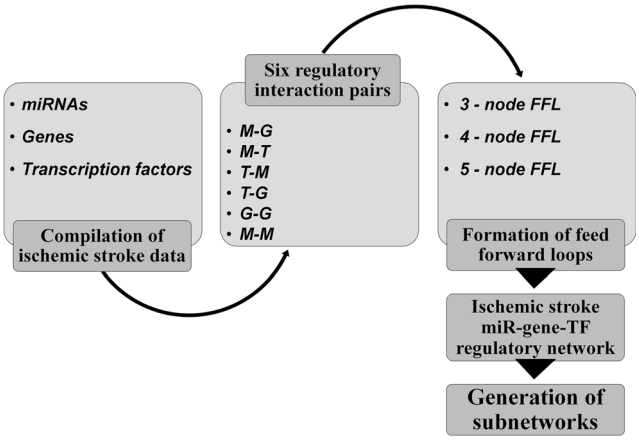



**Fig. 2** Workflow diagram. Thirty-one functional miRNAs, 831 gene targets, and 69 transcription factors were retrieved to form six regulatory interaction pairs. miRNA-gene (M-G) and miRNA-TF (M-T) pairs were acquired from the miRTarBase, TF-miRNA (T-M) from the chIPBase, TF-gene (T-G) from TRED, and gene-gene (G-G) from GeneMANIA plugin for Cytoscape v3.5.0. miRNA-miRNA (M-M) pairs were formed based on the common gene targets between the two miRNAs and their function in ischemic stroke processes. Depending on the regulatory module, the pairs were grouped to form the miRNA, TF, and composite feed-forward loops (FFLs). The composite FFLs were converged to form the major ischemic stroke regulatory network from which subnetworks were extracted depending on the distribution of nodes and links. The subnetworks were analyzed with the interconnections between miRNA, TFs, and gene targets

### Core miRNAs and Regulatory Components in Post-Stroke Induced Neurogenesis Using a Five-Node FFL

To learn the process-specific applicability of our five-node FFL and to predict the core regulatory components functional in post-stroke induced neurogenesis, we selected 14 neurogenesis-related miRNAs from the compiled set of 31 ischemic stroke miRNAs. A composite five-node FFL was formed according to the procedure described above, and a subnetwork was extracted by identifying the network hubs that formed a five-node FFL. Edge betweenness was evaluated and analyzed to determine the significant miRNA-miRNA interactions.

## Results

The development of a comprehensive regulatory network for ischemic stroke required the formation of feed-forward loops (FFLs) with the following components: ischemic stroke-related genes, miRNAs, and TFs. A comprehensive literature survey and GeneCards database [9] provided a list of unique 828 ischemic stroke genes that was subjected to an enrichment analysis using Gene Ontology Consortium [10, 11]. The biological processes in the enrichment result were found to be closely associated with the ischemic stroke pathology. Thirty-one functionally significant miRNAs that were up-/downregulated following ischemic stroke were compiled for the study with the help of an extensive literature search and miRNA-disease databases [12–14].

Six regulatory relationships (miRNA-gene, miRNA-TF, TF-miRNA, TF-gene, gene-gene, and miRNA-miRNA) were formed to generate three-, four-, and five-node FFLs. The five-node feed-forward loop with miRNA-miRNA interaction is a novel regulatory interaction loop introduced in this study.

### Interaction Pairs Between Ischemic Stroke miRNA, Genes, and TF

We retrieved 422 miRNA-gene pairs using miRTarBase, and out of 828 ischemic stroke genes, 232 were found to be validated targets for 31 miRNAs. BCL2, VEGFA, and IGF1R were the top targeted genes. Among 31 miRNAs, miR-21 and miR-17-5p target the highest number of genes. We also obtained 69 relevant miRNA-TF pairs using miRTarBase. miR-21 and miR-34a were found to target the greatest number of TFs, and E2F1 was targeted by the largest number of miRNAs.

TF-miRNA pairs were acquired from chIPBase and 132 pairs of TF-miRNA were listed. The transcription factor NFKB was found to regulate the highest number of miRNAs. miR-15a-5p and miR-21 were the top regulated miRNAs in the list. Four hundred two TF-gene pairs were retrieved using TRED, where the transcription factors NFKB and SP1 were identified to target the highest number of ischemic stroke genes and BCL2 was the most targeted gene.

Gene-gene coexpression data were acquired from GeneMANIA plugin for Cytoscape v3.5.0. The coexpressed genes were arranged based on the score of relevance, and we obtained gene-gene pairs for ischemic stroke. The miRNA-miRNA pair was selected such that there were, at least, two common target genes between the two miRNAs. A thorough literature search was carried out to identify the ischemic stroke-related pathways and processes between the common genes. Further, we used KEGG database to confirm the relationship between the common genes and finally acquired 117 significant miRNA-miRNA pairs.

### Generation of Three-, Four-, and Five-Node FFLs

A three-node FFL consists of miRNA-TF, TF-miRNA, TF-gene, and miRNA-gene interaction pairs. We obtained 80 unique three-node FFLs with 17 miRNA FFLs, 73 TF FFLs, and 10 composite FFLs. The three-node miRNA and TF FFL is shown in the Supplemental Figs. 1 and 2. The composite three-node FFL comprised miR-15a-5p, miR-146a, miR-17-5p, miR-21, and miR-320a as the principal miRNAs with two transcription factors, NFKB and STAT (Fig. 3a). CCND1,

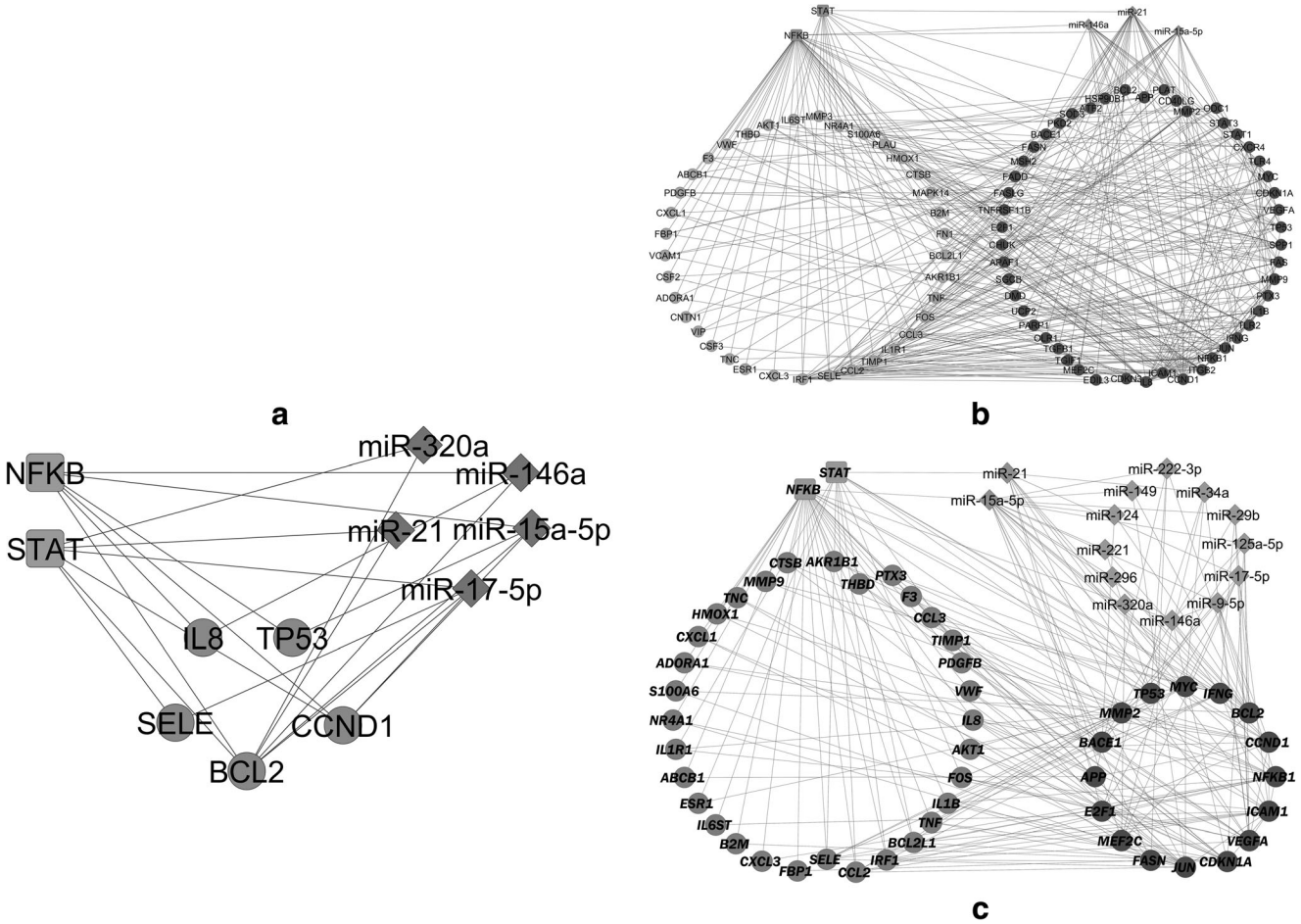



**Fig. 3** Composite FFLs derived for ischemic stroke. Squares represent transcription factors, diamonds represent miRNAs, and circles represent common target genes. **a** Three-node composite FFL. **b** Four-node composite FFL. **c** Five-node composite FFL

TP53, BCL2, SELE, and IL8 were identified to be the target genes in a three-node composite FFL.

Four-node FFL includes five interaction pairs with an additional gene-gene coexpression as compared to three-node FFLs. A total of 2871 four-node FFLs were identified, out of which there were 614 miRNA FFLs, 2551 TF FFLs, and 294 composite FFLs. Supplemental Figs. 3 and 4 represent four-node miRNA and TF FFLs, respectively. It was noted that the composite FFL consisted of only three miRNAs (miR-146, miR-15a-5p, and miR-21) and two transcription factors, NFKB and STAT (Fig. 3b).

A novel five-node FFL was generated by incorporating additional miRNA-miRNA interaction pair into a four-node FFL. We identified 1347 five-node FFLs, with 372 miRNA FFLs, 1159 TF FFLs, and 184 composite FFLs. A five-node miRNA and TF FFL is demonstrated in Supplemental Figs. 5 and 6. The composite FFL enlisted 14 significant miRNA-miRNA pairs with miR-146a, miR-15a-5p, and miR-21 with the transcription factors NFKB and STAT (Fig. 3c). All the composite FFLs had NFKB and STAT as the key transcription factors.

## miRNA-Gene-TF Regulatory Network and Subnetworks

A miRNA-gene-TF regulatory network was built by converging three-, four-, and five-node composite FFLs (Fig. 4), and we obtained subnetworks based on the distribution of nodes and links (Fig. 5a–h). The first subnetwork (Supplemental Fig. 7) consisted of NFKB transcription factor linked to miR-146a and miR-15a-5p with a subset of 28 ischemic stroke-related target genes. We confirmed the relevance of these genes in ischemic stroke by gene enrichment analysis using Enrichr[21] and obtained gene ontology information that stated the biological function of these target genes in processes closely related to ischemic stroke, such as response to oxygen levels and response to hypoxia. NFKB exhibited the highest score in the data retrieved from TRANSFAC by Enrichr, and this confirmed the interaction between the genes and NFKB in

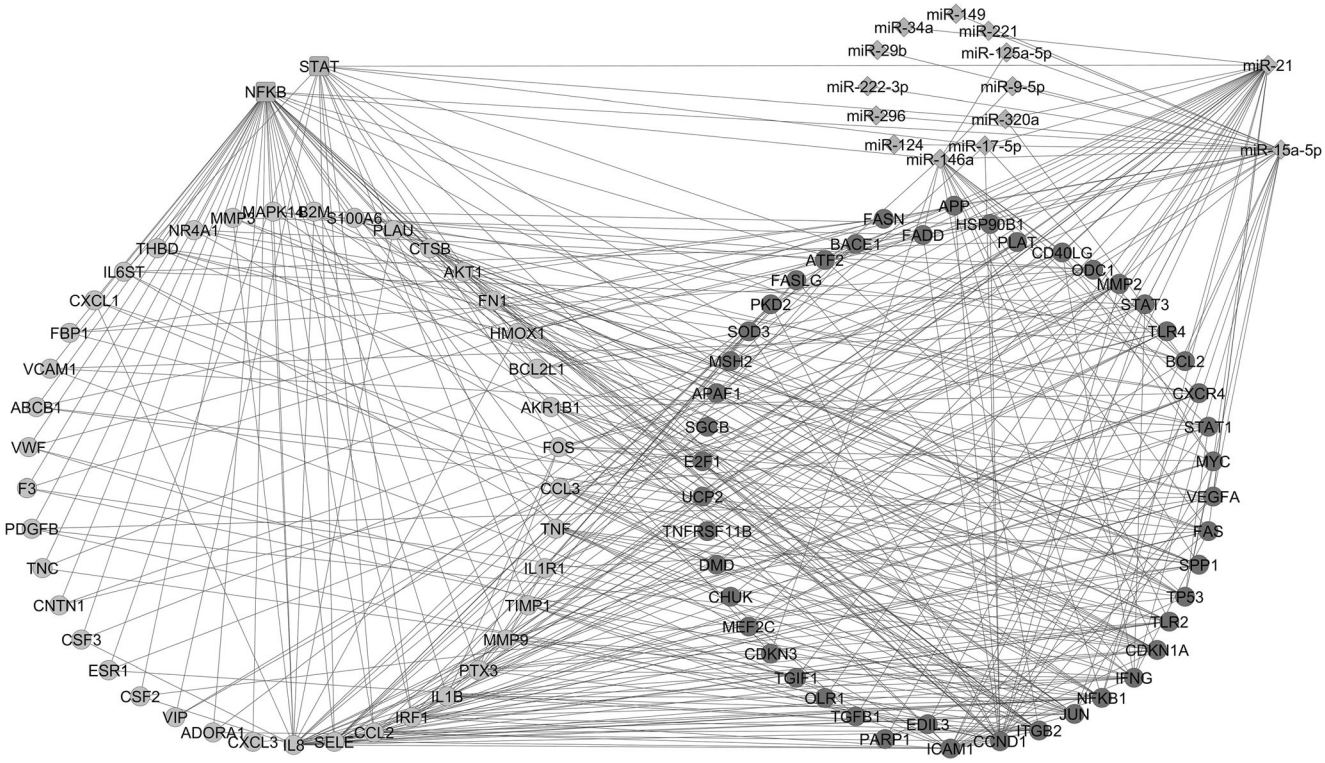



**Fig. 4** miRNA-gene-TF regulatory network. The three-, four-, and five-node composite FFLs were converged together to form an extensive ischemic stroke miRNA-TF regulatory network. The entire network is regulated by two chief transcription factors NFKB and STAT

the generated subnetwork. This subnetwork was further simplified to obtain the major interconnections with NFKB, miR-146a, miR-15a-5p, and four target genes (TP53, CCND1, IL8, and BCL2). BCL2 was found to be paired with NFKB, miR-146a, and miR-15a-5p (Fig. 6a). Similarly, we generated a simplified subnetwork with STAT as the transcription factor (Fig. 6b). This subnetwork comprised three miRNAs (miR-21, miR-320a, and miR-17-5p) and three target genes (BCL2, SELE, and CCND1). miR-21 acted as the central miRNA interacting with miR-320a as well as miR-17-5p. As in the previous subnetwork, BCL2 was targeted by all the major components (miRNAs and TF).

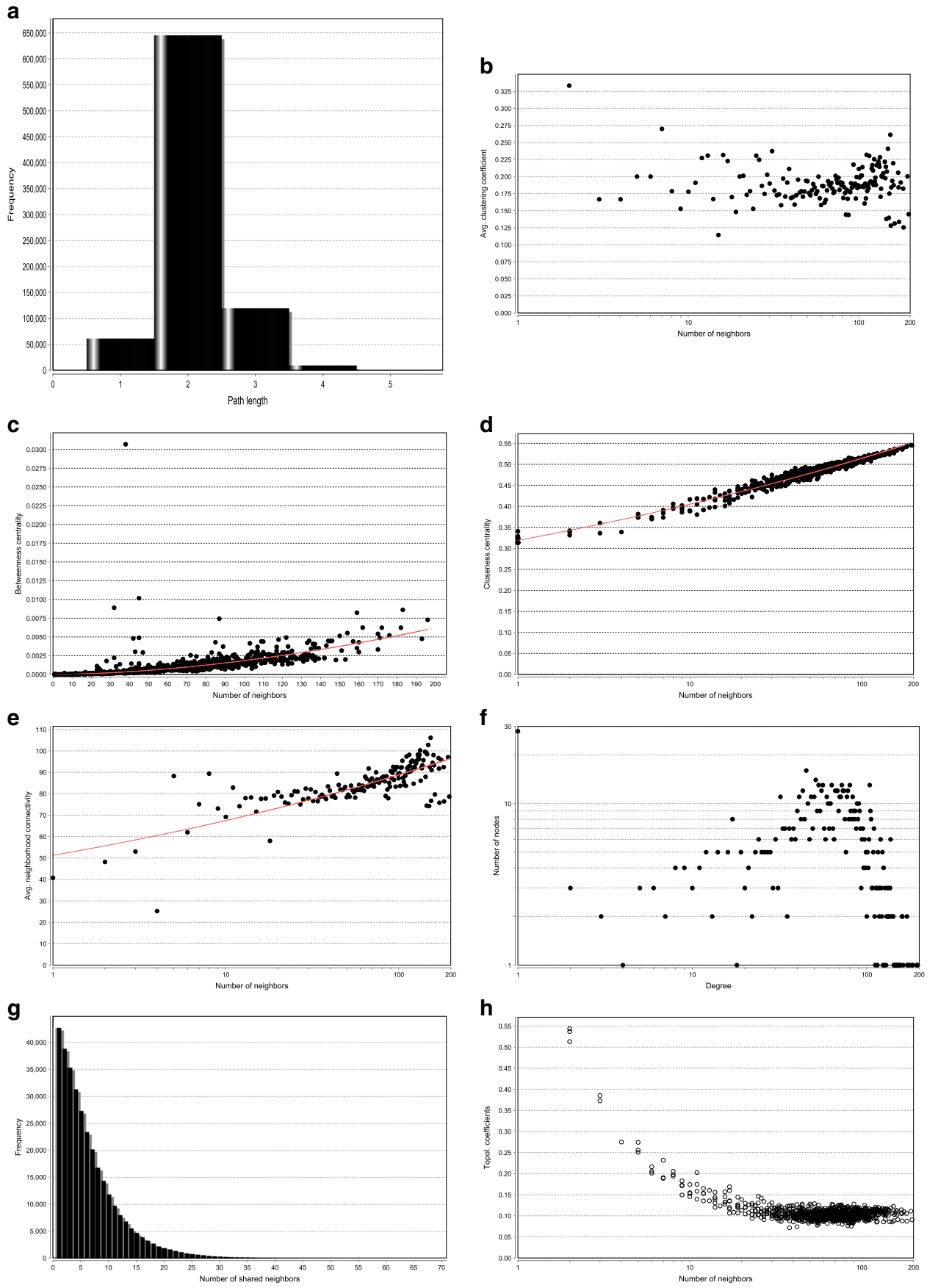
### miR-9 and miR-124 Formed the Core miRNAs Regulating Post-Stroke Neurogenesis

To evaluate the process-specific efficiency of a five-node FFL, we predicted network interactions that could be functional in post-stroke neurogenesis. Out of 31 miRNAs compiled for the study, 14 miRNAs were identified to be associated with neurogenesis. A composite five-node FFL was generated resulting in 915 nodes with interactions already described. Network hubs were extracted, and miR-9-5p, miR-124, and miR-17-5p emerged as the principal miRNAs interacting with the maximum number of genes and TFs (Fig. 7a). Edge betweenness parameter was used to identify the most significant miRNA-miRNA interaction since edges/links have greater relevance than the nodes in process-specific network inference (Table 1). It is considered that the higher the edge betweenness the higher the relevance of the interaction. miR-9 and miR-124 exhibited the highest edge betweenness and were considered to be the central miRNAs regulating post-stroke neurogenesis. The nodes and edges connected with miR-9 and miR-124 were extracted, and we inferred a subnetwork that satisfied the interactions proposed in the composite five-node FFL (Fig. 7b). The inferred network comprised TLX (transcription factor), HDAC4, and BCL2 genes.

## Discussion

In the present study, we identified the miRNA-gene-TF regulatory relationship to obtain a profound insight into the

**Fig. 5** Graphical representation of network parameters. The merged FFLs ▶ were analyzed, and network parameters were evaluated based on the distribution of their nodes and links. **a** Average short path length, **b** average clustering coefficient, **c** betweenness centrality, **d** closeness centrality, **e** average neighborhood connectivity, **f** degree distribution of nodes, **g** frequency of number of shared neighbors, **h** topological coefficients


a
Frequency
Path length
b
Avg. clustering coefficient
Number of neighbors
c
Betweenness centrality
Number of neighbors
d
Closeness centrality
Number of neighbors
e
Avg. neighborhood connectivity
Number of neighbors
f
Number of nodes
Degree
g
Frequency
Number of shared neighbors
h
Topol. coefficients
Number of neighbors

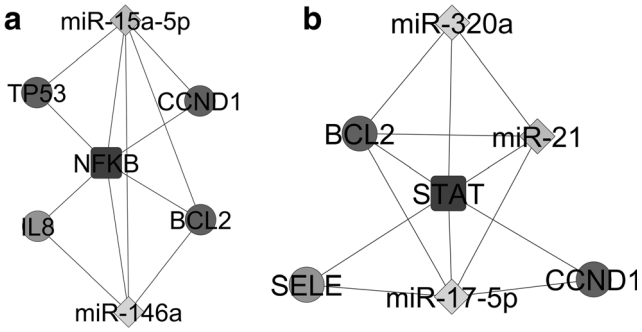



**Fig. 6** NFKB and STAT subnetwork. **a** Simplified NFKB subnetwork with the principal miRNAs and target genes in the innate inflammatory response. **b** STAT subnetwork with principal miRNAs and target genes involved in neuronal survival and regeneration

molecular mechanism of ischemic stroke. Six regulatory interactions were employed in this study (miRNA-gene, miRNA-TF, TF-miRNA, TF-gene, gene-gene, and miRNA-miRNA) to build three-, four-, and a novel five-node feed-forward loop. Each interaction pair was studied extensively to collect the principal miRNAs, TFs, and target genes associated with ischemic stroke. BCL2, VEGFA, and IGF1R were the most targeted genes in miRNA-gene pairs. BCL2 was targeted by 28 miRNAs in this study and ample reports state the impact of BCL2 in ischemic stroke [22–25]. BCL2 is anti-apoptotic and exhibits differential expression following ischemia [22, 24]. For instance, the post-ischemic overexpression of miR-497 downregulated BCL2 and resulted in an increased lesion volume [26]. The overexpression of miR-21, miR-210, miR-124, and miR-181a confer neuroprotection through an increased BCL2 expression [27–30]. VEGF generates endogenous brain responses following ischemia such as neuronal survival and neurogenesis resulting in the neurovascular remodeling [31]. IGF1R is another important gene target that supersedes the neurotoxic effect of estrogen treatment post-ischemia in middle-aged female rats [32].

miR-21, miR-17-5p, miR-34a, and miR-15a-5p were some of the principal miRNAs identified from the miRNA-gene, miRNA-TF, and TF-miRNA pairs. miR-21 was detected to be the most occurring miRNA in all the interaction pairs. The overexpression of miR-21 reduced the ischemic stroke lesion size in vivo [33]. In addition, the miR-21 expression is associated with the etiology of ischemic stroke through proliferative vascular diseases and is abundantly expressed in the atherosclerotic arteries [27, 34]. Similarly, the overexpression of miR-17-92 cluster promotes neural progenitor cell proliferation and neuronal regeneration following ischemia [35]. On the other hand, miR-15a-5p must be downregulated to render neuroprotection through the activation of peroxisome proliferator-activated receptor (PPAR) and subsequent BCL2 overexpression [36]. From TF-miRNA and TF-gene data, our study primarily identified NFKB as one of the important transcription factors regulating the maximum number of miRNAs and genes in ischemic stroke. Increasing evidence suggests that NFKB induces proinflammatory factors (cytokines and chemokines), producing an innate inflammation and regulated apoptosis through BCL2 expression [37]. Hence, miR-21, BCL2, and NFKB were inferred to be the chief components of the four interaction pairs (miRNA-gene, TF-gene, miRNA-TF, and TF-miRNA).

The next phase of this study involved the generation of three-, four- and novel five-node FFLs with miRNA, transcription factor, and target genes as the major components (Fig. 3). Each FFL was divided into miRNA FFL, TF FFL, and composite FFL. The composite FFLs were considered for the analysis as this provides complete information on three-, four-, and five-node FFLs. The three-node composite FFL consisted of miR-21, miR-15a-5p, miR-17-5p, miR-146a, and miR-320a (Fig. 3a), whereas the number of principal miRNAs in the four-node composite FFL was reduced to miR-21, miR-15a-5p, and miR-146a (Fig. 3b). However, the total number of interactions in the four-node FFL was greater due to a large gene-gene coexpression data for each pair of miRNA-TF.

It is particularly important to consider miRNA-miRNA interaction in ischemic stroke because a number of miRNAs are differentially expressed following ischemia and some of these miRNAs function together [2, 3]. There has been limited evidence of synergistic activity of miRNAs in ischemic stroke, and hence, prediction of miRNA-miRNA interaction might open novel prospects to understand the ischemic stroke pathological and neuroprotection processes. miRNA-miRNA pairs were incorporated to generate five-node FFLs forming a complete regulatory network to comprehend molecular processes associated with ischemic stroke. miRNA-miRNA pairs were established based on the common target genes between two miRNAs. For instance, the miRNA-miRNA interaction between miR-15a-5p and miR-146a was predicted through common target genes, BCL2, and NFKB1. KEGG pathway analysis showed that BCL2 and NFKB1 were involved in ischemic stroke-related pathways such as hypoxia inducible factor-1 (HIF-1) and phosphatidylinositol-3-kinase (PI3-AKT) signaling pathway. HIF-1 signaling elicits neuronal protection by delaying the neuronal death following an ischemic stroke in vitro as well as in vivo [38]. The PI3-AKT signaling pathway is well known to regulate the neuronal cell fate and function in neuroprotection through ischemic post-conditioning [39]. For instance, the synergism between miR-21 and miR-17-5p was predicted based on seven common target genes (BCL2, E2F1, ICAM1, MYC, MMP2, PTEN, VEGFA). Enrichr analysis of these genes shows that they are significantly expressed in response to low oxygen levels and are crucial in cerebral ischemia. The significant miRNA-miRNA pairs were then linked to genes and TF to form a five-node FFL. The five-node feed-forward loop with miRNA-

miRNA interaction ensures an effective means to depict a complete regulatory network (Fig. 3c).

The composite FFLs (three-, four-, and five-node) were converged to obtain a regulatory network for ischemic stroke, one of the major outputs of the current study (Fig. 4). For the analysis of the complex regulatory network, we extracted two main subnetworks with NFKB and STAT as the chief regulatory transcription factors (Fig. 6a, b). The NFKB subnetwork comprised two miRNAs (miR-146a and miR-15a-5p) and four target genes (BCL2, CCND1, IL8, and TP53) (Fig. 6a). miR-146a is NFKB dependent and instigates innate immune responses [40], but its direct relevance in ischemic stroke has not been studied yet. miR-15a-5p is known to induce apoptosis and plays an important role in the NFKB non-canonical pathway during macrophage differentiation. The decrease in miR-15a-5p reduces the macrophage hyperactivity and

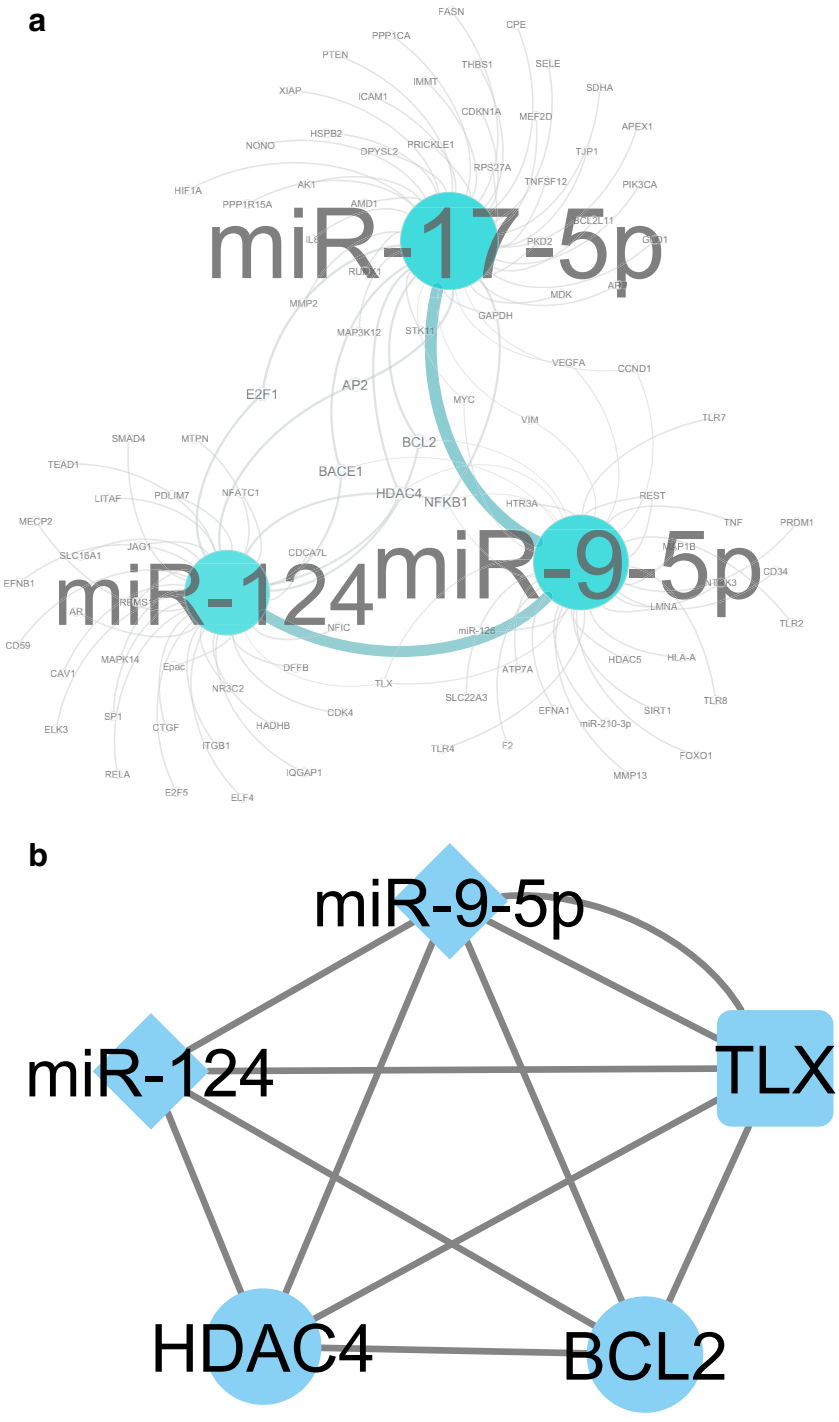



**Fig. 7** miRNA regulatory network specific for post-stroke induced neurogenesis. **a** Network hubs with miR-9-5p, miR-124, and miR-17-5p as the principal miRNAs interacting with the maximum number of genes and TFs involved in post-stroke neurogenesis. **b** The nodes and edges connected with miR-9 and miR-124 were extracted to infer a subnetwork that satisfied the interactions proposed in the composite five-node FFL

**Table 1** Edge betweenness for miRNA-miRNA interactions

| miRNA-miRNA interaction | Edge betweenness |
|---|---|
| miR-124 (interacts with) miR-9-5p | 647.0953678 |
| miR-9-5p (interacts with) miR-34a | 214.3711996 |
| miR-17-5p (interacts with) miR-9-5p | 179.7108985 |
| miR-133a (interacts with) miR-9-5p | 151.1780092 |
| miR-125a-5p (interacts with) miR-9-5p | 136.8090763 |
| miR-9-5p (interacts with) miR-210-3p | 129.4670129 |
| miR-181a (interacts with) miR-9-5p | 123.7451442 |
| miR-145 (interacts with) miR-9-5p | 118.1002844 |
| miR-15a-5p (interacts with) miR-9-5p | 111.6258679 |
| miR-126 (interacts with) miR-9-5p | 109.3953838 |
| miR-9-5p (interacts with) miR-29b | 108.5471709 |
| miR-9-5p (interacts with) miR-146a | 98.79707219 |

promotes the proinflammatory stimuli through NFKB target genes [41]. These studies suggest that miR-146a and miR-15a-5p are closely associated with NFKB to generate inflammatory responses. Ischemic brain injury is often accompanied by acute or chronic inflammatory processes and subsequent recruitment of inflammatory cells in the brain [42]. Therefore, the NFKB subnetwork focuses on the inflammatory and innate immune response following ischemic stroke.

Three miRNAs (miR-320a, miR-17-5p, and miR-21) and three target genes (BCL2, CCND1, and SELE) formed the STAT subnetwork (Fig. 6b). Numerous reports suggest the role of STAT in neuronal survival and regeneration [43, 44]. Neuronal injury activates STAT and renders neuroprotection through the JAK-STAT signaling pathway, which in turn, activates the transcription of pri-miR-21 in spinal cord injury [45]. STAT also promotes the expression of the miR-17-92 cluster that induces neural progenitor proliferation post-stroke [35, 46]. One of the studies demonstrated the action of anti-miR-320a in reducing the ischemic lesion size with the increase in the expression of the gene JAK [47]. JAK functions in combination with STAT [45], and our subnetwork predicts the relationship between miR-320a and STAT. Thus, the STAT subnetwork highlights the neuronal repair and regeneration with its corresponding gene targets and miRNAs.

BCL2 and CCND1 were the common target genes that occurred in both the subnetworks (Fig. 6a, b). CCND1 is an important regulator of cell cycle and promotes cell proliferation [48]. The cell cycle machinery has an indispensable role in the ischemic stroke [48], but there have been contradictory studies with CCND1 as the target gene. CCND1 is related to the excitotoxic neuronal death and is upregulated during neuronal apoptosis [48, 49]. CCND1 is shown to be overexpressed following ischemia/reperfusion while certain studies have shown the implications of CCND1 in neuronal survival [49]. In the first subnetwork, CCND1 is connected to miR-15a-5p and NFKB (Fig. 6a) whereas in the second subnetwork CCND1 is found to be interacting with miR-17-5p and STAT (Fig. 6b). Interestingly, downregulation of miR-15a-5p promotes neuroprotection whereas miR-17-5p is upregulated to promote neural progenitor cell proliferation leading to neuronal regeneration post-ischemia [35, 36]. Our study predicts that CCND1 might prove to be a beneficial target for miR-15a-5p and miR-17-5p that could either promote neuronal survival or induce neuronal regeneration post-ischemia. "Cytochrome c release from mitochondria", "response to ischemia" and "glucose starvation" were identified to be the biological processes associated with both the subnetworks (Fig. 6a, b), and these processes are in congruence with the ischemic stroke pathology.

Further in this study, miR-9 and miR-124 were identified to be the core regulators of post-stroke induced neurogenesis. Previous reports state that miR-124a downregulation following cerebral ischemia could increase neural progenitor cell proliferation while the introduction of miR-124a mitigates proliferation and enhances neuronal differentiation [50]. Hence, miR-124a is known to induce post-stroke neurogenesis by balancing the neuronal proliferation and differentiation. Nonetheless, the role of miR-9 in post-ischemic neurogenesis is unknown. miR-9 is reported to be downregulated in an in vivo ischemic stroke model where the introduction of miR-9 agomir provides neuroprotection post-ischemia by targeting BCL2L11 [51]. Our model established a link between miR-9 and miR-124, which could possibly mean that miR-9 could also stimulate post-stroke neurogenesis in conjunction with the TLX activity. TLX forms a feedback regulatory loop with miR-9 to maintain a balance between neural stem cell proliferation and differentiation [52]. Therefore, it is predicted that TLX regulates the expression of miR-124 and miR-9-5p to induce neurogenesis post-ischemia in an adult brain. In addition, the inferred model suggested that miR-9-5p, miR-124, and TLX regulate the expression of their target genes, BCL2 and HDAC4.

In conclusion, the present study introduced a novel five-node FFL to comprehend the regulatory relationships between miRNAs, TFs, and their gene targets. The key regulatory components identified in the form of subnetworks comprised miRNAs, TFs, and genes associated with innate inflammatory response and neuronal survival mechanism following ischemic stroke. In addition, the five-node network model identified miR-9 and miR-124 as the core regulatory miRNAs in post-stroke neurogenesis. Further studies are underway to experimentally validate the deduced interactions in the network model. This network model could be extended to elucidate the regulatory components involved in the pathophysiology of other diseases.

**Author Contributions** RKG, NSS, and FSM contributed to all components of the work (design, experimentation, analysis, and writing).

**Funding Information** This study was funded by (a) the Department of Biotechnology, Government of India "Bioinformatics Infrastructure Facility for Biology Teaching through Bioinformatics (BIF-BTBI)" (grant number: BT/BI/25/001/2006 dated 25 March 2011) and (b) the Kerala State Council for Science, Technology and Environment, Science Research Scheme (grant number: 018/SRSLS/2014/CSTE).

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interests.

## References

1. Ladecola C, Anrather J (2011) Stroke research at a crossroad: asking the brain for directions. Nat Neurosci 14:1363–1368
2. Jeyaseelan K, Lim KY, Armugam A (2008) MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. Stroke 39:959–966
3. Saugstad JA (2010) MicroRNAs as effectors of brain function with roles in ischemia and injury, neuroprotection, and neurodegeneration. J Cereb Blood Flow Metab 30:1564–1576
4. Nampoothiri SS, Menon HV, Das D, G K R (2016) ISCHEMIRs: finding a way through the obstructed cerebral arteries. Curr Drug Targets 17:800–810
5. Lavrik IN, Zhivotovsky B (2014) Systems biology: a way to make complex problems more understandable. Cell Death Dis 5:e1256
6. Werner HM, Mills GB, Ram PT (2014) Cancer systems biology: a peek into the future of patient care? Nat Rev Clin Oncol 11:167–176
7. Mangan S, Alon U (2003) Structure and function of the feed-forward loop network motif. Proc Natl Acad Sci U S A 100: 11980–11985
8. Sun J, Gong X, Purow B, Zhao Z (2012) Uncovering microRNA and transcription factor mediated regulatory networks in glioblastoma. PLoS Comput Biol 8:e1002488
9. Safran M, Dalah I, Alexander, Rosen N, Iny Stein T, Shmoish M, Nativ N, Bahir I et al (2010) GeneCards Version 3: the human gene integrator. Database (Oxford) 2010:baq020
10. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K et al (2000) Gene ontology: tool for the unification of biology. Nat Genet 25:25–29
11. The Gene Ontology Consortium (2015) Gene ontology consortium: going forward. Nucleic Acids Res 43:D1049–D1056
12. Jiang Q, Wang Y, Hao Y, Juan L, Teng M, Zhang X (2009) miR2Disease: a manually curated database for microRNA deregulation in human disease. Nucleic Acids Res 37:D98–D104
13. Ruepp A, Kowarsch A, Theis F (2012) PhenomiR: microRNAs in human diseases and biological processes. Methods Mol Biol 822: 249–260
14. Li Y, Qiu C, Tu J, Geng B, Yang J, Jiang T, Cui Q (2014) HMDD v2.0: a database for experimentally supported human microRNA and disease associations. Nucleic Acids Res 42:D1070–D1074
15. Yang JH, Li JH, Jiang S, Zhou H, Qu LH (2013) ChIPBase: a database for decoding the transcriptional regulation of long non-coding RNA and microRNA genes from ChIP-Seq data. Nucleic Acids Res 41:D177–D187
16. Jiang C, Xuan Z, Zhao F, Zhang MQ (2007) TRED: a transcriptional regulatory element database, new entries and other development. Nucleic Acids Res 35:D137–D140
17. Hsu SD, Tseng YT, Shrestha S, Lin YL, Khaleel A, Chou CH, Chu CF, Huang HY et al (2014) miRTarBase update 2014: an information resource for experimentally validated miRNA-target interactions. Nucleic Acids Res 42:D78–D85
18. Warde-Farley D, Donaldson SL, Comes O, Zuberi K, Badrawi R, Chao P, Franz M, Grouios C et al (2010) The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. Nucleic Acids Res 38:W214–W220
19. Xu J, Li CX, Li YS, Lv JY, Ma Y, Shao TT, Xu LD, Wang YY et al (2011) MiRNA-miRNA synergistic network: construction via co-regulating functional modules and disease miRNA topological features. Nucleic Acids Res 39:825–836
20. Kanehisa M, Goto S (2000) KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28:27–30
21. Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, Clark NR, Ma'ayan A (2013) Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics 14:128
22. Martinou JC, Dubois-Dauphin M, Staple JK, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D et al (1994) Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron 13:1017–1030
23. Hata R, Gillardon F, Michaelidis TM, Hossmann KA (1999) Targeted disruption of the bcl-2 gene in mice exacerbates focal ischemic brain injury. Metab Brain Dis 14:117–124
24. Chen J, Simon RP, Nagayama T, Zhu R, Loeffert JE, Watkins SC, Graham SH (2000) Suppression of endogenous bcl-2 expression by antisense treatment exacerbates ischemic neuronal death. J Cereb Blood Flow Metab 20:1033–1039
25. Webster KA, Graham RM, Thompson JW, Spiga MG, Frazier DP, Wilson A, Bishopric NH (2006) Redox stress and the contributions of BH3-only proteins to infarction. Antioxid Redox Signal 8:1667–1676
26. Yin KJ, Deng Z, Huang H, Hamblin M, Xie C, Zhang J, Chen YE (2010) miR-497 regulates neuronal death in mouse brain after transient focal cerebral ischemia. Neurobiol Dis 38:17–26
27. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T (2002) Identification of tissue-specific microRNAs from mouse. Curr Biol 12:735–739
28. Devlin C, Greco S, Martelli F, Ivan M (2011) miR-210: more than a silent player in hypoxia. IUBMB Life 63:94–100
29. Sun Y, Gui H, Li Q, Luo ZM, Zheng MJ, Duan JL, Liu X (2013) MicroRNA-124 protects neurons against apoptosis in cerebral ischemic stroke. CNS Neurosci Ther 19:813–819
30. Ouyang YB, Lu Y, Yue S, Giffard RG (2012) miR-181 targets multiple Bcl-2 family members and influences apoptosis and mitochondrial function in astrocytes. Mitochondrion 12:213–219
31. Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA (2002) Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc Natl Acad Sci U S A 99:11946–11950
32. Selvamani A, Sathyan P, Miranda RC, Sohrabji F (2012) An antagomir to microRNA Let7f promotes neuroprotection in an ischemic stroke model. PLoS One 7:e32662
33. Buller B, Liu X, Wang X, Zhang RL, Zhang L, Hozeska-Solgot A, Chopp M, Zhang ZG (2010) MicroRNA-21 protects neurons from ischemic death. FEBS J 277:4299–4307
34. Ji R, Cheng Y, Yue J, Yang J, Liu X, Chen H, Dean DB, Zhang C (2007) MicroRNA expression signature and antisense-mediated depletion reveal an essential role of microRNA in vascular neointimal lesion formation. Circ Res 100:1579–1588
35. Liu XS, Chopp M, Wang XL, Zhang L, Hozeska-Solgot A, Tang T, Kassis H, Zhang RL et al (2013) MicroRNA-17-92 cluster mediates the proliferation and survival of neural progenitor cells after stroke. J Biol Chem 288:12478–12488
36. Yin KJ, Deng Z, Hamblin M, Xiang Y, Huang H, Zhang J, Jiang X, Wang Y et al (2010) Peroxisome proliferator-activated receptor

delta regulation of miR-15a in ischemia-induced cerebral vascular endothelial injury. J Neurosci 30:6398–6408
37. Harari OA, Liao AK (2010) NF-κB and innate immunity in ischemic stroke. Ann N Y Acad Sci 1207:32–40
38. Shi H (2009) Hypoxia inducible factor 1 as a therapeutic target in ischemic stroke. Curr Med Chem 16:4593–4600
39. Liu J, Xu Q, Wang H, Wang R, Hou XY (2013) Neuroprotection of ischemic postconditioning by downregulating the postsynaptic signaling mediated by kainate receptors. Stroke 44:2031–2035
40. Taganov KD, Boldin MP, Chang KJ, Baltimore D (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A 103:12481–12486
41. Li T, Morgan MJ, Choksi S, Zhang Y, Kim YS, Liu ZG (2010) MicroRNAs modulate the noncanonical NF-κB pathway by regulating IKKα expression during macrophage differentiation. Nat Immunol 11:799–805
42. Jin R, Yang G, Li G (2010) Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. J Leukoc Biol 87:779–789
43. Dziennis S, Alkayed NJ (2008) Role of signal transducer and activator of transcription 3 in neuronal survival and regeneration. Rev Neurosci 19:341–361
44. Ahn YH, Lee G, Kang SK (2006) Molecular insights of the injured lesions of rat spinal cords: Inflammation, apoptosis, and cell survival. Biochem Biophys Res Commun 22:560–570
45. Bhalala OG, Pan L, Sahni V, McGuire TL, Gruner K, Tourtellotte WG, Kessler JA (2012) microRNA-21 regulates astrocytic response following spinal cord injury. J Neurosci 32:17935–17947
46. Kohanbash G, Okada H (2012) MicroRNAs and STAT interplay. Semin Cancer Biol 22:70–75
47. Sepramaniam S, Armugam A, Lim KY, Karolina DS, Swaminathan P, Tan JR, Jeyaseelan K (2010) MicroRNA 320a functions as a novel endogenous modulator of aquaporins 1 and 4 as well as a potential therapeutic target in cerebral ischemia. J Biol Chem 285: 29223–29230
48. Rashidian J, Iyirhiaro G, Aleyasin H, Rios VI, Callaghan S, Bland RJ, Slack RS, During MJ et al (2005) Multiple cyclin-dependent kinases signals are critical mediators of ischemia/ hypoxic neuronal death *in vitro* and *in vivo*. Proc Natl Acad Sci U S A 102:14080–14085
49. Li Y, Chopp M, Powers C, Jiang N (1997) Immunoreactivity of cyclin D1/cdk4 in neurons and oligodendrocytes after focal cerebral ischemia in rat. J Cereb Blood Flow Metab 17:846–856
50. Liu XS, Chopp M, Zhang RL, Tao T, Wang XL, Kassis H, Hozeska-Solgot A, Zhang L et al (2011) MicroRNA profiling in subventricular zone after stroke: MiR-124a regulates proliferation of neural progenitor cells through Notch signaling pathway. PLoS One 6:e23461
51. Wei N, Xiao L, Xue R, Zhang D, Zhou J, Ren H, Guo S, Xu J (2016) MicroRNA-9 mediates the cell apoptosis by targeting Bcl2l11 in ischemic stroke. Mol Neurobiol 53:6809–6817
52. Zhao C, Sun G, Li S, Shi Y (2009) A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. Nat Struct Mol Biol 16:365–371