Functional annotation of differentially expressed fetal cardiac microRNA targets: implication for microRNA-based cardiovascular therapeutics

Sharad Saxena¹ · Anubhuti Gupta¹ · Vaibhav Shukla² · Vibha Rani¹

Received: 30 April 2018 / Accepted: 17 November 2018 / Published online: 21 November 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Gene expression pattern of a failing heart depicts remarkable similarity with developing fetal heart. Elucidating genetic as well as epigenetic mechanisms regulating the gene expression during cardiac development will improve our understanding of cardiovascular diseases. In the present study, we aimed to validate and characterize differentially expressed known microRNAs (miRNA) obtained from next generation sequencing data of two fetal cardiac developmental stages (days 4th and 14th) from chicken (*G. gallus domesticus*) using bioinformatic approaches. Potential mRNA targets of individual miRNA were identified and classified according to their biological, cellular, and molecular functions. Functional annotation of putative target genes was performed to predict their association with cardiovascular diseases. We identified a total of 19 differentially expressed miRNAs between 4th and 14th day sample from the data sets obtained by next generation sequencing. A total of nearly 1522 potential targets ranging from 15 to 270 for each miRNA were predicted out of which 1221 were unique, while 301 were overlapping. Gene ontology and KEGG analysis revealed that majority of these target genes regulate critical cellular and molecular processes including transcriptional regulation, protein transport, signal transduction, matrix remodeling, Ras signaling, MAPK signaling, and TGF-beta signaling pathways indicating the complex nature of microRNA-mediated gene regulation during cardiogenesis. We found a significant association between potential target genes and cardiovascular diseases validating a link between fetal cardiac miRNAs and regulation of cardiovascular disease-related genes. These important findings may lay a foundation for further understanding the regulatory mechanisms operative in gene re-programming in the failing heart.

Keywords Fetal cardiac miRNA · Chicken heart development · Bioinformatics · Gene re-programming

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13205-018-1520-8>) contains supplementary material, which is available to authorized users.

✉ Vibha Rani
vibha.rani@jiit.ac.in
Sharad Saxena
s.a28@hotmail.com
Anubhuti Gupta
anubhutigupta2010@gmail.com
Vaibhav Shukla
vaibhavsols@gmail.com

¹ Transcriptome Laboratory, Centre for Emerging Diseases, Department of Biotechnology, Jaypee Institute of Information Technology, A-10, Sector-62, Noida, Uttar Pradesh 210309, India

² School of Life Sciences, Manipal University, Manipal, Karnataka 576104, India

Introduction

Heart function is requisite for perfect embryogenesis, and therefore, heart is one of the earliest differentiating and functioning organs in vertebrates (Espinoza-Lewis and Wang 2012). Differentiation of fetal cardiomyocyte progenitor cells is dependent on tightly regulated genetic as well as epigenetic factors. Most of these underlying molecular mechanisms are considered to be conserved from insects to vertebrates (Srivastava and Olson 2000). Recent studies suggest that the molecular events taking place during cardiac development re-activate in the course of pathological cardiomyocyte hypertrophy, cardiomyocyte apoptosis, myocardial fibrosis, cardiac dysfunction, and heart failure (Abu-Issa and Kirby 2007). Reactivation of fetal gene programming occurs to combat the stress and to maintain the haemostasis in cardiac cells (Nandi and Mishra 2015). Therefore, identification

and characterization of novel molecular mechanisms underlying cardiogenesis will provide better understanding of cardiac fetal gene programming as well as adaptive responses during heart failure. Genetic factors regulating cardiac development and cardiovascular diseases (CVDs) have been vigorously reported in recent studies; however, the understanding of epigenetic factors with respect to cardiac development and CVDs is still lacking. Epigenetic factors modulate the gene expression without altering the nucleotide sequence of genome. MicroRNAs (miRNA) are one such recently reported epigenetic regulators which modulate the expression of > 50% of genes at post-transcriptional level either by cleaving mRNA or by inhibiting translation based on the complementarity between the miRNA and its target (Bianchi et al. 2017). In brief, miRNAs are encoded by nuclear RNA polymerase II into ~2000 nucleotides primary transcripts (pri-miRNAs). Another enzyme Drosha and Pasha cleaves pri-miRNAs into precursor miRNAs (pre-miRNA) of 50–100 nucleotides which gets transported into cytoplasm. In cytoplasm, Dicer modifies the pre-miRNAs into mature miRNA of 18–23 nucleotides which gets incorporated into RISC (RNA-induced silencing complex). One of the strand (guide strand) binds with the 3' UTR of target mRNA and modulates its expression (Saxena et al. 2017a).

Recently, a number of miRNAs have been studied for their role during stage-specific cardiac development in mouse (miR-27) as well as zebrafish (miR-218) (Catalanotto et al. 2016). However, miRNAs have short half-life, and their expression during cardiac development varies across different developmental stages (Mishra et al. 2016). Therefore, elucidating the miRNAs and their biological role at different stages of development will provide a better understanding of their role in cardiac development. Due to the conserved molecular mechanism, cardiac development and its functions are similar among vertebrates. Chicken (*G. gallus domesticus*) is one of the most studied developmental models because of its embryonic morphological similarities with human at early stages of development. In addition, its heart has closest anatomy and many similar characteristics with humans (four chambered heart) in non-mammals (Cakstina et al. 2014; Mok et al. 2015). Its whole genome has been sequenced and > 60% genes are closely similar homologues in humans (Eyras et al. 2005). Chicken embryonic development is classified into several development stages by Hamburger and Hamilton (HH). According to HH staging, HH 21–24 (day 4) is the stage during which cardiac remodeling, valve formation, and differentiation of cardiac neural crest cells for outflow tract development begins. Similarly, HH 40 (day 14) is the stage around which cardiac development is completed followed by hatching by HH 46 (day 20) (Martinsen 2005; Wittig and Munsterberg 2016). Therefore, analyzing miRNA profile at the initial day and at the completion day of cardiac development will provide a clear picture of differentially expressed miRNAs regulating

the fetal cardiac gene program. In the present study, we aimed to perform in-silico characterization and functional annotation of differentially expressed miRNAs obtained from 4th and 14th day cardiac tissue sample from chicken embryo. We also analyzed the association of fetal cardiac miRNAs with CVDs to extend our understanding of fetal gene as well as miRNA re-expression during CVDs.

Materials and methods

miRNA collection and determination of their primary features

In our previous study, we have sequenced small RNA libraries from chicken (*G. gallus domesticus*) heart tissue at different developmental stages using next-generation sequencing NCBI, GEO accession: GSE69663 (4th day: GSM1705503) (14th day: GSM1705508). For the present study, we extracted differentially expressed known miRNAs with at least 10 reads from 4th (beginning of heart development) and 14th day (completion of heart development) samples. Their hairpin loop structure, minimum free energy, and chromosomal location were determined using MirEval database (<http://mimirna.centenary.org.au/mireval/>). Precursor sequence (FASTA) of each miRNA was uploaded selecting ICGSC_gallus-gallus_4.0 genome. Transcription factor binding sites which potentially regulate these miRNA sequences were also predicted using MirEval database.

miRNA conservation and expression analysis

Each miRNA was searched on miRBase database (<http://www.mirbase.org/search.shtml>) to detect its homologue in humans and other species. Only those miRNAs whose mature sequences perfectly matched with known human miRNAs in the miRBase were considered. All the conserved miRNAs between human and chicken were identified and aligned using Unipro U Gene tool to check the sequence similarities. Up-regulated and down-regulated miRNAs were identified on the basis of expression analysis using log2 RPM value, where 4th day was considered as reference sample. Heat map for the same was generated using Cimmner web tool (<https://discover.nci.nih.gov/cimminer/oneMatrix.do>).

Potential target genes prediction

Potential target genes of up-regulated and down-regulated miRNAs were predicted using Target Scan (score ≤ − 0.45) (http://www.targetscan.org/vert_72/) and miRDB (score ≥ 80) (<http://mirdb.org/custom.html>). Target Scan is an online database based on context model used for

prediction of target gene derived from all contemporary experimental techniques. miRNAs were uploaded using their nomenclature for chicken (*G. gallus domesticus*). Similarly, miRDB is an online database based on support vector machines and high-throughput training data sets. Mature sequence of each miRNA was uploaded for chicken (*G. gallus domesticus*). Common potential target genes obtained from both the databases were identified and selected for further analysis.

Target enrichment and their functional annotation

Functional role of each miRNA was predicted using two databases, namely, PANTHER (Protein Analysis through Evolutionary Relationships) (<http://www.pantherdb.org/index.jsp>) and DAVID (Database for Annotation, Visualization and Integrated Discovery) (<https://david.ncifcrf.gov/summary.jsp>). Target genes were classified on the basis of their biological, cellular, and molecular role. Their involvement in KEGG pathways as well as their association with CVDs was also determined. Significant p values were obtained by Fisher's exact test in DAVID. Enriched targets and their pathways (including KEGG pathways) with p values < 0.05 , and enrichment score of > 1.5 were considered. A network of interaction between miRNAs and their significantly enriched pathways was developed by the Cytoscape software.

Potential target co-expression and pathway interaction

Interactions among potential target genes involved in CVDs (obtained from DAVID) were predicted using Genemania (<https://genemania.org/>). Since *gallus-gallus* is not included in the list of organisms, we analyzed the co-expression selecting homo-sapiens as target organism. Interaction network based upon genes co-expression, genetic, and physical interactions was developed and their percentages were determined.

Statistical analysis

Significant p values were obtained by Fisher's exact test from DAVID database to determine the probability that each biological, cellular, and molecular function assigned to the data set is due to chance alone. Enriched targets and their pathways (including KEGG pathways) with p values < 0.05 , and enrichment score of > 1.5 were considered.

Results and discussion

miRNAs exhibits differential expression during progressive fetal cardiac developmental stages

The present study was designed to investigate the role of differentially expressed miRNAs in fetal cardiac tissue and their influence on critical cellular processes involved in cardiac development and CVDs using in-silico approaches. Several robust, sensitive, and updated bioinformatics tools/databases were used for the characterization of miRNA through potential target genes prediction and functional annotation. We extracted differentially expressed miRNAs from two developmental stages (4th and 14th day) of fetal chicken heart tissue identified in our previous report (Rustagi et al. 2015). All the precursor miRNAs (except for miR-193) formed hairpin loop structures with mature sequence lying in one of the arms. Minimum free energy for all the miRNAs was < -18 Kcal/mol, indicating that these miRNAs were real, exhibiting the properties of true miRNA (Warris et al. 2014). Although miR-193 has been detected as circulating miRNA in human plasma samples; however, precursor sequence of miR-193 obtained from our study as well as from miRBase for both chicken and humans did not form hairpin structure (Wong et al. 2015). Cluster analysis depicted that the expression of mir-9, mir-18, mir-20, mir-107, and mir-196 were down-regulated (Cluster 1), while the expression of mir-10, mir-19, mir-26, mir-27, mir-92, mir-100, mir-101, mir-122, mir-126, mir-146, mir-148, mir-193, mir-199, and let-7 were up-regulated as the fetal cardiac development progresses. Based upon their clustering, up-regulated miRNAs were divided into two clusters (cluster 2a: mir-10, mir26, mir-92, mir-148, and cluster 2b: mir-19, mir-27, mir-100, mir-101, mir-122, mir-126, mir-146, mir-193, mir-199, and let-7) to ease gene ontology depiction (Fig. 1). Similar results have been reported in human cardiac miRNA expression profiling, where the expression of mir-18, mir-20, and mir-107 decreased in later stages of development. Likewise, the expression of mir-10, mir-26, mir-126, mir-146, mir-193, and let-7 was increased during later stages of cardiac development (Zhou et al. 2014). Until date, a very limited number of miRNAs studies have been carried out on chicken model system and a comprehensive cardiac miRNAs profiling during development is still lacking. To the best of our knowledge, for the first time, we are reporting the presence of mir-9 and mir-122 in fetal cardiac tissue of any organism. Until now, mir-9 and mir-122 have been detected as tissue specific miRNAs (mir-9 in brain and mir-122 in liver) (Ludwig et al. 2016). mir-9 has been reported to suppress the expression of myocardin,

Fig. 1 Heat maps showing differential expression of miRNAs from day 4th (beginning of heart development) and day 14th (completion of heart development) considering 4th day as reference. Except for mir-7, 29, 34, and 300, every miRNA showed differential expression pattern

a protein that is required for cardiomyocyte survival and maintenance of heart function (Chen et al. 2008; Huang et al. 2009b), therefore, has the potential to be used as therapeutic target during cardiac hypertrophy (Wang et al. 2010a, b). Likewise, mir-122 was detected in plasma of patients with acute myocardial infarction and hence qualifies to be potential biomarker (Li et al. 2015).

Most of the miRNAs belonged to distinct chromosomes or were far apart within the same chromosome. However, few down-regulated as well as up-regulated miRNAs were found to be on the same chromosome located in close proximity, therefore, exhibiting similar expression pattern (Table 1). The differential expression pattern justified the previous notions that miRNAs within same chromosomal location share common expression and vice versa (Liang et al. 2007). Conservation of miRNAs in different species is one of the theoretical criteria to be an ideal miRNA (Lewis et al. 2003). All the miRNAs aligned 100% with hominids (hsa), therefore, can be said as evolutionary conserved and not organism specific (Fig. 2). Since most of the mRNAs are conserved targets of miRNAs (Bartel 2004),

it can be inferred that these miRNAs might be involved in similar biological processes across all the organisms.

Fetal cardiac miRNAs regulates multiple genes simultaneously

Biological role of miRNAs can only be predicted through their potential target genes prediction. There are several criteria such as seed match without gaps between the miRNA and target mRNA, minimum free energy to measure the miRNA–mRNA binding efficiency, and target-site abundance within mRNA which needs to be considered for target gene prediction (Shukla et al. 2017). Two different tools (miRDB and Target Scan) were used to cover all the important criteria for target prediction. A total of 1522 potential targets ranging from 15 to more than 270 for each miRNA were obtained out of which 1221 were unique, while 301 were the targets of more than one miRNA (Fig. 3). Detection of common and unique gene targets justified the notion that a single miRNA can target multiple numbers of genes,

Table 1 Depicting chromosomal distribution, minimum free energy (MFE), and potential transcription factor regulating miRNAs obtained from miREval database

miRNAs	Chromosomal distribution (<i>Gallus gallus</i>)	Minimum free energy (kcal/mol)	Potential transcription factor regulating miRNAs (Mireval database)
Down-regulated			
mir-9	chrZ:60293378–60293449	– 28.2	Myf, SP1, IRF2, ZAP1, IRF1, RREB1, Zfx, NHLH1, ELF5, REST, RREB1
mir-18	chr1:147253683–147253776	– 18.7	Myf, FOXA1, Foxa2, HMG-IorY, FKH1, PEND
mir-20	chr1:147253380–147253450	– 30.9	Myf, FOXA1, Foxa2, HMG-IorY, FKH1, PEND
mir-107	chr6:18945423–18945481	– 21.9	REST, TTX-3, HNF1A, Sox2, IRF1, RREB1
mir-196	chr2:32864686–32864741	– 22.9	RREB1, Pax4, SP1, RUNX1, SOX9, TFAP2A, Pax4
Up-regulated			
let-7	chr24:3338934–3339010	– 28.5	FOXA1, RORA_1, SFP1, NR2F1, REST
mir-10	chr7:15780543–15780616	– 29.7	Myf, HAP4, PPARG, Trl, DOT6, AZF1, HIF1A
mir-19	chr1:147253250–147253316	– 29.7	Ddit3, FOXA1, Foxa2, PEND, SFL1, Pax4
mir-26	chr2:4537770–4537838	– 33.5	PLAG1, Znf143, SP1, PLAG1, TEAD1, Pax4, RREB1, Zfx
mir-27	chrZ:41508282–41508358	– 40.2	NHLH1, STAT1, AGL3, HAP4
mir-92	chr1:147253137–147253194	– 27.3	Ddit3, FOXA1, Foxa2, PEND, SFL1, Pax4
mir-100	chr24:3330846–3330906	– 20.1	Myf, Myc, EWSR1-FLI1, Foxd3, IRF1
mir-101	chr8:27164036–27164092	– 30.6	SP1, Pax5, NRG1, ESR1, TLX1, AZF1, MZF1
mir-122	chrZ:761057–761122	– 32.2	NFE2L2, TH12, AZF1, Pax5, TEAD1, Gata1, SFL1
mir-126	chr17:7748473–7748541	– 29.8	Myf, ESR1, Zfx, RAP1, RREB1, Spz1, INSM1
mir-146	chr4:88268261–88268339	– 42	Myf, RORA2, kni, CUP2, RREB1
mir-148	chr2:32253590–32253651	– 24.4	PLAG1, abi4, SP1, Pax5, TFAP2A, STP1, IME1
mir-193	Not mappable	NA	NA
mir-199	chr17:5034560–5034628	– 29.4	Myf, NRG1, INO2, TAL1, STP2, RUNX1, TEA1

NA data not available

while a single gene can be regulated by several miRNAs (Saxena et al. 2017b).

Role of several target genes or their families have been well established in heart development in animal model. For instance, inactivation of *BMPR2*, *TGFBRI*, *TGFB2*, and *TGFB3* has been associated with different subtypes of heart defects (Beppu et al. 2009; Langlois et al. 2010; Goishi et al. 2003). *HMGB3*, a member of HMG sub-family of HMG proteins, plays a crucial role in cardiac development (Monzen et al. 2008). Similarly, *VEGFC* governs the formation of cardiac outflow tract and maintains the proliferation of cardiomyocyte cells (van den Akker et al. 2012). BCL family mediates apoptosis, and a general phenomenon involved in both development and diseases (Vogler 2012). We also observed several target genes such as *MMP-2*, *-8*, *-11*, *-16*, *23* and *ADAM10*, *ADAM22* that regulate another crucial phenomenon involved in altering heart structure (morphogenesis), known as extra cellular matrix (ECM) remodeling. *MMP-2* is one of the most studied MMP involved in hypertension, hypertrophy, and fibrosis. Suppression of *MMP-2* leads to dysregulation in ECM remodeling causing hypertrophy and fibrosis (van Linthout et al. 2008). George et al. established matrix

metalloproteinase-2 (*MMP-2*) as an independent predictor of mortality in patients with chronic heart failure (George et al. 2005). The known spectrum of substrates for *MMP-2* contains not only collagens or elastin but also the contractile proteins such as troponin I and myosin 1, making *MMP2* a very important diagnostic and prognostic marker of heart failure (DeCoux et al. 2014). miR-20 identified in our study shows probable bindings with *MMP-2*, and, therefore, could be used as a possible tool to modulate *MMP-2* expression. Identification of transcription factors regulating miRNAs is as critical as the identification of miRNAs regulating gene expression (Ruffalo and Bar-Joseph 2016). We detected several potential transcription factors such as *MYF* (activate myogenesis), *FOXA1* (initiates DNA-protein binding), *GATA1* (erythroid development), *RREB1* (RAS-response), *PAX4* (Paired Box Gene), etc. (Table 1). Majority of these transcription factors play a very critical role in embryonic development and well as diseases (Wang et al. 2010a, b). Therefore, our target prediction raised the significance of further experimental validation of miRNA/mRNA interaction for determining the influence of each miRNA on target genes expression.

Fig. 2 Homologue of each chicken miRNA with known human miRNAs searched on miRBase database. All the differentially expressed miRNAs showed significant similarity with human miRNAs

***Gallus gallus* cardiac miRNAs showed nucleotide bias as well as length variation**

The percentage of the four nucleotides that appeared at each position of the identified miRNAs in *Gallus gallus* were analyzed (Fig. 4a). Uracil (U) was the most biased nucleotide in the first position (68.42%), followed by Adenine (A) (21.05%), while Cytosine (C) or Guanine (G) were the least frequently used nucleotides at the first position with 5.26%, each. Further analysis showed that U also had high frequency at the 10th, 19th, and 21th positions with percentages of 47.36% each and the lowest rates of 15.78% and 10.52% at the 2nd and 14th positions, respectively. The dominant bias towards U at these positions may contribute to the regulatory mechanism of the molecule. In addition, we

analyzed the first nucleotide bias in all identified miRNAs (Fig. 4b) and U was also the most abundant at the 5' end with a percentage of 33.33% in miRNAs with 21 nucleotides, 63.63% in miRNAs with 22 nucleotides, and 100% in miRNAs with 23 nucleotides. These results coordinated with the previous studies that miRNAs usually begin with a U at the 5' terminus (Lau et al. 2001) and are in accordance with the typical characteristic of animal miRNAs sequences (Huang et al. 2017).

The graph of length distribution of precursor miRNA (Fig. 5a) shows that majority of pre-miRNAs have length between 61 and 80 nucleotides followed by 51–60 nucleotides. Increased content of A and U bases in the sequence of pre-miRNA decreases the stability of the secondary structure, making it easier for RISC to synthesize mature

Fig. 3 Number of unique and common potential target genes of down-regulated (cluster-1) and up-regulated (cluster-2) and miRNAs obtained from Target Scan and miRDB databases

miRNA. Graphical analysis of length distribution of mature miRNA sequences (Fig. 5b) shows that most of the miRNA sequences were 22 nucleotides long (57.89%), in accordance with the standard size of animal miRNAs followed by 23 nucleotides (26.32%), whereas only 15.79% miRNAs have 21 nucleotides length (Samad et al. 2018).

Functional annotations with gene ontology and KEGG pathways

Cardiac development is a calibrated, timed, and complex process which includes multiple signaling pathways, biological processes, and their interactions. To determine the role of miRNAs in regulation of critical pathways, gene ontology (GO) and KEGG analysis were performed using

Fig. 4 **a** Nucleotide bias at each position of the identified miRNAs in *Gallus gallus*. U was the most biased nucleotide in the first position followed by A, C or G. **b** Bias of the first nucleotide in *Gallus gallus* cardiac miRNAs

Fig. 5 **a** Graph of length distribution of precursor miRNA. **b** Graphical analysis of length distribution of mature miRNA sequences

PANTHER as well as DAVID databases. PANTHER is a comprehensive software system which categorizes target genes to specific molecular, cellular, and biological processes (Mi et al. 2013). DAVID is a gene-oriented database that enriches the biological summary of a single gene, picks significantly enriched targets, and performs gene ontology (GO) as well as KEGG analysis. It also reveals the association of genes with diseases such as cardiovascular diseases and metabolic disorders (Huang et al. 2009a). GO analysis revealed that targets genes of both cluster-1 and -2 miRNAs were significantly overrepresented in more than 50 biological processes, 20 molecular functions and 25 cellular components with p values < 0.05 , and enrichment score of > 1.5 . The largest number of target genes regulated by both up- and down-regulated miRNAs was predicted to regulate critical biological, cellular, and molecular functions (Fig. 6). The biological functions include cellular, metabolic, and developmental processes, cellular functions include organelle, membranes, macromolecular complex, and extracellular matrix, and molecular functions include binding, catalytic activity, transporter activity, and signal activity. KEGG analysis revealed that for down-regulated miRNAs, there were several

significantly enriched pathways such as Rap1 signaling pathway, Ras signaling pathway, MAPK signaling pathway, cell cycle, mTOR signaling pathway, sphingolipid metabolism, and insulin resistance. Similarly, for up-regulated miRNAs, ECM–receptor interaction, PI3K–Akt signaling pathway, TNF signaling pathway, MAPK signaling pathway, TGF-beta signaling pathway, adrenergic signaling in cardiomyocytes, and insulin secretion were the most enriched pathways. All of the above pathways have been reported to be highly conserved and involved in diverse repertoire of biological events including cell proliferation, differentiation, metabolism, motility, and survival (Wang 2007; Rose et al. 2010; Proud 2004). Interaction network among miRNAs and intersected enriched KEGG pathways depicted the occurrence of extensive cross-talk which might suggest the roles of these miRNAs in cardiac development, function, and diseases (Fig. 7). Since gene interaction regulates health and diseases, we analyzed the gene co-expression networks of target genes associated with CVDs. We observed that the co-expression level of most genes was $> 50\%$ indicating their association with common regulating elements such as miRNAs. (Table S1). miR-9 has been found to suppress myocardin expression

Fig. 6 Gene ontology (GO) and KEGG analysis using PANTHER and DAVID databases. The y-axis shows classification of potential target gene under three GO terms (biological, cellular, and molecular functions) and x-axis shows the percentage of target genes involved in each function

Fig. 7 Interaction network among miRNAs and intersected significantly enriched KEGG pathways obtained from DAVID

that further induce the fetal cardiac gene expression and cardiac hypertrophy as compensatory mechanisms (Wang et al. 2010a, b). miR-20 is another developmental miRNA found to be a potential target against CVDs (Ai et al. 2016). Likewise, miR-92 and miR-126 have been established as efficient biomarker for detection of vulnerable CVDs patients (Niculescu et al. 2015; Khanaghaei et al. 2016). We observed significant association between miR-9, miR-18, miR-20, miR-19, miR-26, miR-27, miR-92, miR-100, miR-101, miR-126, miR-148, miR-193, and miR-199 with CVDs, metabolic disorders, and developmental diseases (p values < 0.05). Several cardiac marker genes such as *MHC*, *BRG*, *PARP*, *ANP* and transcription factors such as *GATA*, *MEF* also exhibit similar expression between fetal and failing heart (Nandi and Mishra 2015). The reason for switching of gene re-program is the difference in environmental conditions of heart cells. While a normal heart is exposed to a balanced nutrition accompanied by sufficient oxygen for digestion, a pathological heart under hypoxic, hypertrophic or atrophic conditions undergoes extensive remodeling in manner a fetal heart undergoes cardiac remodeling (Cox and Marsh 2014). We observed *GATA* to be a putative target of mir-27 and mir-126 while *MEF* to be a putative target of mir-9. Based on our findings and taking into account the previous studies, we may infer that a very small number of miRNAs have been identified and characterized for their role in cardiac development and CVDs until date. Differentially expressed miRNAs characterized in our study may certainly provide important clues regarding gene re-programming during disease conditions that might assist in development of cardiovascular therapeutics via epigenetic tools. Future studies are in progress

to validate the expression of candidate microRNAs and identifying their roles in cardiac hypertrophic conditions, where re-expression of fetal genes is witnessed.

Conclusion

We have validated and characterized a pool of nineteen fetal cardiac miRNAs through most sensitive and specific bioinformatics tools. We identified several miRNAs as potential epigenetic regulators in multi-level, cross-linked cardiac developmental network as well as CVDs. Gene ontology, KEGG, and Genemania analysis have shown that these epigenetic factors regulates critical cellular, molecular biological processes and pathways. Our initial in-silico findings are expected to provide valuable references for the future research of characterizing novel miRNAs for their role in cardiac development and as a new emerging epigenetic drug for plausible future cardiovascular therapeutics.

Acknowledgements This work was supported by the research grant awarded to Dr. Vibha Rani by the Department of Biotechnology, Government of India (BT/PR3642/AGR/36/709/2011) and by CSIR (Council of Scientific & Industrial Research, Government of India) File no: 09/1132 (0004)/18-EMR-I to Sharad Saxena. We acknowledge Jaypee Institute of Information Technology (JIIT), for providing the infrastructural support.

Compliance with ethical standards

Conflict of interest There is no conflict of interest between the authors.

References

- Abu-Issa R, Kirby ML (2007) Heart field: from mesoderm to heart tube. *Annu Rev Cell Dev Biol* 23:45–68
- Ai F, Zhang Y, Peng B (2016) miR-20a regulates proliferation, differentiation and apoptosis in P19 cell model of cardiac differentiation by targeting smoothed. *Biol Open* 5:1260–1265
- Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116:281–297
- Beppu H, Malhotra R, Beppu Y et al (2009) BMP type II receptor regulates positioning of outflow tract and remodeling of atrioventricular cushion during cardiogenesis. *Dev Biol* 331:167–175
- Bianchi M, Renzini A, Adamo S, Moresi V (2017) Coordinated actions of microRNAs with other epigenetic factors regulate skeletal muscle development and adaptation. *Int J Mol Sci* 18:840
- Cakstina I, Riekstina U, Boroduskis M et al (2014) Primary culture of avian embryonic heart forming region cells to study the regulation of vertebrate early heart morphogenesis by vitamin A. *BMC Dev Biol* 14
- Catalanotto C, Cogoni C, Zardo G (2016) MicroRNA in control of gene expression: an overview of nuclear functions. *Int J Mol Sci* 17:1712
- Chen J-F, Wang S, Wu Q et al (2008) Myocardin marks the earliest cardiac gene expression and plays an important role in heart development. *Anat Rec Integr Anat Evol Biol* 291:1200–1211
- Cox EJ, Marsh SA (2014) A systematic review of fetal genes as biomarkers of cardiac hypertrophy in rodent models of diabetes. *PLoS One* 9:e92903
- DeCoux A, Lindsey ML, Villarreal F et al (2014) Myocardial matrix metalloproteinase-2: inside out and upside down. *J Mol Cell Cardiol* 77:64–72
- Espinoza-Lewis RA, Wang DZ (2012) MicroRNAs in heart development. *Curr Top Dev Biol* 100:279–317
- Eyras E, Reymond A, Castelo R et al (2005) Gene finding in the chicken genome. *BMC Bioinform* 6:131
- George J, Patal S, Wexler D et al (2005) Circulating matrix metalloproteinase-2 but not matrix metalloproteinase-3, matrix metalloproteinase-9, or tissue inhibitor of metalloproteinase-1 predicts outcome in patients with congestive heart failure. *Am Heart J* 150:484–487
- Goishi K, Lee P, Davidson AJ et al (2003) Inhibition of zebrafish epidermal growth factor receptor activity results in cardiovascular defects. *Mech Dev* 120:811–822
- Huang DW, Sherman BT, Lempicki RA (2009a) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4:44–57
- Huang J, Min Lu M, Cheng L et al (2009b) Myocardin is required for cardiomyocyte survival and maintenance of heart function. *Proc Natl Acad Sci USA* 106:18734–18739
- Huang Y, Ren HT, Xiong JL, Gao XC, Sun XH (2017) Identification and characterization of known and novel microRNAs in three tissues of Chinese giant salamander base on deep sequencing approach. *Genomics* 109(3):258–264
- Khanaghaei M, Tourkianvalashani F, Hekmatimoghaddam S et al (2016) Circulating miR-126 and miR-499 reflect progression of cardiovascular disease; correlations with uric acid and ejection fraction. *Heart Int* 11:e1–e9
- Langlois D, Hneino M, Bouazza L et al (2010) Conditional inactivation of TGF- β type II receptor in smooth muscle cells and epicardium causes lethal aortic and cardiac defects. *Transgenic Res* 19:1069–1082
- Lau NC, Lim LP, Weinstein EG, Bartel DP (2001) An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* 294:858–862
- Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. *Cell* 115(7):787–798
- Li X, Yang Y, Wang L, Qiao S, Lu X, Wu Y, Xu B, Li H, Gu D (2015) Plasma miR-122 and miR-3149 potentially novel biomarkers for acute coronary syndrome. *PLoS One* 10(5):e0125430
- Liang Y, Ridzon D, Wong L, Chen C (2007) Characterization of microRNA expression profiles in normal human tissues. *BMC Genomics* 8(1):166
- Ludwig N, Leidinger P, Becker K et al (2016) Distribution of miRNA expression across human tissues. *Nucleic Acids Res* 44:3865–3877
- Martinsen BJ (2005) Reference guide to the stages of chick heart embryology. *Dev Dyn* 233:1217–1237
- Mi H, Muruganujan A, Thomas PD (2013) PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. *Nucleic Acids Res* 41:D377–D386
- Mishra S, Yadav T, Rani V (2016) Exploring miRNA based approaches in cancer diagnostics and therapeutics. *Crit Rev Oncol Hematol* 98:12–23
- Mok GF, Alrefaei AF, McColl J et al (2015) Chicken as a developmental model. In: eLS, pp 1–8
- Monzen K, Ito Y, Naito AT et al (2008) A crucial role of a high mobility group protein HMGA2 in cardiogenesis. *Nat Cell Biol* 10:567–574
- Nandi SS, Mishra PK (2015) Harnessing fetal and adult genetic reprogramming for therapy of heart disease. *J Nat Sci* 1:4–6
- Niculescu LS, Simionescu N, Sanda GM, Carnuta MG, Stancu CS, Popescu AC, Popescu MR, Vlad A, Dimulescu DR, Simionescu M, Sima AV, Jeyaseelan K (2015) MiR-486 and miR-92a identified in circulating HDL discriminate between stable and vulnerable coronary artery disease patients. *PLoS ONE* 10(10):e0140958
- Proud CG (2004) Ras, PI3-kinase and mTOR signaling in cardiac hypertrophy. *Cardiovasc Res* 63:403–413
- Rose BA, Force T, Wang Y (2010) Mitogen-activated protein kinase signaling in the heart: angels versus demons in a heart-breaking tale. *Physiol Rev* 90:1507–1546
- Ruffalo M, Bar-Joseph Z (2016) Genome wide predictions of miRNA regulation by transcription factors. *Bioinformatics* 32:i746–i754
- Rustagi Y, Jaiswal HK, Rawal K, Kundu GC, Rani V (2015) Comparative characterization of cardiac development specific microRNAs: fetal regulators for future. *PLoS One* 10(10):e0139359
- Samad AF, Nazaruiddin N, Murad AM, Jani J, Zainal Z, Ismail I (2018) Deep sequencing and in silico analysis of small RNA library reveals novel miRNA from leaf *Persicaria minor* transcriptome. *3 Biotech* 8:136
- Saxena S, Rustagi Y, Jain A, Dubey S, Rani V (2017a) microRNAs-mediated MMPs regulation: novel mechanism for cardiovascular diseases. *Proteases Hum Dis* 497–513
- Saxena S, Jain A, Rani V (2017b) MicroRNA-mediated MMP regulation: current diagnostic and therapeutic strategies for metabolic syndrome. *Curr Gene Ther* 17:214–227
- Shukla V, Varghese VK, Kabekkodu SP et al (2017) A compilation of Web-based research tools for miRNA analysis. *Brief Funct Genomics* 16:249–273
- Srivastava D, Olson EN (2000) A genetic blueprint for cardiac development. *Nature* 407:221–226
- van Linthout S, Seeland U, Riad A et al (2008) Reduced MMP-2 activity contributes to cardiac fibrosis in experimental diabetic cardiomyopathy. *Basic Res Cardiol* 103:319–327
- van den Akker NMS, Caolo V, Molin DGM (2012) Cellular decisions in cardiac outflow tract and coronary development: an act by VEGF and NOTCH. *Differentiation* 84:62–78
- Vogler M (2012) BCL2A1: the underdog in the BCL2 family. *Cell Death Differ* 19:67–74

- Wang Y (2007) Mitogen-activated protein kinases in heart development and diseases. *Circulation* 116:1413–1423
- Wang J, Lu M, Qiu C, Cui Q (2010a) TransmiR: a transcription factor-microRNA regulation database. *Nucleic Acids Res* 38:D119–D122
- Wang K, Long B, Zhou J, Li PF (2010b) miR-9 and NFATc3 regulate myocardin in cardiac hypertrophy. *J Biol Chem* 285:11903–11912
- Warris S, Boymans S, Muiser I, Noback M, Krijnen W, Nap JP (2014) Fast selection of miRNA candidates based on large-scale pre-computed MFE sets of randomized sequences. *BMC Res Notes* 7:34
- Wittig J, Münsterberg A (2016) The early stages of heart development: insights from chicken embryos. *J Cardiovasc Dev Dis* 3:12
- Wong LL, Armugam A, Sepramaniam S et al (2015) Circulating microRNAs in heart failure with reduced and preserved left ventricular ejection fraction. *Eur J Heart Fail* 17:393–404
- Zhou J, Dong X, Zhou Q et al (2014) MicroRNA expression profiling of heart tissue during fetal development. *Int J Mol Med* 33:1250–1260