

# MicroRNAs in congenital heart disease

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**Abstract:** Congenital heart disease (CHD) is a broad term which encompasses a spectrum of pathology, the most common phenotypes include atrial septal defects (ASDs), ventricular septal defects (VSDs), patent ductus arteriosus (PAD) and tetralogy of Fallot (TOF). The impact of CHD is profound and it is estimated to be responsible for over 40% of prenatal deaths. MicroRNAs (miRs) are small, highly conserved, non-coding RNAs which have complex roles in a variety of pathophysiological states. miRs are post-transcriptional negative regulators of gene expression. Individual miRs are known to exert effects in multiple target genes, therefore the altered expression of a single miR could influence an entire gene network resulting in complex pathological states. Recent evidences suggest a role in the dysregulation of miRs in CHD. Mouse knock out models have contributed to our knowledge base revealing specific patterns of miR expression in cardiovascular physiology and pathological states. Specific miRs necessary for embryonic cardiac development have been revealed. Dysregulation of these miRs has been shown to cause structural abnormalities in the heart and vasculature, thus furthering our understanding of the processes which result in CHD. These advances have provided new insight into the signalling pathways responsible for CHD. Furthermore, this new appreciation for miRs in the development of CHD has uncovered their potential for new therapeutic targets where modulated miR activity may reduce the burden of disease. Here, we summarize current knowledge of the cause-effect relationships of miRs in CHD and consider their potential as a therapeutic targets and biomarkers in this clinical setting.

**Keywords:** MicroRNAs (miRs); congenital heart disease (CHD); heart development; biomarkers

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## Introduction

The discovery of non-coding RNAs has provided new insight into the mechanisms that underpin human congenital and acquired diseases. This review will focus in microRNAs (miRs) and congenital heart disease (CHD).

miRs are small, evolutionally conserved, non-coding RNA molecules which have been shown to negatively regulate gene expression (1). Initially identified in animals they are now recognised to be widely distributed in the eukaryotic kingdom and are commonly found in vertebrates. It is estimated that in excess of 1,000 miRs are expressed

in humans Furthermore, bioinformatic analyses suggests that the miRs have the potential to regulate 30% of human genes through a series of complex signalling pathways (2). Moreover, miRs can co-ordinately regulate the stability of multiple target genes. Thus, aberrant expression of miRs can affect multiple intracellular signalling pathways and are associated with many diseases such as cancer, diabetes and heart disease (3-5).

Furthermore, miRs are now known to be key components to the embryonic development of the heart, normal cardiovascular function and cardiac pathophysiology in multiple cell lineages (6-12).

**Table 1** Forms and severity of congenital heart disease as categorised by EUROCAT (19)

Category	Description
Severity I	Single ventricle, hypoplastic left heart, hypoplastic right heart, Ebstein's anomaly, tricuspid atresia
Severity II	Pulmonary valve atresia, common arterial truncus, atrioventricular septal defects (AVSD), aortic valve atresia/stenosis, transposition of great vessels, tetralogy of Fallot, total anomalous pulmonary venous return, coarctation of aorta; without additional CHD anomalies classified as very severe
Other	Ventricular septal defect (VSD), atrial septal defect (ASD), pulmonary valve stenosis (PVS) without additional CHD anomalies classified as very severe or severe; VSD only: VSD without other cardiac or non-cardiac anomalies

A tabulised form of the congenital heart disease (CHD) as defined in the EUROCAT special report on CHD (19). There are a few subtypes of CHD, which are not included in the above table, but these are uncommon and are not included in the standard EUROCAT subgroups (19).

The Global Burden of Disease Study [2013] estimated that almost 30% of all deaths worldwide were caused by cardiovascular disease (13). Along the spectrum of adult cardiovascular diseases, congenital pathology is often the aetiology. Therefore, a new approach to the identification and treatment of CHD is necessary to reduce the prevalence of disease in young and old populations. Of all congenital malformations, CHD comprises of the majority of cases with a prevalence rate of 8 in every 1,000 infants (14). Over 40% of prenatal deaths can be attributed to CHD (15). The incidence of CHD has been associated with both increased neonatal and maternal morbidity. The prevalence of CHD varies widely and is more diffuse in Europe than Northern America (16,17). The Euro Heart Survey suggests that up to 19% of patients with CHD undergo surgery or a catheter-based intervention (18). Common CHD phenotypes include, atrial septal defect (ASD), ventricular septal defect (VSD), patent ductus arteriosus (PDA), tetralogy of Fallot (TOF), transposition of the great arteries (TGA), pulmonary valve atresia (PA), coarctation of the aorta (COA) and tricuspid atresia (TA) (see *Table 1* for a summary of CHD types). Furthermore, these phenotypes can also manifest in syndromic patterns with genetic associations, such as atrioventricular septal defects (AVSD) in patients with Down Syndrome (20).

This review will evaluate the evidence for the association of miRs in CHD, explore the cause-effect relationship in disease states and discuss their potential as therapeutic biomarkers.

### miR biogenesis: an overview

The following paragraph will be an overview of miR biogenesis, for more detail we recommended a reading

by Gama-Carvalho *et al.* (21), miR biogenesis begins with a long 5'-capped and poly A tailed, primitive form of miRNA (pri-miR) transcript configured into a hairpin structure which are derived from protein coding genes or independent non-coding transcriptional unit (22,23). These miR producing genes or are transcriptionally regulated like other protein coding genes but often contain polycistronic clusters.

Maturation of pri-miRs is initiated in the nucleus of a cell to produce precursor miR (pre-miR) which is transported to the cytosol or the endoplasmic reticulum to be cut into its mature form (approximately 22 nucleotides long) by Dicer, a RNase III endonuclease. Dicer activity is critical to miRNA biogenesis and impacts cardiac physiology. In an attempt to investigate the biological importance of miRNAs, mutation or disruption of Dicer has been employed by various groups as a broad method to prevent miRNAs production. Both *in vitro* and *in vivo*, evidence exists to support a role for Dicer-dependent miRNAs in vascular signaling and multi-system roles related to angiogenesis (24-28). Indeed, selective deletion of Dicer impacts the regulation of cardiac morphogenesis, electrical conduction, and cell-cycle control (8). In addition, dilated cardiomyopathy associated with heart failure, and spontaneous cardiac remodeling is found with the deletion of Dicer (29,30). More broadly, Suárez *et al.* excellently review the literature on miRs in the regulation of angiogenesis, with specific mention to dicer selective knockout models in cellular and animal models (31).

The mature miR is a single strand of RNA which has the potential to be recruited to the RNA-induced silencing complex (RISC), which also comprises Argonaute (Ago) proteins. In the RISC, the miR can repress the expression

**Table 2** MicroRNAs implicated in congenital heart disease

MicroRNA	Species	Congenital heart defect	References
miR-133a-1/miR-1-2; miR-133a-2/miR-1-1	Mice	VSD, chamber dilatation	Zhao <i>et al.</i> (8); Liu <i>et al.</i> (34), Catalucci <i>et al.</i> (9)
miR-1-1/miR-181c	Human cardiac tissue	VSD	Li <i>et al.</i> (35)
miR-92	Mouse embryos	VSD	Catalucci <i>et al.</i> (9)
miR-17-92 cluster	Mice	VSD	Ventura <i>et al.</i> (36)
19b, 29c	Human maternal blood	VSD	Zhu <i>et al.</i> (37)
let-7e-5p, miR-155-5p, miR-222-3p, miR-379-5p, miR-409-3p, miR-433, miR-487b	Human plasma	VSD	Li <i>et al.</i> (38)
miR-196	Foetal human heart samples	Cardiac septation, morphogenesis, valve formatio	Goddeeris <i>et al.</i> (39)
miR-99a, let-7c, miR-125b-b, miR-155, miR-802	Human DNA	Down Syndrome	Latronico <i>et al.</i> (40)
miR-19b, miR-22, miR-29c, miR -375, miR -421	Human maternal blood	TOF	Zhu <i>et al.</i> (37), O'Brien <i>et al.</i> (41)
miR-26a, miR-95, miR-30b and miR-141	Human aortic valves; porcine valvular interstitial cells	BAV	Nigram <i>et al.</i> (42); Yanagawn <i>et al.</i> (43)

List of microRNAs implicated in congenital heart disease (CHD). miR, microRNA; VSD, ventricular septal defect; TOF, tetralogy of Fallot; BAV, bicuspid aortic valve.

of its messenger RNA (mRNA) targets. Each miR has the potential to repress the expression of multiple genes. miR achieves this by first recognising a complimentary (or semi complimentary) “seed sequence” containing 8 nucleotides in the 5' untranslated region (5'UTR) to miR binding sites of the 3' untranslated region (3'UTR) of the target mRNA. Ultimately, the targeted mRNA repression can be achieved by mRNA degradation, transcript deadenylation, translation inhibition or sequestration of the mRNAs in the processing body (P-body) (32). miRs can also be released extracellularly and are present in virtually any biological fluid. In comparisons to mRNAs, miRs are more resistant to degradation because of several mechanisms of protection, for example their being engulfed within extracellular vesicles or conjugated to lipoproteins or Ago proteins (33). *Table 2* summarized the miRs so far implicated in development of CHD.

### Ventricular septal defects (VSD)

A VSD is a discontinuation in the septal wall dividing the left and right ventricles of the heart. VSDs may be present at birth or can be acquired after myocardial infarction.

VSDs account for approximately 20–40% of CHD but 80% of the surgical workload (33,44). Large defects may present with severe heart failure in infancy. However, small defects may remain asymptomatic. VSDs lead to a left to right shut of circulation producing left ventricular volume overload resulting in pulmonary hypertension (45).

MiR-1-1 and miR-181c have been implicated in the pathogenesis of VSDs (35). MiR-1 is a regulator of bone morphogenic protein receptor type II (BMP2) and gap junction protein alpha 1 (GJA1) while miR-181c can regulate sex determining region Y (SRY)-box 9 (SOX9). In human cardiac tissue with VSDs, elevated levels of GJA1 and SOX9 coincided with reduced expression of miR-1-1, and elevated miR-181c expression was associated with down regulation of BMP2 (35).

Over-expression of miR-1 plays a fundamental role in ventricular cardiomyocyte proliferation and prevents expansion of the ventricular myocardium (46). Hand2 (a transcription factor that promotes ventricular cardiomyocyte expansion) is a target for miR-1. In addition, this study showed that knockouts of miR-1 results in a reduced pool of proliferating ventricular cardiomyocytes mass in the developing heart (46). Furthermore, haplo insufficiency

of miR-1 or miR-133a is associated with an increased risk of VSD via a process of Hand2 and serum response factor (SRF) respectively (9). In addition to this, a reduction in miRNA-1 and miR-133 expression is associated with cardiac hypertrophy in murine models and human diseases associated with cardiac hypertrophy (9). Similarly, miR-92 deficiency is associated with VSDs in mouse embryos (9).

Targeted deletion of the *miR-1-2* gene in mice produces 50% embryonic lethality as a result of VSDs. The surviving *miR-1-2* homozygous mice exhibited a diverse range of phenotypes including, rapid dilation of the heart and ventricular dysfunction with of atrial thrombi (8). *MiR-133a-1/miR-1-2* and *miR-133a-2/miR-1-1* are myocyte enhancer factor (MEF)-2 dependant enhancers which have been shown to be activate in the linear heart tube during mouse embryogenesis and controls transcription in the cardiac chambers (34). Both *miR-133a-1/miR-1-2* and *miR-133a-2/miR-1-1* genes are expressed in the intraventricular septum and the ventricular myocardium (9,34). Interestingly, singular deletion of either *miR-133a-2* or *miR-133a-1* in mice does not result in pathology. However, combined deletion of *miR-133a-2* and *miR-133a-1* produced late embryonic and neonatal deaths due to VDS and dilatation of the cardiac chambers (47). Similarly, targeted deletion of the *miR-17-92* family of miRs results in neonatal lethality from lung hypoplasia and VSDs (36).

A Chinese study, investigated circulating miR in 20 patients with VSDs compared with 8 VSD-free controls (38). This group identified 1 miR significantly up-regulated (hsa-miR-498) and 7 miRs which were down-regulated in the VSD group (let-7e-5p, miR-155-5p, miR-222-3p, miR-379-5p, miR-409-3p, miR-433, miR-487b). Gene ontology analysis in this study suggested that right ventricle morphogenesis were the potential target of these miRs. Specifically, this group predicted NOTCH1, HAND1, ZFPM2, and GATA3 as mRNA targets of let-7e-5p, miR-222-3p and miR-433 (39).

Foetal human heart samples have been found to contain mir-196a at gestational age 12–14 weeks (48). Mir-196a is implicated in HOXB8-Shh signalling which is utilised throughout cardiac septation, morphogenesis and valve formation (48). Therefore, miR-196a dysregulation could have a role in the formation of atrioventricular septal defects (AVSDs) and cardiac valve dysfunction.

### Syndromic congenital heart disease (CHD)

In a population not affected by prenatal diagnosis, 40–60%

of babies born with Down syndrome have CHD (49,50). Down syndrome is characterised by a number of clinical signs and symptoms, it is often diagnosed via fluorescence in situ hybridisation (FISH) which demonstrates trisomy of chromosome 21. Down syndrome has now been linked to five miRs, including, miR-99a, let-7c, miR-125b-2, miR-155 and miR-802 (40). These miRs have been identified on human chromosome 21. In addition, these miRs have been found to be over-expressed in cardiac tissue of patients with trisomy 21 (40). Furthermore, DiGeorge syndrome which results from the deletion of critical region 8 on chromosome 22 (22q11.2) is responsible for the encoding a component of the RNA-induced slicing complex essential for miR biogenesis, leading to haploinsufficiency of this complex (51). Many patients with DiGeorge Syndrome have associated CHD. This association suggests that multiple miRs are implicated in this syndrome and that dysfunction of miRNA expression could contribute to a gene dosage sensitivity to this disease (51,52).

Embryological links between cardiac and neuronal-craniofacial defects exists at the molecular level and clinically. Deletions of *Dicer* in neural crest cells result in a range of sever cardio-facial-crest defects. These syndromes include Noonans Syndrome, DiGeorge Syndrome, LEOPARD syndrome, cardio-facio-cutaneous syndrome and Costello syndrome (53-55).

### Cyanotic congenital heart disease (CHD)

More recently, miRs have been investigated into the aetiology of cyanotic CHD (41). TOF is the most common form of cyanotic CHD and represents 5–7% of all CHD, with males and females equally affected (56-58). The term TOF describes the tetrad of (I) mostly large and non-restrictive VSDs; (II) an over-riding aorta; (III) right ventricular outflow obstruction; and (IV) right ventricular hypertrophy (59). TOF is now recognised as a spectrum of diseases which share similar intracardiac pathology. The exact cause of TOF is unknown. However, there is a growing understanding of the importance of 22q11 in its incidence. For example, Di George syndrome and velocardiofacial syndrome, both of which have 22q11 deletions, is frequently co-diagnosed in those with TOF (60).

O'Brien *et al.* identified an association with non-syndromic TOF, miRs and spliceosomal RNAs (41). This group identified 61 miRs to have significant changes in expression levels in children with TOF compared with normally developing children. Interestingly, the levels of

miR expression in children with TOF remained similar to those in the foetal myocardium. This group looked at gene expression critical to cardiac development and their correlation to miR expression in TOF myocardium. They found that in children with TOF, splicing variants were observed in 51% of genes critical to cardiac development. They identified 33 miRs which were significantly down-regulated in TOF myocardial tissue compared to the normally developing myocardium (41). Together these findings suggest central roles for miRNAs and their spliceosomal function in TOF.

Later this group identified an inverse correlation between the expression of miR-421 and SOX4 in patients with TOF. SOX4 is a key regulator of the Notch pathway, which has been implicated in cardiac function, suggesting that miR-421 is a potential contributor to TOF (61).

### Bicuspid aortic valve (BAV)

BAV are a leading cause of calcific aortic stenosis and insufficiency which results in a high prevalence of thoracic aortic aneurysms in this patient group, BAV is a common congenital cardiac defect which has a population presence of 1–2% (62).

Recently, Yanagawa *et al.* have identified distinct miR profiles a small cohort of human BAV leaflets in comparisons with control patients with a tricuspid aortic valve (TAV). This group identified 8 miRs which were up-regulated and 27 miRs which were down-regulated in patients with BAV, compared to patients with TAV. Most significantly, expression of miR-141 was down-regulated 14.5 fold in patients with BAV (43).

Nigam *et al.* further investigated the association of miRs and BAV (42). In this study, the authors investigated miR expression in aortic valve leaflets of patients with aortic stenosis and those with aortic insufficiency in nine patients undergoing aortic valve replacement. They were able to show that miR-26a and miR-195 levels were significantly reduced and miR-30b expression to be reduced by 62% ( $P < 0.06$ ) using quantitative reverse transcription-polymerase chain reaction. Following this they identified that human aortic valve interstitial cells treated with miR-26a or miR-30b mimics reduced miR levels of calcification-related genes, such as BMP2, alkaline phosphatase (ALPL) and SMAD1 and of SMAD3. Interestingly, aortic valve interstitial cells treated with miR-195 showed increased mRNA levels of calcification-related genes, specifically BMP2 and RUNX2.

### miR-mediated regulation in CHD

miR mediated signalling in the formation of CHD may include multiple pathways. Intracellular signalling activated by transforming growth factor beta (TGF- $\beta$ ) have a key role in cardiovascular development and specifically in cardiogenesis. Studies in both humans and animal models have indicated that altered TGF- $\beta$  activity results in a variety of CHDs including, double outlet right ventricle, septal defects and an overriding tricuspid valve (63,64). Although not essential for cardiac development, inactivation of the genes encoding the TGF- $\beta$  type 1 (TGFB1) or type 2 receptors (TGFB2) in cardiac myocytes leads to severe valvuloseptal defects (65,66). Interestingly, inactivation in cardiomyocytes was not shown to lead to obvious cardiac defects in embryos by this group. Transgenic evidence suggests that constitutively activated TGFB1 arrests cardiac development at the looping stage and results in ventricular hypoplasia (67). In addition, human genetic studies have supported the significance of altered TGF- $\beta$  signalling in CHD. For example, mutations in the genes encoding for TGFB1 and TGFB2 are associated with Marfan syndrome and Loeys-Dietz syndrome, both of which are implicated in CHD (68-71). Furthermore, there is evidence to show that mutations in TGFB2 and SMAD3 are associated with syndromic aortic aneurysms (72-74).

Peng *et al.* have shown that inactivated Dicer1 in mice at midgestation leads to severe myocardial wall defects (75). These mutant hearts display abnormal cell proliferation, apoptosis, and expression of contractile proteins. Expression of TGFB1 is up-regulated in mutant hearts and inhibition of TGFB1 reduces the defect observed in cardiomyocyte apoptosis. To add another layer of complexity, TGFB1 mRNA is regulated by multiple miRNAs at different stages of cardiogenesis (75-77).

In human cardiac tissue, Akt is highly expressed. Akt is a protein which is known to have critical application in the regulation of cardiac development including proliferation, metabolism, angiogenesis and survival through a process of phosphorylation of downstream substrates that control the apoptotic machinery (78-80). Akt mediated signalling is complex, and involves a system of miRs, PIWI (P-element-induced wimpy testis) interacting RNAs (piRNAs) and their associated proteins (78-83). In the embryonic heart Akt3 is highly expressed, whereas Akt1 is predominantly expressed in the adult heart (79).

As previously discussed, abnormal miR-155 activity is implicated in patients with Down Syndrome. A study

investigating miR-155 in human cardiomyocyte progenitor cells has showed that increased expression of miR-155 can inhibit necrosis. However, they observed that necrotic cell death was not induced by inhibiting endogenous miR-155. Their study also suggested that increased miR-155 levels did not impact the expression patterns of cell survival and apoptotic related genes. Therefore, miR-155 inhibits necrosis mediated by repressing the receptor interacting protein 1 (RIP1), but independently of the Akt pro-survival pathway (81).

Other miRs known to be implicated in CHD have also been linked to the Akt signalling pathway. For example, miR-92 is thought to activate the Akt pathway through inhibiting its negative regulator PHLPP2 (84). MiR-92 increases resistance to apoptosis and deficiency of miR-92 resulting in apoptosis, which may induce the formation of the VSD phenotype (84). The miR-17-92 cluster which is highly expressed in the murine myocardium may protect the heart by diminishing the apoptosis and alleviating ischemia (84). Furthermore, overexpression of MiR-1 targets Akt, via an insulin sensitive pathway which may be partially responsible for the formation of VSDs (85,86). MiR-26a and miR-22 targets PTEN leading to activation of Akt which may precipitate complex CHD, including TOF and BAV (87-89).

MiR mediated signalling is likely to be complex and driven by multiple factors (79). However, this evidence suggests that miR mediated signalling in the myocardium may provide critical information leading to novel therapeutic targets in CHD.

### miRs as a biomarker

MiRs are attractive clinical biomarkers as they remain stable in blood, urine and other biological fluids and evade RNA degrading enzymes (90-93). After using sequencing by oligonucleotide ligation and detection (SOLiD) sequencing to systemically screen maternal serum miRNAs, Zhu *et al.* hypothesised that miRs in the maternal serum could act as a candidate biomarker for the prenatal detection of foetal CHD in early pregnancy (37). This group studied 60 women in total (30 control women with normal pregnancies and 30 pregnant women who have foetuses with CHD) and identified four significantly up-regulated miRs (miR-19b, miR-22, miR-29c, miR-375) in mothers carrying foetuses with CHD. Sensitivity for these biomarkers ranged from 55.6–77.8% and specificity ranged from 66.7–88.9%. Furthermore, a combination of the four differentially expressed biomarkers was showed to be a more efficient marker for CHD detection. Of note, miR-

19b and miR-29c were significantly up-regulated in VSDs and all four miRs were up-regulated in TOF. Furthermore, miR-22 may be specifically upregulated in TOF. The results of this study are very important because they suggest that specific miR are associated with types of CHD, furthermore they explore the use of serum detection is a possible method for prenatal diagnosis. However, this idea is its infancy and there are certainly some limitations to this study regarding the sample size, huge heterogeneity of CHD and possibly variability within the mother populations themselves. Further research is required to accurately explore the possibility that miR can be used in the clinical practice for prenatal detection in CHD.

### Discussion

The aetiology of CHD is likely to be a multifactorial process with contributions from anomalous gene expression and processing, epigenetic factors and a variety of environmental factors. It is considered that miRs over and under expression and co-expression have specific and generalised effects on cell signalling pathways involved in CHD. Despite our expanding knowledge base of the genetic basis and signalling pathways involved in vertebrate cardiac formation there are still huge gaps that require further investigation.

Previous studies have identified a central role for miRs in embryonic cardiogenesis (e.g., miR-1 and mir-133-a/b). However, it is likely that miRs have multiple effects in embryology across different cell lineages and also in disease progression.

In light of recent advances in our knowledge base regarding miR expression and function in human and animal studies, there are still significant roles of miRs in physiology and pathophysiological process we have yet to discover.

It is hoped that a simple blood or urine test may be a novel diagnostic biomarker for the detection of CHD. miR detection from placental tissues from foetuses with CHD and from maternal peripheral blood suggests a role for serum biomarkers as an early way to detect such CHD. Measuring these abundant molecules in minimally-invasive tests on easily accessible maternal and children samples may provide highly specific and sensitive future role in the prenatal and postnatal detection of CHD.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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