The role of cardiac transcription factor NKX2-5 in regulating the human cardiac miRNAome

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MicroRNAs (miRNAs) are translational regulatory molecules with recognised roles in heart development and disease. Therefore, it is important to define the human miRNA expression profile in cardiac progenitors and early-differentiated cardiomyocytes and to determine whether critical cardiac transcription factors such as NKX2-5 regulate miRNA expression. We used an NKX2-5 eGFP reporter line to isolate both cardiac committed mesoderm and cardiomyocytes. We identified 11 miRNAs that were differentially expressed in NKX2-5-expressing cardiac mesoderm compared to non-cardiac mesoderm. Subsequent profiling revealed that the canonical myogenic miRNAs including MIR1-1, MIR133A1 and MIR208A were enriched in cardiomyocytes. Strikingly, deletion of NKX2-5 did not result in gross changes in the cardiac miRNA profile, either at committed mesoderm or cardiomyocyte stages. Thus, in early human cardiomyocyte commitment and differentiation, the cardiac myogenic miRNA program is predominantly regulated independently of the highly conserved NKX2-5-dependent gene regulatory network.

The heart is the first functional organ to develop in the human embryo, and the organ most commonly affected by disease in infants and adults. Heart development is tightly controlled by an evolutionarily conserved network of transcription factors and disruption of this network can result in a variety of congenital heart malformations. MicroRNAs (miRNAs), short (19–22 base pair) RNA regulatory molecules, add another layer of regulatory precision to reinforce core cardiac transcriptional networks. Furthermore, the cardiogenic regulatory framework is reinforced by the direct control of certain microRNAs by critical myogenic transcription factors, including serum response factor (SRF), myocyte enhancer factor-2 (MEF2c) and GATA4. In addition to their key roles in heart development, miRNAs are critical to maintaining cardiac tissue homeostasis and function. For example, global perturbation of the cardiac miRNAome via deletion of the microRNA processing enzymes Dicer and DGCR8 leads to dilated cardiomyopathy. In view of this, miRNAs emerged as a new class of functional regulators of cardiomyogenesis and heart disease. Given their important role in both heart development and function, careful compellation of the human cardiomyocyte miRNAome will facilitate studies in a number of areas in cardiac biology. Human pluripotent stem cells offer an opportunity to study the expression profile of miRNAs in human heart muscle cells.

Here, we have used a well-established NKX2-5 cardiac reporter line and loss-of-function model to determine whether the human cardiac miRNAome is directly dependent on NKX2-5, a transcriptional factor that is essential for mammalian heart development. We show that the cardiomyogenic miRNA program is activated early during human cardiomyocyte differentiation in vitro. The expression of the myogenic miRNAs, MIR1-1, MIR133A1, MIR208A and MIR499A, was established by day 10 of pluripotent stem cell differentiation into the cardiac lineage. However, NKX2-5 was dispensable for maintenance of the human cardiomyocyte miRNAome.
with no differentially expressed miRNAs identified in either cardiac committed mesodermal progenitors or immature differentiated cardiomyocytes. These data suggest that, in the main, establishing the cardiomyogenic miRNA program occurs independently of the highly conserved core NKX2-5 gene regulatory network.

**Results**

**Defining miRNA expression profiles in hESC derived human cardiac progenitors.** We utilized the NKX2-5\(^{−/−}\) hESC reporter cell line, in which enhanced GFP (eGFP) is expressed from the NKX2-5 locus\(^{19}\), to isolate hESC-derived cardiac mesodermal progenitors and cardiomyocytes by flow cytometry. Using a monolayer differentiation protocol\(^{22,23}\), GFP\(^+\) myogenic-lineages emerged between days 6 to 14 of the differentiation (Fig. 1a). To obtain a profile of miRNAs during early cardiomyocyte differentiation, cells were harvested and sorted into three populations: NKX2-5 negative mesoderm (day 6 GFP\(^{neg}\) cells), cardiac mesodermal progenitors (day 6 GFP\(^{+}\) cells) and early cardiomyocytes (day 10 GFP\(^{+}\) cells) for sequencing of small RNA species (Fig. 1b,c). A summary of small RNA sequencing data is shown in Supplementary Table 1. The average number of high-quality reads in the day 6 GFP\(^{neg}\), day 6 GFP\(^+\) and day 10 GFP\(^+\) populations were 2.01 \(\pm\) 0.10 \(\times\) \(10^6\) (n = 3), 1.71 \(\pm\) 0.08 \(\times\) \(10^6\) (n = 3), and 2.35 \(\pm\) 0.08 \(\times\) \(10^6\) (n = 3), respectively. Small nucleolar RNAs (snoRNAs) were also detected in day 6 GFP\(^+\) and GFP\(^{neg}\) populations (1.18 \(\pm\) 0.24 \(\times\) \(10^6\) (n = 3) and 3.19 \(\pm\) 1.7 \(\times\) \(10^6\) (n = 3), reads respectively) and day 10 cardiomyocytes (1.6 \(\pm\) 0.17 \(\times\) \(10^6\) (n = 3)). Thus, other small non-coding RNA populations such as snoRNAs are also present during human cardiac differentiation, but their functions are currently unclear.

Unsupervised hierarchical clustering indicated that the miRNA profiles of day 6 GFP\(^+\) (cardiac mesoderm progenitors) and GFP\(^{neg}\) populations were more closely related to each other than to day 10 cardiomyocytes (Fig. 1d and Supplementary Table 2). The similarity of miRNA expression profiles between the NKX2-5 positive and negative populations at day 6 most likely reflects the recent emergence of the NKX2-5\(^{+}\) cells from the pool of mesodermal cells. Differential expression analysis between day 6 GFP\(^+\) and GFP\(^{neg}\) cells identified seven miRNAs enriched in the GFP\(^+\) population (Fold change \(>2\) with adjusted p-value \(<0.05\)) (Fig. 1e; Supplementary Table 2). Among the seven overrepresented miRNAs, six have been reported to have important roles in murine cardiac development. For example, MIR133A1 and MIR133A2 play critical roles in promoting pre-cardiac mesoderm, while suppressing endodermal and neuroectodermal lineages\(^{24}\). We also observed higher levels of MIR125B1 in d6 cardiac progenitors, which plays a role in mesoderm development\(^{25}\) and regulates MEF2d in the HL-1 atrial myocyte cell line. Q-PCR of the cardiomyogenic miRNAs MIR1-1, MIR133A1 and MIR208A showed these were more highly expressed in day 6 cardiac mesoderm while MIR499 was not differentially expressed between GFP\(^{neg}\) and GFP\(^+\) cells (Fig. 1g,h). In addition, four miRNAs (MIR122, MIR126, MIR1247 and MIR127) were expressed at higher levels in GFP\(^{pos}\) mesodermal progenitors than GFP\(^+\) cells. (Fig. 1e; Supplementary Table 2). These miRNAs have been implicated in a wide range of non-cardiac processes such as liver homeostasis, hematopoiesis and self-renewal of cancer stem cells\(^{26-28}\).

To identify miRNAs that may be important drivers of cardiomyocyte differentiation, we compared the expression profile of day 6 and d10 GFP positive cells (Fig. 1d,f). This analysis identified a total of 112 differentially expressed miRNAs (Supplementary Table 2). Studies in the mouse have identified a core miRNA network involved in cardiac myogenic function\(^7\). The known myomiRs (miRNA-1,-1-2,133b and -208)\(^{9}\) were all expressed at higher levels at day 10 compared to day 6. Moreover, MIR499, a miRNA located within the intron of myosin heavy chain 7b (MYH7b), which encodes a cardiomyocyte sarcomeric protein, showed the highest differential expression (5.62 fold) in day 10 cardiomyocytes\(^{9}\). Q-PCR profiling of miRNA expression in day 10 GFP\(^+\) cardiomyocytes confirmed these key miRNAs are more highly expressed in cells committed to the cardiomyocyte lineage (Fig. 1h). MIR-1-1, MIR133A1, MIR208A and MIR145 expression levels are further upregulated in the day 10 NKX2-5\(^{pos}\)/GFP\(^+\) populations relative to day 6 populations. The data indicates that expression of canonical myogenic miRNAs is established early in cardiomyocyte differentiation.

A set of miRNAs that regulate DNA synthesis were more highly expressed in the mesoderm progenitor stage compared to day 10 cardiomyocytes (Supplementary Fig. 1). The Hippo-YAP pathway is an established driver of cardiomyocyte proliferation\(^{30-32}\) and miRNA regulators of this network are differentially expressed between day 6 mesodermal progenitors and the early cardiomyocyte cells at day 10 (Supplementary Fig. 2). For example, MIR30E, which is a known repressor of YAP1 expression\(^{13}\) is more highly (2 fold) expressed in day 10 cardiomyocytes. Conversely, MIR302a (2.2 fold) and MIR302e (2.5 fold) are found in the highly proliferative cardiac progenitor population but are lower in day 10 cardiomyocytes. This finding is consistent with the known function of the miR302–367 cluster in repressing the Hippo pathway components Mst1, Lats3 and Mob1b and, therefore, cardiomyocyte proliferation\(^{31}\). Thus, the NKX2-5\(^{+}\) positive day 6 population expresses a cohort of miRNAs consistent with the highly proliferative nature of this cardiac progenitor population.

**NKX2-5 is not required to establish the cardiac microRNAome.** In order to determine if NKX2-5 is required to establish the human cardiac miRNAome, we isolated cardiac progenitors and early cardiomyocytes from NKX2-5\(^{−/−}\) (NKX2-5\(^{−/−}\) ) hESCs\(^{19}\). Day 6 cardiac mesodermal cells and day 10 cardiomyocytes were isolated by flow cytometry and subjected to small RNA sequencing (Fig. 2a,b). Similar to the heterozygous NKX2-5\(^{+/-}\) cells, the day 10 NKX2-5\(^{−/−}\) cells differentially expressed miRNAs, including members of the myomiRs consistent with the cardiomyogenic cell phenotype (Fig. 2b; Supplementary Table 3). This result was confirmed by Q-PCR for the myomiRs MIR1-1, MIR133A1, MIR208A and MIR499. Strikingly, non-hierarchical clustering and principal component analysis demonstrated that day of differentiation was a larger discriminator between samples than NKX2-5 genotype (Fig. 2c,d; Supplementary Table 3). The canonical cardiac miomyRs, such as MIR1-1, MIR126, MIR133 and MIR208, were unperturbed in NKX2-5 knockout cardiomyocytes (Fig. 2e,f,g). Further, even miRNAs with proximal NKX2-5 binding sites\(^{48}\), such as MIR138-2 were not dysregulated (Fig. 2g). Indeed, we did not identify any miRNAs that required NKX2-5 activity in day 10 cardiomyocytes (Fig. 2e,g) for appropriate expression.
**Figure 1.** MicroRNA expression profiling of NKKX2-5 positive cardiac progenitors and cardiomyocytes. (a) Epifluorescent images of differentiating NKX2-5 expressing cells (Scale bar, 100 µm). (b) Percentages of GFP positive and negative cells at day 6 and 10 of differentiation. Data shows mean ± SEM (n = 3) determined by flow cytometry. (c) Flow cytometric purification of cardiac progenitors and cardiomyocytes based on eGFP expression from the NKX2-5 locus. Percentage of cells are indicated on plots. (d) Heat map of unsupervised hierarchical clustering of microRNA sequence profile showing that samples cluster according to day of differentiation (log2 RPM values). (e) Dot plot representation of miRNA-seq absolute expression (log2 RPM values) for miRNAs at day 6 of differentiation. Dashed lines mark 2-fold differential expression level. (f) Dot plot representation of miRNA-seq absolute expression (log2 RPM values) comparing day 6 and day 10 GFP positive cells. Dashed lines mark 2-fold differential expression level. (g) Q-PCR profiling supports miRNA sequencing data and confirms upregulation of canonical myogenic miRNAs in GFP+ cells at day 6. (h) Q-PCR of myomiRs demonstrates they are more highly expressed in day 10 cardiomyocytes than day 6 GFP+ cells.

**Discussion**

Using differentiating human pluripotent stem cells as a model we describe the miRNAome of pre- contractile cardiac progenitor cells and contracting cardiomyocytes. Emerging NKX2-5 positive cardiac progenitors are capable of giving rise to three cell lineages, namely cardiac muscle, endothelial and smooth muscle cells. The microRNA profile of these cardiac progenitors is similar to NKX2-5 negative mesoderm, which likely reflects the recent separation (less than 24 hours) of these lineages. However, the canonical cardiac miRNAs (referred to
as the myomiRs, are upregulated in the NKK2-5 positive day 6 population, consistent with the eventual differentiation of these cells toward cardiomyocytes. By day 10 of differentiation the cardiomyocytes had upregulated 112 microRNAs, many of which had been previously identified as key drivers of myocardial function in the mouse. This increase may be a reflection of phenotypic complexity occurring as cardiac progenitors mature into functional cardiomyocytes and require the on-going regulation of the definitive cardiomyocyte gene expression program.

A highly conserved network of transcription factors drives both cell specification and spatio-temporal patterning during heart development. At present, our understanding of the regulatory relationships between cardiac transcription factors and the cardiac miRNAome is limited, though systematic profiling provides a platform to further define these linkages. Given its vital role in heart development, we hypothesized that the transcription factor NKK2-5 was necessary for the regulation of the cardiomyogenic microRNAs. Here we searched for NKK2-5 dependent microRNAs and, surprisingly, found that no microRNAs were differentially expressed between NKK2-5 heterozygote and null cardiomyocytes. For example, in immature human cardiomyocytes in vitro, NKK2-5 is not required to up-regulate microRNAs, such as miR-1, that have been identified to play important roles in mouse heart development. Therefore, the phenotype observed in NKK2-5 null cultures is not due to perturbed microRNA expression but rather the disruption of the cardiac myogenic transcriptional network, including HEY2.

Figure 2. NKK2-5 is dispensable for establishing the appropriate human cardiomyocyte microRNA profile. (a) Flow cytometric purification of NKK2-5 null (NKK2-5−/−) cardiac progenitors and cardiomyocytes based on eGFP expression from the NKK2-5 locus. Percentage of GFP+ cells shown on plots. (b) Dot plot representation of miRNA-seq expression (log, RPKM values) for microRNAs in NKK2-5−/− GFP positive cells at day 6 and day 10 of differentiation. Dashed lines mark 2-fold differential expression level. (c) Q-PCR profiling supports miRNA sequencing data and shows upregulation of canonical myogenic microRNAs during differentiation in NKK2-5 null cells. (d) Multidimensional scaling plot of miRNA expression profiles from differentiating NKK2-5 heterozygote cells (GFP+, GFP+) and NKK2-5 null (d6 or d10 GFP+) at day 6 and 10 of differentiation. (e) Heat map of unsupervised hierarchical clustering of microRNA sequence profile (log2 RPM values). (d,e) show that samples cluster according to day of differentiation and not NKK2-5 genotype. (f) Scatter plot of miRNA-seq expression (log, RPM values) for microRNAs at day 6 of differentiation from NKK2-5−/−GFP+ and NKK2-5−/− cells showing no significantly differential expressed microRNAs. (g) Scatter plot of miRNA-seq absolute expression (log, RPM values) for microRNAs at day 10 of differentiation from NKK2-5−/−GFP+ and NKK2-5−/− cardiomyocytes showing that no microRNAs are NKK2-5 dependent. Yellow dot denotes MIR138-2.
Thus, it is possible that the key developmental transcriptional networks driving cardiomyogenesis do not directly establish the miRNAome and that early stage heart development can tolerate miRNA dysregulation.4

A conditional knockout of Dicer, which results in a blockade of miRNA production, using Nkx2-5 as a driver of Cre recombinase resulted in embryonic lethality at 12.5 days post-coitum (dpc), with the embryos showing pericardial edema and poorly developed ventricular myocardium.39 However, the early stages of cardiomyogenesis were not disrupted in Nkx2-5 driven Dicer knockouts with embryos at 11 dpc having normal appearance.38 These findings suggest that cardiac microRNAs have restricted, non-critical roles early in heart development, in contrast to the essential roles that transcription factors within the cardiac gene regulatory network play. Furthermore, multiple studies implicate cardiac miRNAs in the pathophysiology of stress responsive cardiac hypertrophy.40–44 Taken together these data suggest miRNAs have key roles in cardiomyocyte function and physiology, possibly as regulators of stress-responsive gene expression programs, rather than development. In this context, human pluripotent stem cell models provide an avenue to investigate the role of miRNAs in the pathogenesis of human myocardial disease and assess efficacy of miRNA-based therapeutics in a pre-clinical setting.

A caveat to this study is that differentiating human pluripotent stem cells lack the precise spatio-temporal cues present in the embryo. Therefore, it may be that the miRNA sequence profiles presented here do not fully reflect those found in the developing human foetus. In addition, this study focuses on very early stages of cardiogenesis and the regulatory role of NKX2-5 may change over developmental time. A second limitation is that miRNA expression may be perturbed in Nkx2-5<sup>−/−</sup> cardiomyocytes, however, we have previously demonstrated that Nkx2-5 heterozygosity does not impact cardiomyocyte differentiation at this early stage.4,45 It is possible that later in cardiomyocyte development Nkx2-5 is important for maintaining the appropriate mix of miRNAs in the heart. Nevertheless, our data suggest that in the early stages of human heart development the miRNAome does not require Nkx2-5. Conversely, other key regulators of cardiac muscle development such as MEF2c, TBX5 and GATA4<sup>−/−</sup>, which are not disrupted in Nkx2-5 null cardiomyocytes,45 may have a role in regulating cardiomyogenic miRNAs. Studies focussed on the regulatory nexus between the cardiac gene regulatory network,19,47,48 and the miRNAome in the human context will be required to fully understand the transcriptional regulation of the cardiac microRNA network.

Methods

Ethical approvals. All experiments were approved by the Royal Children’s Hospital Research Ethics Committee (HREC 33001 A). Methods were carried out in accordance with the relevant guidelines and regulations provided by National Health and Medical Research Council (National Statement on Ethical Conduct in Human Research).

Cell culture and cardiac differentiation. All cell culture reagents were purchased from Thermo Fisher unless stated otherwise. HES3 Nkx2-2<sup>−/−</sup> and Nkx2-2<sup>−/−</sup>/Nkx2-5<sup>−/−</sup> cells were routinely passaged using TrypLE Select and maintained on tissue culture flasks, as previously described.41 To induce differentiation, hESCs were dissociated into single-cell suspension using TrypLE Select and seeded onto Geltrex coated (1:100 dilution) culture plates at 2.5 × 10<sup>4</sup> cells/cm<sup>2</sup> in basal differentiation media consisting of RPMI (Thermo 61870), B27 minus vitamin A (Thermo 12587) and 50 µg/ml ascorbic acid (Sigma A92902), further supplemented with 10 µM CHIR99021 (Tocris Bioscience 4423) and 80 ng/mL Activin A (Peprotech). Following 24 and 96 h of induction, media was replaced with basal media supplemented with 5 µM IWR-1 (Sigma I0161) and from day 5 onwards, differentiating cultures were maintained in basal media only until harvested for analysis.

FACS analysis. To isolate cardiac-cell lineages from non-cardiac cell lineages, live cell sorting based on eGFP expression of differentiating cultures at either day 6 or day 10 were performed. Differentiating hESCs were dissociated, filtered through a 40 µM cell strainer and resuspended in PBS containing 2% fetal calf serum and 1 µg/mL propidium iodide. Flow cytometric gates of GFP positive and negative cells were set using negative control HESCs cultured in basal media containing 100 ng/ml bFGF. Sorted GFP positive and negative cells on day 6 and day 10 of differentiation were collected and snap frozen prior to RNA extraction. Cell sorting was done using BD Influx<sup>™</sup> cell sorters (BD Biosciences) and flow cytometric data was analyzed using Flowlogic software (Inivai Scientific).

Next generation sequencing and bioinformatics analysis. Total RNA from GFP sorted populations on day 6 and day 10 differentiating cultures were prepared in triplicate using the miRNAeasy Mini (Qiagen 217004). A minimum amount of 500 ng of total RNA was analyzed for RNA integrity and submitted for sequencing. Small RNA libraries were prepared using Trueq Small RNA Sample Prep Kit (Illumina) and small RNA sequencing was performed on the Illumina Hi-Seq platform using the Illumina CASAVA 1.8.2 software (Australian Genomic Research Facility). Short RNAs were sequenced as 50 base pair single end reads and all adapter and primer sequences were removed using cutadapt<sup>™</sup>. Bowtie aligner was used to map the 17-26 base pair single end reads to the human reference genome (hg19) allowing one mismatch. The uniquely mapped reads were summarized across microRNAs with featureCounts (Rsubread v1.20.6<sup>™</sup> using ensemble miRNA gene annotation. Lowly expressed miRNAs were filtered out (less than 10 counts per million in fewer than three samples). The data was Voom transformed with cyclic loess normalization, and differential expression assessed using empirical Bayes moderated t-tests from the R Bioconductor limma package (Version 3.20.9) through the statistical language R. Unbiased hierarchical clustering was performed using standard complete linkage and Euclidean distance. Data has been deposited on GEO (GSE134852).

Quantitative PCR. To analyze miRNA gene expression levels, quantitative real time PCR was performed on differentiating cultures at day 6 and day 10. 1 µg of RNA was reversed transcribed using SuperScript<sup>®</sup> III
(Invitrogen) and miRNA expression levels were determined using Taqman Gene Expression Assays with Taqman Universal PCR Master Mix (ThermoFisher) on the ABI 7300 Real-Time PCR detection system (Applied Biosystems). Transcript expression levels were normalized to the averaged expression of the reference gene snRNA RNU24, and gene relative quantification was calculated using the 2^(-ΔΔCt) method.

**Data availability**

The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or the GEO database (http://www.ncbi.nlm.nih.gov/geoi/, accession codes GSE134852) or from the corresponding author (DAE) upon reasonable request.

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Competing interests

The authors declare no competing interests.

Additional information

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