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MicroRNAs in a hypertrophic heart: from foetal life to adulthood

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ABSTRACT

The heart is the first organ to form and undergoes adaptive remodelling with age. Ventricular hypertrophy is one such adaptation, which allows the heart to cope with an increase in cardiac demand. This adaptation is necessary as part of natural growth from foetal life to adulthood. It may also occur in response to resistance in blood flow due to various insults on the heart and vessels that accumulate with age. The heart can only compensate to this increase in workload to a certain extent without losing its functional architecture, ultimately resulting in heart failure. Many genes have been implicated in cardiac hypertrophy, however none have been shown conclusively to be responsible for pathological cardiac hypertrophy. MicroRNAs offer an alternative mechanism for cellular regulation by altering gene expression. Since 1993 when the function of a non-coding DNA sequence was first discovered in the model organism *Caenorhabditis elegans*, many microRNAs have been implicated in having a central role in numerous physiological and pathological cellular processes. The level of control these antisense oligonucleotides offer can often be exploited to manipulate the expression of target genes. Moreover, altered levels of microRNAs can serve as diagnostic biomarkers, with the prospect of diagnosing a disease process as early as during foetal life. Therefore, it is vital to ascertain and investigate the function of microRNAs that are involved in heart development and subsequent ventricular remodelling. Here we present an overview of the complicated network of microRNAs and their target genes that have previously been implicated in cardiogenesis and hypertrophy. It is interesting to note that microRNAs in both of these growth processes can be of possible remedial value to counter a similar disease pathophysiology.

Key words: cardiac hypertrophy, hypertrophic heart rat, microRNAs, apoptosis, cardiogenesis, biomarkers, anti-sense therapy.

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I. INTRODUCTION

The human body ages naturally and as it does biological changes occur which do not necessarily mean deterioration of health leading to death (Berzlanovich *et al.*, 2005). Rather, these changes may result in accumulated cellular damage in due course which makes an individual more susceptible to disease (López-Otín *et al.*, 2013). For instance, endothelial dysfunction due to hypertension, dyslipidaemia, smoking and oxidative stress can result in atherosclerotic changes in coronary vessels (Nabel & Braunwald, 2012). Similarly, cardiovascular risk factors such as insulin resistance, obesity, hypertension and behavioural risk factors including depression, smoking and physical inactivity can result in Alzheimer's disease and dementia (Barnes & Yaffe, 2011; Teipel, 2013).

Aging can be viewed from a biochemical perspective as well. Telomeres protect stem cells from chromosomal degradation, however with gradual decline of telomerase activity successive cycles of proliferation result in shortening of telomere length resulting in decreased proliferation and cellular aging (Flores & Blasco, 2010). In addition, several other molecular processes have been suggested to contribute to aging such as mitochondrial dysfunction, genomic instability, epigenetic modifications and progenitor cell depletion (López-Otín *et al.*, 2013).

Non-coding ribonucleic acids (ncRNAs) that in the past were regarded as merely 'junk' oligonucleotide sequences are now an entire research field of interest. MicroRNAs are one such set of ncRNAs that regulate gene expression primarily by binding to their complementary mRNA sequence and influencing gene expression (Esteller, 2011). Various families of microRNAs are thought to be conserved during evolution in humans and plants and have been shown to play an essential role in development, growth and maintenance (Cuperus, Fahlgren & Carrington, 2011; Wang *et al.*, 2011; Yi & Fuchs, 2011). Several studies have suggested that levels of microRNAs change with time, for example during pregnancy and in age-related neurodegeneration (Gilad *et al.*, 2008; Aw & Cohen, 2012). Thus researching microRNA-led regulation of gene expression with time is of importance in the understanding of mechanisms behind various acquired and heritable diseases.

One such group of disorders is adaptive cardiac hypertrophy. It can be a product of physiological or pathological influences on the structure and function of the myocardium including varied ventricular mass, measures of contractile proteins, degree of fibrosis, calcium handling and neuronal makeover (Hill & Olson, 2008). Cardiac hypertrophy is an increase in cardiac cell mass which can ultimately result in fatal cardiovascular events (Levy *et al.*, 1990; Ghali, Liao & Cooper, 1998). The Framingham Heart Study identifies left ventricle (LV) hypertrophy as the most important cardiovascular risk factor after age (Levy *et al.*, 1990). The adaptation occurs as a compensatory response to physiological or pathological stress on the myocardium which leads to growth of terminally differentiated cardiomyocytes (Grossman, 1980; Frey & Olson, 2003). In either case hypertrophy results in ventricular dysfunction but not all individuals exposed to the risk factors have the same degree of increase in cardiac mass (Gardin *et al.*, 1995). In addition, numerous clinical studies involving twins and families have given evidence of the role that heritable genes have in determining heart size (Mayosi, 2002).

The focus of this review is to describe the involvement of microRNAs in cardiac growth and development. In addition, the role of microRNAs will be discussed in the context of physiological and pathological cardiac hypertrophy. Lastly, the importance of microRNAs in modern-day medicine in relation to their value as tools of diagnosis and therapy of cardiac hypertrophy will be emphasized.

II. CARDIAC HYPERTROPHY: THE GOOD AND THE BAD

(1) Physiological *versus* pathological hypertrophy

As the human body ages, the heart goes through various physiological and pathological alterations. Post-neonatal cardiac growth and cardiac hypertrophy associated with pregnancy or regular athletic exercise are part of the natural growth process. On the other hand incidence of heart

failure increases with age primarily due to hypertension and coronary heart disease with associated left ventricular hypertrophy (Kannel & Belanger, 1991). Hypertrophic changes are a common denominator in the equation of cardiac function and have two forms: concentric and eccentric. Concentric hypertrophy can result from increased forced contractility particularly in response to higher peripheral resistance seen in hypertension or valvular stenosis and after excessive muscular exercise done by wrestlers and weight lifters (Maillet, van Berlo & Molkentin, 2012). Eccentric hypertrophy, by contrast, is seen chronically after an ischaemic episode, in dilated cardiomyopathy, during pregnancy and in endurance athletes (Maillet *et al.*, 2012).

There are some distinct features differentiating between physiological and pathological hypertrophy. An increase in apoptosis, necrosis, type I collagen deposition in the myocardium, up-regulation of foetal gene expression, down-regulation of genes encoding contractile protein, decreased fatty acid oxidation and enhanced glucose metabolism are hallmarks of pathological hypertrophy (Bernardo *et al.*, 2010).

microRNAs, not surprisingly regulate various biological pathways resulting in altered cardiac mass. The complex signalling pathways recognized to be involved in cardiac hypertrophy will be discussed later in this review along with the relevant microRNAs predicted to manipulate them.

(2) An animal model differentiating between physiological and pathological hypertrophy

The hypertrophic heart hat (HHR) is a rodent model that develops left ventricular hypertrophy independently of hypertension (Harrap *et al.*, 2002). The model was successfully developed by cross breeding the spontaneously hypertensive rat (SHR) with the Fischer (F-344) rat. A number of genes potentially associated with concentric cardiac hypertrophy were demonstrated to be altered in the HHR following microarray analysis (Dwyer *et al.*, 2008). For instance, DnaJ homolog subfamily A member 3 (*DNAJA3*) was overexpressed in young HHRs. *DNAJA3* encodes for Ras GTPase-activating protein binding protein (RasGAP) which has a central role in hypertrophic signalling (Trentin *et al.*, 2001). Similarly, guanine nucleotide binding protein gene (*GNG5*), *Rab12* – a Ras (rat sarcoma) oncogene family member – and mitogen activated protein kinase 1 (*MAPK1*) were overexpressed, these genes are involved in the Ras/mitogen-activated protein kinase (MAPK) signalling pathway that results in cardiac hypertrophy (Dwyer *et al.*, 2008). Four important genes from the microarray data were validated *via* real-time polymeric chain reaction (PCR) and suggested to be involved with cardiac hypertrophy (Dwyer *et al.*, 2008). These genes were: natriuretic peptide precursor type B (*NPPB*), a marker of left ventricular hypertrophy (Jaffe, Babuin & Apple, 2006), *MAPK1*, a downstream component of the Ras signalling pathway (Heineke & Molkentin, 2006), Tropomyosin 1 (*TPM1*), involved in myofibril assembly (McKeown *et al.*, 2014), and TRAF family member–associated NF-kB activator (*TANK*), activator of the pro-hypertrophic nuclear factor kB (*NF-kB*) signalling pathway (Cabal-Hierro *et al.*, 2014).

An interesting finding has come to light using the HHR model. It has been revealed that the number of cardiomyocytes in neonatal HHRs are fewer compared to the normal heart rats (NHRs) of corresponding age, resulting in smaller hearts at birth and compensatory hypertrophy as the rat ages with increased cardiac output (Porrello *et al.*, 2009). Further evidence points towards apoptosis and early terminal differentiation of cardiomyocytes with phosphatidylinositol 3 kinase-pyruvate dehydrogenase kinase, isoenzyme 1-protein kinase B (PI3K-PDK1-PKB/Akt) and MAPK signalling pathways being up- and down-regulated, respectively, in HHR neonates, possibly as a protective mechanism against apoptosis to prevent further reduction in cardiomyocyte numbers (Porrello *et al.*, 2009). The PI3K-PDK1-PKB/Akt signalling pathway has been well established as a positive modulator of physiological hypertrophy but has also been implicated in pathological hypertrophy (Bernardo *et al.*, 2010). An increased number of binucleated cardiomyocytes and decreased cellular proliferation was also noted indicating an early exit from the cell cycle (Porrello *et al.*, 2009). This previous research has shown that there is a disturbance in cell growth and altered apoptotic-signalling mechanisms that need further investigation.

III. MICRORNAS AT A GLANCE

It was initially believed that 18 microRNAs and their families make up more than 90% of all cardiac microRNAs in mice aged 6–8 weeks (Rao *et al.*, 2009). However, with recent improvements in deep sequencing technology and the ever-growing microRNA database, there has been an exponential increase in the number of microRNAs thought to regulate expression of cardiac genes (Hu *et al.*, 2012). From the list of common microRNAs (Rao *et al.*, 2009; Hu *et al.*, 2012), miR-1, miR-27b, miR-30e, let-7i and miR-208a were found to be differentially expressed during early cardiac hypertrophy in mice with transverse aortic constriction (TAC) (Hu *et al.*, 2012). Two of these important microRNAs are discussed later in this review, miR-1 (Section IV.1) and miR-208a (Sections IV.2 & VI.3).

Fig. 1 provides an overview of the many microRNAs that are believed to regulate both normal cardiogenesis and pathological cardiac hypertrophy as a result of cardiovascular risk factors and compensatory remodelling.

(1) Processing and function

The role of microRNAs was first described in *Caenorhabditis elegans* where silencing of mRNA *LIN-14* by *LIN-4* at various time points during growth resulted in normal development of the worm from an embryo (Lee, Feinbaum & Ambros, 1993). The microRNA was transcribed from the worm's own genome and hence it was suggested they have an

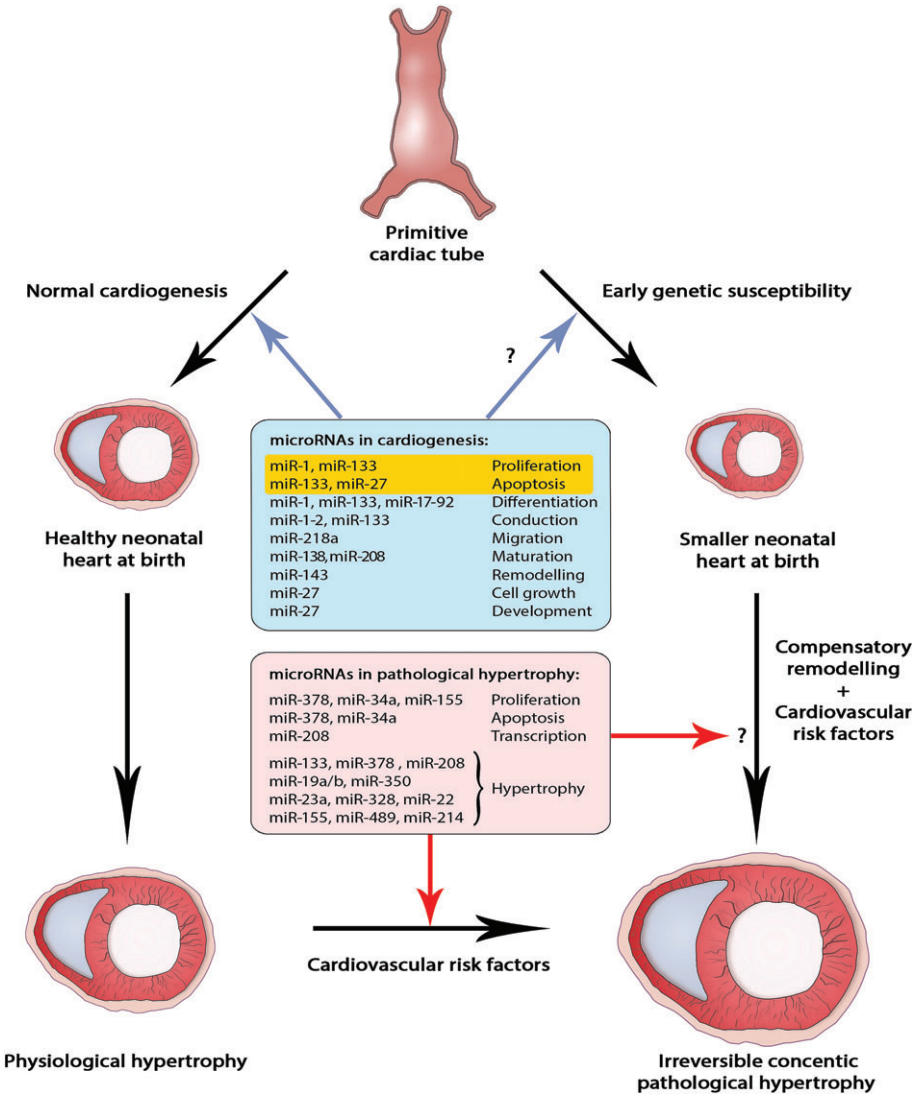

Fig. 1. Regulation of cardiogenesis and cardiac hypertrophy by microRNAs. During cardiogenesis, various cellular processes regulated by microRNAs result in the formation of a normal neonatal heart from a primitive cardiac tube. This is followed by physiological growth of the heart to compensate for an increase in demand for cardiac output. However numerous cardiovascular risk factors can result in variable expression of genes and microRNAs that lead to irreversible pathological hypertrophy. It has been suggested that dysregulation of certain genes during cardiogenesis results in apoptosis and premature exit of cardiac cells from the cell cycle. This leads to a reduced number of terminally differentiating cardiomyocytes in the heart of a neonate at birth, resulting in a smaller heart. The heart thus has to compensate by hypertrophic remodelling, predisposing an individual to cardiovascular disease, particularly hypertension with consequential irreversible concentric pathological hypertrophy. It is yet to be determined which microRNAs can result in a smaller heart with reduced number of cardiac cells at birth with consequential compensatory pathological hypertrophy. These microRNAs may be of interest for future research.

auto-regulatory function in gene expression, which is now widely accepted.

Most of the mammalian microRNA transcription sequences are part of introns of protein-coding transcripts but some are found in exons of non-coding regions as well (Rodriguez *et al.*, 2004). microRNAs are initially transcribed by RNA polymerases as longer molecules called pri-microRNAs in the presence of transcription factors similar to those involved in protein coding (Lee *et al.*, 2004; Borchert, Lanier & Davidson, 2006). Pri-microRNAs are cleaved into smaller sequences called pre-microRNAs by the protein Drosha, which are further cleaved by the protein Dicer into microRNAs (Knight & Bass, 2001; Lee *et al.*, 2003). A select group of folded introns with conserved splice sites have been identified that have a similar structure to pre-microRNAs, hence skip being cleaved by Drosha and are instead further processed by Dicer, thus suggestive of an alternative pathway of biogenesis for certain microRNAs (Ruby, Jan & Bartel, 2007). Then a hairpin loop that joins the complementary strands is removed by

RNA polymerase III and allows the RNA-induced silencing complex (RISC) to separate the two strands (Kim & Nam, 2006). Exportin-5, a protein of the nuclear membrane, transports the pre-microRNA out of the nucleus; knockdown of this protein results in accumulation of pre-microRNA in the nucleus (Yi *et al.*, 2003). Similar results can be seen in tumours with deficient expression of this protein (Melo *et al.*, 2010).

RISC further acts as a cofactor with the microRNA by forming a multi-protein RNA-silencing complex (Holley & Topkara, 2011). The complex binds to a complementary mRNA and suppresses the function of the corresponding gene(s) in several ways: blocking initiation and elongation, forcing premature termination of translation, deadenylation of mRNAs to prevent their reuse and most importantly degradation of the mRNA (Eulalio, Huntzinger & Izaurralde, 2008; Filipowicz, Bhattacharyya & Sonenberg, 2008). Most of the repression mediated by microRNAs is the result of mRNA decay due to perfect continuous pairing of the seed region found 2–8 nucleotides from the 5′ end of the microRNA that is critical for target specificity (Lee & Shin, 2012). If the target mRNA has adequate complimentary sequences the complex will cleave it, resulting in substantial repression of the mRNA, otherwise if there is an appropriate collection of complimentary sequences only translation will be blocked with demonstrable disruption of the target sequence leading to a lesser degree of repression (Bartel, 2004; Baek *et al.*, 2008).

(2) Fine tuning of microRNAs

MicroRNAs, being regulators of gene expression, are in turn regulated through a feedback-loop system that has components at different levels. RNA-binding proteins DiGeorge Syndrome critical region gene 8 (*DGCR8*) and thyroid hormone binding proteins (TRBP) critically interact with Drosha and Dicer, respectively, to modulate microRNA processing (Chendrimada *et al.*, 2005; Han *et al.*, 2009). Processing is also regulated by stimulatory pathways and accessory proteins such as LIN-28, p68 and p72 (Winter *et al.*, 2009; Davis-Dusenbery & Hata, 2010; Krol, Loedige & Filipowicz, 2010). In addition, core proteins of the microRNA-RISC complex, argonaute RISC catalytic component (AGO) and trinucleotide repeat-containing gene 6A protein (GW182) required for efficient mRNA silencing, are also targets of regulation along with several modulating factors (Winter *et al.*, 2009; Krol *et al.*, 2010; Braun, Huntzinger & Izaurralde, 2013).

Editing of the microRNA gene sequence by adenosine deaminases that act on RNAs (ADARs) inhibits Dicer-mediated cleavage with reduced production of mature microRNAs that results in more stringent loading of mature microRNA onto the RISC complex and may even redirect the complex to another target mRNA (Tomaselli *et al.*, 2013). Moreover, modifications at the 3′ end of a microRNA such as adenylation, uridylation or O-methylation can alter its stability in a positive manner (Krol *et al.*, 2010). Interestingly, even an increased cell density can result in more cell–cell interactions which increase the efficiency of Drosha and the RISC complex, thus up-regulating microRNA function (Hwang, Wentzel & Mendell, 2009). Lastly, in addition to the above-mentioned regulatory mechanisms there are auto-regulatory feedback loops in which the mRNA suppressed holds the code necessary for transcription factors required in biogenesis of the microRNA upstream (Siomi & Siomi, 2010). An example is the feedback loop that exists between miR-1 and insulin like growth factor 1 (IGF1) *via* PKB/Akt. miR-1 targets IGF1, which in turn down-regulates the pro-hypertrophic PI3K-PDK1-PKB/Akt signalling pathway, allowing forkhead family transcription factor Forkhead box O3a (FOXO3A) to increase expression of miR-1 (Elia *et al.*, 2009). Overexpression of PKB/Akt and FOXA3A in neonatal rat cardiomyocytes leads to decreased and increased expression of miR-1, respectively (Elia *et al.*, 2009).

IV. MICRORNAS REGULATE CARDIOGENESIS

Expression profiling of the developing heart has revealed that levels of mature microRNAs vary during important developmental milestones (Cao *et al.*, 2012). Comparison of the levels between normal and pathological states helps to identify the role of vital and relevant microRNA congenital anomalies such as the teratology of Fallot (O'Brien *et al.*, 2012).

The heart is the first organ to form and it is vital that the developmental process continues uninterrupted for normal morphogenesis and function. The primitive heart starts as a single tube derived from mesodermal cells and is called the primary heart field (PHF). It also functions as a framework for cells of the second heart field (SHF), with the two field cells interacting through several regulated complex signalling networks resulting in a mature four-chambered heart (Srivastava, 2006). During embryogenesis various cell types participate in a complex network of interactions with specific microRNAs concentrated in a particular cell giving it a unique functional identity (Small & Olson, 2011). Table 1 lists a number of microRNAs involved in mechanisms of cardiogenesis and their target genes, which are discussed below.

(1) miR-1/133a: major players in differentiation and proliferation

miR-1 is the first and best studied microRNA in cardiogenesis and is continually expressed from the eighth day (E.8) in embryonic mice (Cordes & Srivastava, 2009). In vertebrates miR-1 is transcribed with miR-133 as a cluster from a common polycistronic transcript (Chen *et al.*, 2005). Polycistronic transcripts have cistrons (loci) that are expressed together by the same transcription factor(s). It is suggested the locus of miR-1/133a duplicated during evolution to yield three related microRNA clusters; miR-1-1/133a-2, miR-1-2/133a-1 and miR-206/133b (Chen *et al.*, 2005).

Table 1. Many microRNAs play a vital role in regulating a range of biological processes necessary for normal progression of cardiogenesis. miR-1 and miR-133 target numerous genes and affect an array of cellular pathways such as differentiation and proliferation, which are fundamental processes during cardiac development. Other microRNAs are equally important as they regulate pathways that ensure the heart develops as a viable organ. + and − indicate a positive or negative regulatory effect, respectively, of the microRNA on a particular cellular pathway

Pathway	miR-1	miR-133	miR-1-2	miR-218a	miR-138	miR-143	miR-17-92	miR-499
Differentiation	+, Nkx2.5 +, α-MHC +, WNT +, FGF	+, Nkx2.5 +, α-MHC					+, ISL1	
Proliferation	−, HAND2	−, SRF +, NELFA −, CyclinD						
Apoptosis		−, SRF −, CyclinD −, Caspase9						
Conduction		−, KCNQ1 −, KCNH2	+, IRX5 +, KCND2					
Migration				+, ROBO1				
Maturation					+, CSPG2 +, NOTCH1B			+, MYH7B
Remodelling						+, ADD3		

Transcription of these clusters is regulated by serum response factor (SRF), myocyte enhancer factor 2 (MEF2) and a basic helix-loop-helix (bHLH) protein, myoblast determination (MYOD) (Zhao, Samal & Srivastava, 2005; Liu *et al.*, 2007).

miR-1 and miR-133 work in confluence to divert embryonic stem cell differentiation towards a mesodermal cell lineage instead of endodermal or neuroectodermal lineages by reducing the expression of Nkx2.5 and α-myosin heavy chain (α-MHC) (Takaya *et al.*, 2009). Nkx2.5 is a homeodomain factor which represses proliferation of cells in the SHF and outflow tracts (Prall *et al.*, 2007), whereas α-MHC encodes for contractile content in the heart after birth. On the other hand, the two microRNAs work in conflict during maturation of cardiomyocytes from mesodermal precursor cells with miR-1 fostering and miR-133 antagonizing the process (Ivey *et al.*, 2008).

Overexpression of miR-1 results in death at E13.5 in mice (Zhao *et al.*, 2005). The microRNA is suggested to induce early exit of cardiomyocytes from the cell cycle by limiting the function of heart and neural crest derivatives expressed 2 (HAND2), a bHLH protein required for growth of the right and trabeculation of the left ventricle (Zhao *et al.*, 2005). Overexpression of miR-1 also demonstrates the important regulatory role it plays in succession of the cardiac cell lineage. miR-1 promotes differentiation of embryonic stem cells as well as progenitor cardiac cells into cardiomyocytes instead of smooth muscle or endothelial cells by suppressing int/Wingless (WNT) and fibroblast growth factor (FGF) signalling pathways during early cardiogenesis (Lu *et al.*, 2013). By contrast, deletion of miR-1-2 from the hearts of mice resulted in fatal ventricular septal defects in late embryonic life in half the embryos, with the surviving mutant hearts exhibiting continuous mitosis whereas the adult heart undergoes only a single cycle of cellular division immediately after birth before exiting the cell cycle (Zhao *et al.*, 2007).

However, a study by Wei *et al.* (2014) demonstrated that absence of miR-1 did not result in the previously mentioned cardiac malformations and hence embryonic lethality, instead the mice developed chamber enlargement after P12 with all them subsequently dying by P17. A probable explanation for the later appearance of the fatal phenotype due to miR-1 suppression found by Wei *et al.* (2014) compared to Zhao *et al.* (2007) is a change in the gene-editing technology used that allows relatively more accurate snipping of target sequences with reduced silencing of nearby genes. However, miR-1 knockout mice suffer the same fate as their previous counterparts, indicating a different pathophysiology. A new target gene for miR-1 has been discovered, estrogen-related receptor β (ERRβ) that is responsible for induction of the foetal cardiac gene program, including cellular proliferation, glycolysis, glycogenesis and expression of sarcomere-associated genes (Wei *et al.*, 2014). It has been suggested that inhibition of glucose metabolism allows the embryonic heart to transit into the postnatal stage with β-oxidation of fatty acids as the predominant source of energy, in addition to expression of adult sarcomere/contractile proteins instead of foetal genes (Wei *et al.*, 2014). Therefore the role of miR-1 is crucial during cardiogenesis and in regulating cardiac cell proliferation, maturation and metabolism.

A similar disease-state overexpression of miR-1 can be seen in mutant mice when there is deletion of miR-133a-1 and miR-133a-2 that results in ventricular septal defects and dilated cardiac chambers (Liu *et al.*, 2008). The small percentage of mutant mice that do survive to adulthood eventually die of dilated cardiomyopathy and heart failure evident by increased cardiac markers of stress: myosin heavy chain 7 (*MYH7*), atrial natriuretic factor (*ANF*) and B-type natriuretic peptide (*BNP*) (Liu *et al.*, 2008). These knockout mice had up-regulated expression of SRF and cyclin D along with increased cardiomyocyte proliferation, disorganization of sarcomeres, fibrosis and apoptosis (Liu *et al.*, 2008). Absence of miR-133a in mice also activates smooth muscle genes due to lack of inhibition of SRF

resulting in less mature hearts as these genes are normally activated temporarily during heart tube formation (Liu *et al.,* 2008).

By contrast, overexpression of miR-133a inhibits proliferation leading to death from hypoplastic ventricular walls and septal defects (Liu *et al.,* 2008). However, it has also been reported that miR-133 promotes proliferation by inhibition of SRF (Chen *et al.,* 2006). A possible explanation suggested by Liu *et al.* (2008) is that SRF can be a positive and negative regulator of proliferation depending on the availability of suitable co-factors and external stimuli. Overexpression of the microRNA also inhibits stress-induced cardiomyocyte hypertrophy by targeting members of the Rho family – Ras homolog family member A (*RhoA*) and *Saccharomyces cerevisiae* S288c (*Cdc24*) – which encode for GTP-binding proteins involved in cytoskeletal and myofibrilliar rearrangement (Care *et al.,* 2007). miR-133a also targets negative elongation factor complex member A (*NELFA*), an RNA polymerase II regulator which plays a role in the activation of transcription of foetal cardiac genes such as myosin heavy chain 6 (*Myh6*), and *Myh7* (Care *et al.,* 2007). The three targeted genes under physiological conditions result in cardiac hypertrophy thus suggesting a delicate balance of cellular proliferation and growth mediated by miR-133a.

mir-133a is believed to affect several other cellular pathways. The microRNA prevents apoptosis possibly through down-regulating caspase-9, regulates extracellular matrix of the myocardium by targeting connective tissue growth factor (CTGF) and controls metabolism by targeting kruppel-like factor 15 (KLF15) which in turns limits glucose transport 4 (GLUT4) (Liu & Olson, 2010).

(2) Other significant microRNAs

mir218-a is believed to be involved in cardiac morphogenesis with overexpression resulting in inadequate heart tube looping, an uneven ventricle wall thickness and severally dysmorphogeneic atrial chambers which are sometimes merely string-like in structure (Chiavacci *et al.,* 2012). miR-218a and the host gene slit homolog 2 (*SLIT2*) are regulated by T-box transcription factor (TBX5) with microRNA expression directly proportional to transcription factor levels (Chiavacci *et al.,* 2012). miR-218a is underexpressed in very early stages of heart development but later allows passage of precursor cells within the heart fields by targeting roundabout axon guidance receptor homolog 1 (*ROBO1*) (Chiavacci *et al.,* 2012). Overexpression of the microRNA results in delayed migration and the above-mentioned cardiac defects whereas down-regulation did not affect cardiogenesis indicating that other microRNAs play a role in this complex process (Chiavacci *et al.,* 2012).

miR-138 has been implicated in ventricle development as well. It is expressed for a limited time during cardiac looping between 24 and 34 h post-fertilization in mice (Morton *et al.,* 2008). The microRNA allows maturation of ventricular cardiomyocytes by limiting synthesis of retinoic acid dehydrogenase, which disrupts the retinoic acid signalling pathway (Morton *et al.,* 2008). This in turn prevents expression of two genes involved in formation of the atrioventicular canal from spreading into the maturing ventricle: chondroitin sulfate proteoglycan 2 (*CSPG2*), which encodes for a cell adhesion protein versican, and Notch homolog 1b translocation-associated (*Drosophila*) (*NOTCH1B*) (Morton *et al.,* 2008).

Another microRNA, miR-143, has shown to be active in ventricular myocardial cells on the fifth day of embryonic life of zebra fish (*Brachydanio rerio*) where it represses the function of cytoskeletal F-actin capping protein encoded by adductin3 (*ADD3*) which is involved in rearranging F-actin allowing cytoskeletal growth and cellular remodelling (Deacon *et al.,* 2010). The microRNA can also be found in the heart tube of a zebra fish 36–48 h after fertilization where it is believed to be involved in regulating ventricular contractions (Deacon *et al.,* 2010). Deletion of the microRNA results in atrial dilatation and dysfunctional ventricles in fish (Deacon *et al.,* 2010).

A cluster of microRNAs collectively named miR-17-92 is activated downstream of the bone morphogenetic protein (BMP) signalling pathway and is believed to facilitate differentiation of cardiac precursor cells in the SHF by repressing a progenitor gene; insulin gene enhancer protein ISL-1 (*ISL1*) (Wang *et al.,* 2010*b*). Overexpression of the microRNA cluster affects development of the outflow tract whereas deletion results in fatal hypoplastic ventricular walls with septal defects (Wang *et al.,* 2010*b*). Moreover, miR-17-92 allows cardiac cells to proliferate at any age including during embryonic and post-natal development; overexpression of the cluster thus is protective against myocardial infarctions, whereas miR-17-92 knockout mice had evidence of compensatory cardiac cell hypertrophy with decreased numbers of cardiomyocytes (Chen *et al.,* 2013). *In vitro* experiments on rat neonatal cardiomyocytes revealed phosphatase and tensin homolog (*PTEN*) to be the target gene (Chen *et al.,* 2013).

During development of the heart, *MYH7* is transiently expressed until birth and encodes for a slow ATPase β-myosin heavy chain (β-MHC), then another protein α-MHC, which is a fast ATPase coded by *MHY6*, takes over as the predominant MHC in cardiac cells, this transition is mediated by circulating thyroid hormone (Morkin, 2000). In addition, these genes also express intronic microRNAs collectively called MyoMirs: miR-208a and miR-208b, encoded by *MYH6* and *MYH7*, respectively (Callis *et al.,* 2009). A third microRNA of this group, miR-499, encoded by *MYH7B* is believed to up-regulate β-MHC and allows ventricular maturation (Fu *et al.,* 2011). MyoMirs are expressed alongside their host genes thus regulate myosin content of cells and therefore effective muscle contraction (Liu & Olson, 2010). Hence the role of miR-208b is vital during cardiogenesis and thereafter to allow the required levels of myosin to develop for the contractile machinery. These microRNAs will be discussed further in Section VI.3.

microRNAs, as expected, have an important role to play in development of the cardiac conduction system as well. miR-1-2 knockout mice which survive the

embryonic period die of sudden onset arrhythmias with the electrocardiogram (ECG) showing bradycardia, a shortened PR interval and a widened QRS complex (Zhao *et al.*, 2007). A proposed mechanism is that a transcription factor Iroquois homeobox 5 (*IRX5*) is up-regulated which in turn down-regulates potassium voltage-gated channel shal-related subfamily member 2 (*KCND2*) which encodes for potassium channels (Zhao *et al.*, 2007). Similarly, miR-133a also targets genes encoding for potassium channels and associated subunits: potassium voltage-gated channel KQT-like subfamily member 1 (*KCNQ1*) and potassium voltage-gated channel subfamily H member 2 (*KCNH2*) with overexpression of the microRNA resulting in QT prolongation predisposing to arrhythmias and fibrillation (Cordes & Srivastava, 2009; Liu & Olson, 2010).

(3) Effects of microRNA global deletion

Another way to study the influence of microRNAs is to prevent their biogenesis in selective cells and analyse the compound effects of universal deletion. Inhibition of Dicer from epicardial cells in embryonic mice results in pericardial haemorrhage with an anomalous coronary vasculature by E18.5 (Singh *et al.*, 2011). Mesothelial progenitor cells mature into epicardial cells by E10.5 in mouse embryos, which further differentiate into various cellular types including vascular smooth muscle cells in a transformation process termed epithelial-to-mesenchymal transition (EMT) (Singh *et al.*, 2011). Absence of Dicer results in overexpression of E-cadherin and failure of zona occludens 1 (ZO-1), a tight junction protein, to move away from the cell surface thus keeping the cells adhered to each other and inhibiting EMT (Singh *et al.*, 2011). Additionally, a smooth muscle cell marker smooth muscle cell 22 (SMC22) is weakly expressed in the developing coronary vasculature further confirming failure of the transition process (Singh *et al.*, 2011). An abnormal vasculature can predispose to heart failure (Uysal *et al.*, 2014).

Cardiac-specific Dicer knockout mice die within 4 days of birth with histologically evident ventricular dilatation, disorganized sarcomeres and an altered measure of contractile protein expression (Chen *et al.*, 2008). In addition, cardiac function was severely diminished in these mice indicative of dilated cardiomyopathy with eventual heart failure (Chen *et al.*, 2008). Additionally, adolescent and adult mice with induced cardiac-specific Dicer deletion had an escalating increase in expression of foetal genes along with increasing myocardial inflammation, chamber remodelling, dilatation with myocardial hypertrophy, and decreasing cardiac function, all characteristics of progressive heart failure (da Costa Martins *et al.*, 2008). Similarly in a later study, deletion of cardiac Dicer was lethal in embryonic mice. These mice died by E13.75 due to ventricle septal defects associated with the aorta and pulmonary artery exiting from the right ventricle (Saxena & Tabin, 2010). Sema domain immunoglobulin domain (Ig) short basic domain secreted (semaphorin) 3c (SEMA3C) and its regulatory transcription factor were up-regulated in these mutant mice; they play a vital role physiologically in positioning outflow tracts through guiding axons of neuronal cells and promoting migration of neural crest cells (Saxena & Tabin, 2010). In addition, apoptosis is important for cardiogenic remodelling of outflow tracts and was diminished in these knockout mice compared to wild-type mice (Saxena & Tabin, 2010).

Another global Dicer knockdown investigation had similar results showing that murine neural crest cells destined towards cardiogenesis lead to cardiovascular malformations such as ventricular septal defects, great vessel outlets in the right ventricle and misplaced subclavian and carotid arteries (Huang *et al.*, 2010). Interestingly, migration patterns of the neural crest cells into the outflow tracts and cardiac fields from the third, fourth and sixth pharyngeal arches were affected instead of consequential apoptosis in the absence of Dicer (Huang *et al.*, 2010).

V. POSTNATAL HYPERTROPHY: REGULATION OF CELLULAR PROLIFERATION

It is of general belief that cardiomyocytes lose their regenerative and proliferative capacity at birth resulting in physiological hypertrophy, which is the hallmark of postnatal cardiac growth. However, it was revealed that neonatal rat hearts continue to proliferate up to 7 days following birth (Porrello *et al.*, 2011*b*). In addition, a population of cardiac progenitor cells called cardiac side population (CSP) have been identified which have the potential to differentiate into mature cardiac cells (Pfister *et al.*, 2005).

The miR-15 family has been shown to be up-regulated in mouse hearts between 7 and 14 days of age and represses expression of cell cycle genes, particularly checkpoint kinase 1 (*CHEK1*), which halts cardiomyocytes in the G2/M transition phase of the cell cycle (Porrello *et al.*, 2011*a*). The family includes miR-15a, miR-15b, miR-16-1, miR-16-2, miR-195 and miR-497 that are clustered on three different chromosomes with similarities in sequences of their mature microRNAs (Porrello *et al.*, 2011*a*). Overexpression of miR-195 in primary neonatal cardiac cells results in hypertrophy and binucleation with ventricular septal defects and cardiac hypoplasia seen in mice (Porrello *et al.*, 2011*a*). Alternatively, suppression results in increased numbers of cardiomyocytes undergoing a mitosis that was insufficient for the cells to entirely progress through the cell cycle, suggesting other regulatory mechanisms (Porrello *et al.*, 2011*a*).

Similarly, there are several cyclins and cyclin-dependent kinases (CDKs) particularly CDK2 and CDK6 that were shown to be down-regulated in 6-day-old postnatal cardiomyocytes and are targets of miR-29, miR-30 and miR-141 (Zhang *et al.*, 2013*b*). The microRNAs induce senescence in postnatal cardiomyocytes whereas suppression by anti-microRNA treatment in primary neonatal cardiomyocytes at 1 day of age resulted in increased expression of cyclin A2 (*CCNA2*) with more cells entering the cell cycle (Zhang *et al.*, 2013*b*). Moreover, expression of miR-141 along with miR-137 was also decreased in cardiac cells that had regained proliferative capabilities (Zhang *et al.*,

2010). Unfortunately a great deal of research is still required to fill in the gaps in our understanding of the role of microRNAs in physiological cardiac hypertrophy occurring as part of the normal growth process.

VI. MICRORNAS IN PATHOLOGICAL HYPERTROPHY

Pathological hypertrophy is a precursor to heart failure and can occur as a result of various stressors on the myocardium. Fortunately, cardiac hypertrophy can be induced in a laboratory setting by banding the aorta as it descends which forces the myocardium to adapt to an increase in peripheral resistance, or by stimulating cardiac cells using various biochemical agonists that trigger the hypertrophic signalling pathways. Much research is being done to understand the complexity of microRNA-led regulation of cardiac hypertrophy. Microarray analysis has revealed several microRNAs that are up- or down-regulated in the hearts of mice subjected to TAC and cultured rat neonatal cardiomyocytes treated with a hypertrophic agonist such as phenylephrine (PE) or transfected with recombinant adenovirus (van Rooij *et al.,* 2006; Sayed *et al.,* 2007; Tatsuguchi *et al.,* 2007). For instance, expression of miR-21 and miR-18b was down-regulated during hypertrophic changes whereas introduction of the microRNAs inhibited the remodelling process (Tatsuguchi *et al.,* 2007). Another study demonstrated that overexpression of miR-195 in transgenic mice results in cardiac hypertrophy (van Rooij *et al.,* 2006). Here we discuss some microRNAs that are of general interest and have the potential to be of therapeutic value. Table 2 provides a summary of the microRNAs involved in mechanisms of pathological hypertrophy and their target genes.

(1) microRNA-378

miR-378 has been implicated to have a role in cardiac hypertrophy through inhibiting the Ras signalling pathway. This pathway is activated by various receptors and associated ligands grouped under receptor tyrosine kinases (RTKs), including insulin-like growth factor receptor (IGFR), and G protein coupled receptor (GPCR), the ligand for which more importantly in hypertrophy is angiotensin II (Heineke & Molkentin, 2006). Two distinct pathways further mediate pro-hypertrophic signalling through Ras: (*i*) v-raf-1 murine leukaemia viral oncogene homolog 1 – dual specificity mitogen-activated protein kinase kinase 1 – extracellular signal-regulated kinase 1/2″ (Raf1-MEK1/2-ERK1/2) (ERK1 and 2 are also known as MAPK3 and 1, respectively); (*ii*) PI3K-PDK1-AKT. AKT inhibits glycogen synthase kinase (GSK)-3β, with both paths resulting in activation of nuclear factor of activated T-cells (NFAT) and transcription of cardiac hypertrophy genes (Proud, 2004; Heineke & Molkentin, 2006).

Table 2. Several microRNAs have an important regulatory role in the development of pathological cardiac hypertrophy by targeting several genes that encode for proteins, which are part of pathways downstream of hypertrophic stimuli. + and − indicate a positive or negative regulatory effect, respectively, of the microRNA on a particular cellular pathway

Pathway	miR-19a/b	miR-22	miR-23a	miR-34a	miR-133	miR-155	miR-208	miR-214	miR-328	miR-350	miR-378	miR-489
Hypertrophy	+, ATROGIN1	+, PTEN	+, FOXO3A		−, RHOA	+, SOCS1	+, THARP1	+, EZH2	+, SERCA2A	+, p38	−, GRB2	−, MYD88
	+, MURF1	+, PURB	+, MURF1		−, CDC24	+, JARID2	+, MSTN			+, MAPK8	−, IGFR1	
		+, SIRT1			−, NELFA					+, MAPK9	−, ERK2	
		+, HDAC4 +, CAV3			−, MYH6 −, MYH7					+, NFAT	−, KSR1	
Apoptosis	+, ATROGIN1			+, SIRT1						+, p38	+, IGFR1	−, MYD88
	+, α-CryB			+, ALDH2						+, MAPK8	−, CASP3	
	+, Bim			+, BCL2						+, MAPK9		
				+, PNUTS						+, NFAT		
Proliferation				−, SIRT1		+, JARID2					−, IGFR1	
				+, SMAD4								
Migration				+, SMAD4								
Transcription							+, MED13					

miR-378 blocks growth factor receptor bound protein 2 (*GRB2*) which plays an important role in activation of the Ras pathway by being recruited to the cell membrane and interacting with son of sevenless (SOS); levels of the protein were increased in patients with heart failure and in cardiomyocytes stimulated by PE (Nagalingam *et al.*, 2013). Transgenic mice with haploinsufficiency of *GRB2* had attenuated compensatory responses to TAC (Zhang *et al.*, 2003). Inhibition of Grb2 results in down-regulation of both mediating pathways, which was evident by decreased phosphorylation of ERK1/2 and p70S6 (a kinase regulating translation), decreased accumulation of NFAT in the nucleus and increased phosphorylation of AKT/GSK-3β (Nagalingam *et al.*, 2013). By contrast, inhibition of miR-378 induced expression of foetal genes resulting in increased levels of skeletal actin, NPPB and ANF in the presence of PE stimulation (Nagalingam *et al.*, 2013). Another study validated, in addition to *GRB2*, miR-378 targets *IGFR1, ERK2* and kinase suppressor of Ras (*KSR1*), a scaffold protein on which ERK2 activation relies (Ganesan *et al.*, 2013).

Synergistic to the function of the miR-15 family, miR-378 has also been implicated as a potential regulator of the cardiac cell cycle during postnatal life through repression of IGF1R from the seventh postnatal day onwards when expression of the microRNA is induced (Knezevic *et al.*, 2012). The IGF1R signalling cascade is associated with proliferation of cardiomyocytes during foetal life and DNA synthesis in neonatal cells, hence it is important for the growth and survival of cardiac cells (Kardami, 1990). A double feedback-loop arrangement exists between miR-378 and IGF1, hence postnatal induction of miR-378 can be a negative regulator of the cell cycle (Knezevic *et al.*, 2012).

The role of miR-378 in apoptosis is rather inconclusive. As previously mentioned, miR-378 functioned as a negative regulator of the cell cycle and induced apoptosis (Knezevic *et al.*, 2012) whereas another investigation has validated caspase-3 as a target of the microRNA and hence is protective of ischaemia-induced apoptosis in cardiomyocytes (Song & Yuan, 2012). Ganesan *et al.* (2013) observed that during *in vivo* experiments, miR-378 did not induce apoptosis, although it was observed in cultured cells suggesting that cells are more sensitive to apoptotic stimuli *in vitro*.

(2) microRNA-34a

miR-34a belongs to the miR-34 family, which also includes miR-34b and miR-34c. It has been investigated on numerous occasions to play a role in aging and cancer by regulating cellular proliferation and apoptosis. miR-34a can influence the cell cycle through suppression of many genes including sirtuin1 (*SIRT1*), a regulator of the cell cycle and senescence (Yamakuchi & Lowenstein, 2009). miR-34a expression can be induced by p53 as a result of oxidative stress and DNA damage, the microRNA in turn inhibits *SIRT1* which then allows p53 to remain acetylated resulting in arrest of the cell cycle and apoptosis (Yamakuchi & Lowenstein, 2009). Human aortic endothelial cells transfected with miR-34a precursor displayed a higher number of endothelial cells in the G1 phase of the cell cycle indicating senescence and decreased proliferation; in addition these cells had reduced growth (Ito, Yagi & Yamakuchi, 2010; Badi *et al.*, 2015). An interesting observation was that expression of miR-34a increased with age in untransfected cells and also in the hearts of 32-weeks-old mice compared to 4 weeks old which suggests the microRNA has a role in aging, characterized by increased apoptosis and decreased cell proliferation (Ito *et al.*, 2010).

Not surprisingly, another target identified is an anti-apoptotic gene aldehyde dehydrogenase 2 (*ALDH2*), suppression of which after a myocardial infarction (MI) and hypoxia-induced miR-34a expression resulted in increased apoptosis (Fan *et al.*, 2013). miR-34a levels in the serum of post-MI patients and rat models were further elevated establishing the importance of the role of this microRNA in cardiac dysfunction following ischaemia (Fan *et al.*, 2013). Increased apoptosis results in a hastened progression to dilated cardiomyopathy instead of compensatory hypertrophy with inevitable heart failure. Inhibition of *ALDH2* results in activation of the MAPK signalling pathway with increased activity of c-Jun N-terminal kinase (JNK) and p38 leading to apoptosis (Zhang *et al.*, 2011). A complex network of intercommunicating proteins is induced following activation of MAPK-mediated apoptosis, which is beyond the scope of this review.

Increased glucose levels can also induce apoptosis in cardiac cells, which may explain the cellular pathogenesis behind diabetic cardiomyopathy. H9c2 cells grown in high-glucose culture media had elevated levels of miR-34a expression with suppressed levels of yet another anti-apoptotic protein, B-cell CLL/lymphoma 2 (BCL2), resulting in apoptosis (Zhao *et al.*, 2013). Bcl2 has been widely investigated as having an important role in regulating apoptosis and is the target of other microRNAs such as miR-195, miR-24 and miR-365 (Singh & Saini, 2012).

Another study demonstrated that aged mice had elevated levels of miR-34a expression with increased cardiac fibrosis, hypertrophy, shorter telomeres and increased apoptosis whereas inhibition of miR-34a in 18-months-old mice resulted in a reduced number of dead cells and miR-34a knockout mice demonstrated reduced age-associated deterioration of cardiac function (Boon *et al.*, 2013). In addition, mouse models of induced acute myocardial infarction (AMI) with inhibition of miR-34a had improved recovery, evident by decreased fibrosis and improved cardiac contractile function (Boon *et al.*, 2013). The target identified and validated in this study was phosphatase 1 nuclear-targeting subunit (*PNUTS*), which has surfaced as an important component of the DNA damage response and positively regulates cell survival (Boon *et al.*, 2013; Loffredo, Pancoast & Lee, 2013).

Similarly, cardiac fibroblasts isolated from the AMI mouse model demonstrated induction of transforming growth factor β1 (TGF-β1) in response to ischaemia with increased expression of miR-34a which targets mothers against decapentaplegic homolog 4 (*SMAD4*) resulting in cardiac

fibrosis (Huang *et al.*, 2014). Activation of TGF-β1 transforms fibroblasts into cardiac fibroblasts and stimulated expression of miR-34a, which in turn inhibited SMAD4 and allowed collagen deposition by the TGF-β1 signalling pathway in a positive feedback loop (Huang *et al.*, 2014). Overexpression of miR-34a resulted in increased cardiac fibroblast proliferation and migration, indicating that the microRNA promotes cardiac fibrosis in response to a myocardial infarction (Huang *et al.*, 2014). In another study, SMAD4 knockout mice had cardiac hypertrophy, decreased contractile function and increased fibrosis as a result of up-regulated ERK1/2 and MEK1 expression (Wang *et al.*, 2005).

In relation to cardiac hypertrophy, it has been shown that inhibition of the miR-34 family and not miR-34a alone can improve the outcome in mouse models with pre-existing cardiac dysfunction and remodelling (Bernardo *et al.*, 2012). Mice subjected to a myocardial infarction developed hypertrophy and that were then treated with anti-miR-34 on day 2 after infarction had slower progression of ventricular wall remodelling (hypertrophy to dilatation, decreased atrial enlargement, lung congestion and fibrosis) at 8 weeks of age compared to control mice (Bernardo *et al.*, 2012). Similar results were observed in mice subjected to pressure overload *via* TAC that developed hypertrophy with subsequent systolic dysfunction at 5 weeks of age and were then treated with anti-miR-34 for a further 6 weeks (Bernardo *et al.*, 2012).

(3) MyoMirs: microRNA-208

Myo-microRNAs that were discussed in Section IV.2 play a vital role in switching expression of α-MHC to β-MHC. Stressful conditions and disease states such as hypothyroidism induce expression of β-MHC that consequently results in impaired cardiac contraction and function (Morkin, 2000). Overexpression of miR-208a results in concomitant expression of β-MHC and miR-208b whereas blocked expression has an opposite effect thus indicating the dominant role of miR-208a in this microRNA family (Callis *et al.*, 2009). miR-208a/b-led repression of thyroid hormone receptor associated protein 1 (THRAP1), a triidothyronine (T3) receptor co-regulator, and 'myostatin' allow the heart to undergo compensatory hypertrophy and fibrosis in response to stress stimuli (Callis *et al.*, 2009). Thyroid hormone promotes expression of several genes enhancing inotropic (contractile) function of the heart including sarco/endoplasmic reticulum Ca^{2+}-ATPase-2a (SERCA2A), α-MHC, β1-adrenergic receptor, Na^+/K^+-ATPase and K^+ channels while suppressing β-MHC (Li *et al.*, 2014*b*). Myostatin prevents cardiac hypertrophy by inhibiting AMP-activated kinase (AMPK) through the transforming growth factor-β-activated kinase 1 (TAK1); on the other hand, AMPK activation is seen in myocardial infarction, left ventricular pressure overload and cardiac hypertrophy (Biesemann *et al.*, 2014). Hearts of miR-208a transgenic mice were larger, had enlarged chambers with thick ventricular myocardium, increased cell size and poor cardiac function (Callis *et al.*, 2009). A previous study demonstrated that cultured cardiac cells under cyclical mechanical stress created by a vacuum induced TGF-β1, which in turn stimulated miR-208a and downstream β-MHC expression (Wang *et al.*, 2013).

This microRNA also delicately influences cardiac conduction; ECG recordings of miR-208a transgenic mice demonstrated an increased PR interval which indicates an atrioventricular (AV) heart block while loss-of-function mice had absent P waves suggesting atrial fibrillation (Callis *et al.*, 2009). A proposed mechanism for arrhythmias in the loss-of-function model of miR-208a is increased levels of transcription factor GATA-binding protein 4 (GATA4) with decreased expression of homeodomain-only protein (HOP) and subsequently connexion-40, a gap junction protein that allows propagation of electric activity (Callis *et al.*, 2009). miR-208 has also been shown to inhibit mediator complex subunit 13 (MED13) which is required for transcription of genes and can reduce expression of metabolic genes as explained earlier (Carrer *et al.*, 2012).

(4) microRNA-214

miR-214 has been shown actively to induce cardiac hypertrophy *in vivo* and *in vitro* by directly targeting enhancer of zeste homolog 2 (*EZH2*), a major histone methyltransferase belonging to the polycomb repressor complex 2 (PRC2) (Yang *et al.*, 2013). EZH2 allows normal growth of cardiac progenitor cells by stabilizing cardiac gene expression and down-regulating expression of skeletal muscle genes *via* suppressing a homeodomain transcription factor gene, sine oculis homeobox homolog 1 (*SIX1*), thus preventing hypertrophy of cardiac cells in response to stress (Delgado-Olguin *et al.*, 2012). miR-214 and *EZH2* expression levels were analysed in cardiac hypertrophy rat models with a constricted abdominal aorta and cultured cardiomyocytes treated with PE to induce hypertrophy. Results of both experiments demonstrated increased expression of miR-214 with decreased quantities of EZH2 mRNA and protein (Yang *et al.*, 2013). Overexpression of miR-214 in PE-treated cultured cardiac cells resulted in increased cell size and surface area along with elevated levels of hypertrophic markers actin alpha 1 skeletal muscle (ACTA1), MYH7 and natriuretic peptide A (NPPA), whereas suppression of the microRNA had the opposite effect (Yang *et al.*, 2013). Similarly, overexpression of miR-214 in transgenic mice resulted in hypertrophy at 3 months of age with sudden death between 6 and 12 months due to cardiac dysfunction including increased ventricular diameter, thin chamber walls and a progressively decreasing ejection fraction (Yang *et al.*, 2014). By contrast, suppression of miR-214 by an injectable complementary antagonist in mice with transverse aortic constriction resulted in a lesser degree of hypertrophy and associated fibrosis with unaffected cardiac function (Yang *et al.*, 2014).

(5) microRNA-19a/b

Another microRNA family, miR-19a/b that is part of miR-17-92 cluster, has been shown to target multiple

pathways that result in cardiac hypertrophy and induce apoptosis of cardiac cells during stress. Expression of an anti-hypertrophic gene muscle atrophy F-box protein (*ATROGIN-1)* during physiological conditions inhibits the calcineurin/nuclear factor of activated T-cells (CN-NFAT) signalling pathway through ubiquitin-mediated proteolysis and thus prevents cardiac hypertrophy (Li *et al.,* 2004). CN-NFAT is a pro-hypertrophic signalling pathway that is activated by cytosolic Ca^{2+} from the endoplasmic reticulum (Carreño *et al.,* 2006). ATROGIN-1 also targets charged multi-vesicular body protein 2B (CHMP2B), which is part of an endosomal sorting complex (ESCRT), thus promoting autophagy and preventing apoptosis (Zaglia *et al.,* 2014). Another protein, muscle ring finger protein 1 (MURF1) inhibits agonist-stimulated protein kinase C (PKC)-mediated signalling pathway by interacting with the receptor for activated protein kinase C (RACK1), whereas activation of this pathway leads to cardiac cell hypertrophy (Arya *et al.,* 2004). Overexpression of miR-19a/b results in suppression of *ATROGIN-1* and *MURF1* which in turn up-regulates associated pathways leading to increased cardiomyocyte size along with elevated levels of cardiac hypertrophic markers (Song *et al.,* 2014). In addition, miR-19a/b was found to promote proliferation of neonatal cardiac cells by targeting PTEN, a prohypertrophic and tumour suppressor gene, in conjugation with its role as a member of the miR-17-92 cluster (Chen *et al.,* 2013).

On the other hand, miR-19a/b up-regulates αCrystallin-B (*αCryB*) through activation of the CN-NFAT signalling pathway during agonist-induced stress on cardiomyocytes (Song *et al.,* 2014). αCryB is an anti-apoptotic gene and calcineurin-mediated activation is cardio-protective during cellular stress (Bousette *et al.,* 2010). Another pro-apoptotic protein 'Bim' was down-regulated by overexpression of miR-19a/b, which along with αCryB improved cardiac cell survival against endoplasmic reticular stress (Song *et al.,* 2014). In contrast to calcineurin activation of NFAT, stress-activated protein kinases (SAPKs), p38 and MAPK 8 and 9 (JNK 1 and 2), which are part of the MAPK signalling pathway, are negative regulators with evidence suggesting a balance in activation (dephosphorylation) and deactivation (phosphorylation) of the nuclear factor by the two signalling pathways (Molkentin, 2004).

(6) microRNA-350

Overexpression of miR-350 during later stages of the hypertrophic response to TAC was observed, leading to decreased expression of p38 and MAPK8/9 with increased translocation of dephosphorylated NFAT into the nucleus (Ge *et al.,* 2013). Cultured H9c2 cells 48 h after transfection with a miR-350 vector exhibited up-regulation of hypertrophic markers, a gradual change in morphology from spindle to polymorphic, and increased cell surface area, however at the same time they were also observed to shrink in size, suggestive of the apoptosis seen during the later stages of hypertrophy, dilated cardiomyopathy and heart failure (Ge *et al.,* 2013). These results suggest that miR-350 is expressed during the transition phase of cardiac remodelling in response to pressure overload, that is from hypertrophy to dilated cardiomyopathy and eventually death (Ge *et al.,* 2013).

(7) microRNA-23a

The CN-NFAT signalling pathway can trigger expression of miR-23a. In response to stressful stimuli, calcineurin-activated NFAT binds to the promoter region of miR-23a≈27a≈24-2 resulting in expression of miR-23a and subsequent cardiac hypertrophy (Lin *et al.,* 2009). miR-23a suppresses *FOXO3A*, which has a role in numerous cellular and biological process including cardiac hypertrophy (Wang *et al.,* 2012). FOXO3A induces transcription of an antioxidant enzyme catalase which neutralizes reactive oxygen species (ROS) resulting in down-regulation of myocardian (MYOCD), a cofactor for SRF, hence preventing hypertrophy (Tan *et al.,* 2008). Another target of miR-23a in addition to FOXO3A is MURF1, which as previously explained is an anti-hypertrophic protein and thus the microRNA can convey a hypertrophic signal downstream of stress-induced calcineurin activation (Lin *et al.,* 2009).

(8) microRNA-328

Another microRNA, miR-328, when overexpressed in transgenic models, cultured cells or mouse hearts with induced hypertrophy suppresses its target gene, *SERCA2A*, which in turn results in increased intracellular calcium and activation of CN-NFAT signalling pathway (Li *et al.,* 2014*a*). SERCA gene isoforms 1–3 levels gradually increase and then decrease with age with *SERCA2A* being the primary transcript during cardiogenesis and encoding for Ca^{2+}-ATPase pumps in the sarco/endoplasmic membrane that transport Ca^{2+} into the sarco/endoplasm resulting in muscle relaxation (Arai, Matsui & Periasamy, 1994). Thirty-week-old miR-328 transgenic mice display considerably hypertrophied hearts with shortened, hyper-contracted sarcomeres evident of increased intracellular calcium along with structural disorganization of myofilaments (Li *et al.,* 2014*a*).

(9) microRNA-22

miR-22 targets many genes involved in cardiac hypertrophy, cardiomyocyte survival and cardiac aging. miR-22 was shown to repress *PTEN*, which is a negative regulator of PI3K, resulting in activation of the pro-hypertrophic PKB signalling pathway evident by increased cell size and elevated markers of hypertrophy in cultured cells (Xu *et al.,* 2012). As expected, attenuation of miR-22 resulted in decreased hypertrophic changes in response to PE and angiotensin-II stimulation (Xu *et al.,* 2012). The absence of miR-22 also predisposes the heart to chamber dilatation and dysfunction during pressure overload with a rapid transition through the compensatory hypertrophic phase (Gurha *et al.,* 2012). The fast changeover is due to decreased suppression of purine-rich element binding protein B (*PURB*) that in turn

inhibits transcription of positive cofactors of SRF such as MYOCD and also through direct inhibition of cardiac genes that encode for sarcomeric/contractile proteins (Gurha *et al.,* 2012). Important genes regulated by SRF including *SERCA2A*, which as mentioned previously is responsible for Ca^{2+} handling in muscle cells, and *MHY7*, which encodes for the required myosin content during compensatory hypertrophy, were down-regulated (Gurha *et al.,* 2012).

In addition, miR-22 suppresses *SIRT1* and histone deacetylase 4 (*HDAC4*); a cofactor of the MEF2 transcription factor family (Huang *et al.,* 2013). Mice lacking miR-22 had dilated cardiomyopathy earlier than expected instead of a physiological hypertrophy in response to functional stress by various stimuli (Huang *et al.,* 2013). As expected, transgenic mice overexpressing miR-22 had hypertrophic hearts with increased SRF and decreased levels of caveolin-3 (*CAV3*), an inhibitor of hypertrophy (Gurha *et al.,* 2012).

Overexpression of miR-22 in cardiac fibroblasts and vascular smooth muscle cells led to suppression of mimecan, a secretory protein, which resulted in increased chemotaxis and senescence thus playing an important role in cardiac aging (Jazbutyte *et al.,* 2013). Expression levels of the microRNA showed a progressively upward trend with age and increased cardiac fibrosis later in life in conjugation with its role of targeting mimecan (Jazbutyte *et al.,* 2013). Thus, the role of miR-22 appears to be crucial in cardiac morphological makeover in reaction to stress with knockout mice progressing to dilated cardiomyopathy with characteristic cellular death and fibrosis instead of compensatory hypertrophy (Huang & Wang, 2014).

(10) microRNA-155

Interestingly, cardiac hypertrophy can also be induced by paracrine stimuli. Macrophages express high quantities of miR-155 in response to inflammation through Toll-like receptors (TLRs) which stimulates myeloid differentiation factor 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathways (O'Connell *et al.,* 2007). In the absence of miR-155 in macrophages, knockout mice had decreased cardiac inflammation, hypertrophy and dysfunction to pressure overload by TAC, whereas bone marrow transplant restored miR-155 function (Heymans *et al.,* 2013). In addition, ventricular cardiomyocytes allowed to flourish in growth media initially used to culture macrophages injected with miR-155 antago-microRNA showed attenuated growth suggesting cytokine involvement (Heymans *et al.,* 2013). The microRNA target identified was suppressor of cytokine signalling 1 (*SOCS1*) (Heymans *et al.,* 2013), which inhibits janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway-mediated hypertrophy and inflammation (Shi & Wei, 2012). It is believed that phosphorylated STAT3 in macrophages phosphorylates STAT3 in cardiomyocytes through paracrine signalling and induces hypertrophy (Heymans *et al.,* 2013).

Expression of miR-155 in cardiomyocytes was recently validated as opposed to previous views. Absence or inhibition of miR-155 in knockout mice resulted in decreased hypertrophy and cardiac remodelling with slow progression to dilated cardiomyopathy in response to TAC and transgenic calcineurin activation (Seok *et al.,* 2014). miR-155 targets jumonji AT-rich interactive domain 2 (*JARID2*) in cardiac cells (Seok *et al.,* 2014), which previously has been shown to interact with the retinoblastoma protein (Rb) to further suppress E2 transcription factors (*E2F*) function with consequently reduced expression of cyclin D1, cyclin D2 and Cdc2 in the cell cycle and hence decreased proliferation (Jung *et al.,* 2005). The miR-155-JARID2-cardiac hypertrophy axis needs further exploration.

(11) microRNA-489

miR-489 is equally expressed in all cardiac cell types and is anti-hypertrophic by targeting *MYD88*, an important adaptor protein in the TLR-4 mediated NF-kB activation pathway which contributes to the development of cardiac hypertrophy and promotes apoptosis (Ha *et al.,* 2006). *In vitro*, knockdown of the microRNA promoted cardiomyocyte hypertrophy after angiotensin II stimulation whereas overexpression led to reduced cell surface area and decreased expression of hypertrophic markers (Wang *et al.,* 2014). Similar results were seen in transgenic models overexpressing miR-489 and knockdown with antago-microRNAs (Wang *et al.,* 2014). miR-489 in turn is regulated by a long noncoding RNA (lncRNA) named cardiac hypertrophy-related factor (CHRF), which binds directly to the microRNA and is found in cardiac cells during angiotensin II-stimulated hypertrophy, in the hearts of mouse models with constricted aortas and in cardiac tissue from human patients with heart failure (Wang *et al.,* 2014). Thus the axis of CHRF-miR489-MYD88 has the potential for therapeutic application in which presence of a microRNA may improve cardiac function.

VII. DIAGNOSTIC AND THERAPEUTIC VALUE

(1) microRNAs are potential biomarkers

microRNAs can be found circulating in the plasma similar to protein biomarkers that may be specific to a disease or the underlying pathology (Reid, Kirschner & van Zandwijk, 2011; Siddeek *et al.,* 2014). microRNAs are protected from degradation by forming complexes with circulating proteins or inclusion into protective shells such as exosomes and apoptotic bodies (Gupta, Bang & Thum, 2010). Isolated RNA yields of microRNAs are low in the serum and currently the most sensitive method of detecting circulating microRNAs is reverse transcriptase real-time PCR followed by normalization to synthetic *C. elegans* or other endogenous circulating microRNAs (Gupta *et al.,* 2010). However expression levels of these endogenous microRNAs are variable among patients with different risk factors or after exposure to chemicals and drugs (Gupta *et al.,* 2010; Siddeek *et al.,* 2014). Hence the best method to normalize PCR data is arguable.

A number of cardiac microRNAs, including miR-1/133a and miR-208a, have altered serum levels in cardiovascular disease such as AMI, heart failure and coronary artery disease (Gupta *et al.,* 2010). Blood samples collected from rat models of AMI showed that levels of miR-1/133a and miR-208a peaked at 3–12 h and then decreased over the next 12 h with miR-208a being undetectable 24 h after onset; a similar pattern is observed in human patients with AMI (Wang *et al.,* 2010*a*). These trends mimic cardiac biomarkers already in clinical use and thus may have a diagnostic role in the future. Decreased miR-133a levels in the blood can also serve as a predictive marker of cardiac hypertrophy in patients on haemodialysis (Wen *et al.,* 2014).

Several microRNAs were found to have altered levels in the serum of patients with tissue rejection following a heart transplant: miR-10a, miR-31, miR-92a and miR-155; these may serve as non-invasive biomarkers, removing the need for tissue biopsies (Duong Van Huyen *et al.,* 2014). Similarly, miR-199a-5p, miR-27a and miR-29a can aid as potential prognostic markers in patients with hypertrophic cardiomyopathy to assess left ventricular hypertrophy and fibrosis (miR-29a only) (Roncarati *et al.,* 2014). Hypertrophic cardiomyopathy is a heritable disease that may go undetected for many years prior to sudden death and hence regular monitoring of these potential biomarkers may assist in the prevention of fatal events.

(2) Antisense therapy

Antisense therapy uses a gain-of-function or microRNA replacement strategy. The gain of function approach is more widely acceptable and uses synthetic complementary sequences to the microRNA, anti-microRNA oligonucleotides, that disallow formation of the RNA-silencing complex or induce degradation of the microRNA, thus permitting the target mRNA to continue with its function (Bader, Brown & Winkler, 2010). The other approach restores the function of a microRNA by introducing a synthetic mimic, plasmid DNA or cofactor/promoter stimulating agents that can then suppress the function of an undesirable or overexpressed gene (Zhang, Wang & Gemeinhart, 2013*a*).

Synthesizing chemically modified complementary sequences to the precursor or mature microRNA can treat a disease resulting from suppression of mRNAs by microRNAs (Weiler, Hunziker & Hall, 2006). The modifications are meant to increase target specificity and include addition of 2′-O-methyl, 2′z-O-methoxyethyl and 2′-O-modified (locked nucleic acid modified oligonucleotides) groups to the oligonucleotide (Weiler *et al.,* 2006). A study demonstrated the pharmacological characteristics of antagomirs through injecting anti-miR-122 and anti-miR-16 into rats and then analysing their properties in all the major organs. It was found that inhibition of miR-122 was dose dependent and that levels of the inhibitor were detectable until 23 days after injection (Krützfeldt *et al.,* 2005). Additionally, bioavailability of anatgomir-16 was abundant in all tissues except for the brain, possibly due to selective permeability of the blood–brain barrier (Krützfeldt *et al.,* 2005). Lastly, raised blood alanine aminotransferase (ALT) levels, which are an indicator of hepatotoxicity, were undetectable after treatment suggesting that the synthetic oligonucleotides are potentially safe for therapeutic use (Krützfeldt *et al.,* 2005). Delivery of a microRNA mimic or inhibitor is also crucial in gene therapy and can be broadly grouped as synthetic and viral delivery systems, both having their own sets of benefits and limitations. Synthetic delivery systems include liposomes, polyethylenimine (PEI)-based, dendrimers, poly(lactide-co-glycolide) (PLGA) particles and natural polymers like chitosan, protamine and collagen (Zhang *et al.,* 2013*a*). These synthesized particles have certain advantages over viral systems such as modifiable composition, easy synthesis and a reduced immune response (Zhang *et al.,* 2013*a*).

The use of synthetic oligonucleotides as mainstream tools for genetic therapy is inevitable with our continuing understanding of the function and downstream effects of microRNA-led regulation of cellular process and pathways.

VIII. CONCLUSIONS

(1) Aging of the heart is a continuous process with the journey starting from the embryonic formation of the heart tube until the eventual death of cardiac cells due to various pathological insults. Physiological cardiac hypertrophy is part of the normal growth process whereas pathological cardiac hypertrophy is a compensatory response to increased cardiac and circulatory stress that leads to expression of inherent myocardial genes. However, it is now believed that the compensatory response may also result from fewer terminally differentiated cardiomyocytes after birth for which the heart makes up by increasing cell size.

(2) The HHR is a unique model to study cardiac hypertrophy because myocardial remodelling is due to inherent activation of hypertrophic genes in the absence of any external stimuli.

(3) MicroRNAs are recognized to provide another layer of regulatory control on cellular processes. Several microRNAs have previously been investigated in hypertrophic hearts, which can be potentially used as diagnostic and therapeutic tools.

(4) It is necessary to continue investigating the various cellular pathways regulated by these microRNAs and others to understand cardiac adaptation better in the quest of finding that perfect microRNA or combination as the ultimate tool/s for genetic therapy.

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