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MicroRNA profiling during mouse ventricular maturation: a role for miR-27 modulating Mef2c expression

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Aims Non-coding RNA has been recently demonstrated to be a novel mechanism for modulation of gene expression at the post-transcriptional level. The importance of microRNAs in the cardiovascular system is now apparent. Mutations of distinct microRNAs have provided evidence for fundamental roles of microRNAs during cardiovascular development. However, there is limited information about global microRNA profiles during mouse heart development. In this study, we have gained insight from the expression profiles of microRNAs during mouse ventricular development by microarray and qRT-PCR analysis.

Methods and results Our microarray analysis reveals that relatively few microRNAs display either increasing or decreasing expression profiles during ventricular chamber formation. Interestingly, most of the differentially expressed microRNAs display a rather discrete peak of expression at particular developmental stages. Furthermore, we demonstrate that microRNA-27b (miR-27b) displays an overt myocardial expression during heart development and that the transcription factor-encoding gene Mef2c is an miR-27b target.

Conclusion Our data present a comprehensive profile of microRNA expression during ventricular maturation, providing an entry point for investigation of the functional roles of the most abundantly and differentially expressed microRNAs during cardiogenesis.

Keywords MicroRNAs • Cardiogenesis • Microarrays • Mef2c • miR-27b

1. Introduction

Cardiac morphogenesis is a complex process that requires the formation of a four-chambered heart with synchronous contraction from an initially linear peristaltic cardiac tube.[1] During this process, the heart forms prospective atrial and ventricular chambers which soon thereafter are separated into right and left components configuring the fully formed heart.[2] Over the past few years, we have started to understand the complex regulatory mechanisms that govern cardiac morphogenesis. Several transcription factors such as Nkx2.5, Gata4, and Mef2c play pivotal roles in early cardiogenesis.[3] Other transcription factors such as T-box genes (Tbx2, Tbx3, Tbx5, and Tbx18) and Pitx2 exert crucial roles during subsequent cardiac developmental stages.[4]

During cardiac morphogenesis, multiple changes in cardiac-enriched gene expression occur, leading to the identification of molecularly distinct myocardial regions.[5] These changes have been extensively documented for sarcomeric proteins.[6] Several studies have demonstrated that distinct regulatory sequences are able to initiate chamber-specific gene expression at discrete developmental stages, either in atrial or in ventricular domains.[7] However, in several cases, transgene and endogenous gene expression is not always coincidental. These observations thus reinforce the idea that our understanding of cardiac gene regulatory networks is still incomplete.

Non-coding RNA has been recently demonstrated to be an additional pathway modulating gene expression at the post-transcriptional level.[8] MicroRNAs constitute a subset of single-stranded non-coding RNAs (19–25 nucleotides) which are capable

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of interfering with mRNA stability and/or protein translation, since they are able to target and bind to specific homologous RNA sequences on mature mRNAs.[9] MicroRNAs normally target 3′UTRs by recruiting the RNA-induced silencing complex, providing the cellular and molecular basis for mRNA cleavage. At present, the molecular mechanisms of microRNA biogenesis and target binding are well understood;[10] yet, there is limited understanding of microRNA tissue-specific distribution, abundance, and transcriptional regulation.[11,12] Recent data have demonstrated that miR-1 and miR-133 play pivotal roles in cardiac hypertrophy[13] and these microRNAs might also be involved in putative arrhythmogenic events in the adult human heart.[14,15] In the context of cardiovascular development, present knowledge is mainly restricted to microRNA expression profiling in the developing chicken heart,[16] although fundamental roles for microRNAs have also been recently demonstrated by genetic deletion of miR-1-2 in mice.[17]

In this study, we have performed comprehensive microRNA profiling during ventricular chamber development by microarray analysis. Our data reveal that relatively few microRNAs display increasing or decreasing expression profiles during ventricular maturation. Interestingly, most of the differentially expressed microRNAs display a rather discrete peak of expression. Thus, these data provide an entry point to dissect the functional roles of the most abundantly and differentially expressed microRNAs during ventricular development. Moreover, we demonstrate that microRNA-27b (miR-27b) displays an overt myocardial expression during cardiogenesis and regulates Mef2c.

2. Methods

2.1 Tissue samples

C57/BL6 mouse embryos were isolated at three distinct developmental stages [embryonic day (E) 12.5, E15.5, and E18.5] and ventricular chambers corresponding to all the embryos of a single litter were carefully dissected, pooled, snapped frozen in liquid nitrogen, and stored at −80°C until used. In addition, 1-week neonatal ventricular samples were also obtained, pooled ($n = 5$), and processed accordingly. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The work was approved by the University of Jaén Ethics Committee.

2.2 RNA isolation

Total RNA isolation was performed using Trizol reagent (Invitrogen) according to the manufacturer's guidelines. Total RNA was used for microRNA microarray analyses as detailed in Supplementary material online. For qRT-PCR analyses, DNAse treatment was performed during 1 h at 37°C prior to cDNA synthesis either using SuperScript RT III (Invitrogen) or Exiqon microRNA qRT-PCR detection system.

2.3 Microarray analysis

MirVana microarrays (Ambion) were used to profile microRNA signatures at different developmental stages of ventricular chamber formation. Twenty micrograms of total RNA were used to hybridize each microRNA microarray and two distinct microarrays were assessed per developmental stage analysed. Detailed information about the hybridization conditions and statistical analyses performed is given in Supplementary material online.

2.4 *In situ* hybridization

Embryos were hydrated by incubation in graded ethanol/PBS steps until reaching a pure sterile PBS solution. Embryos were subsequently prehybridized and hybridized overnight with LNA-labelled microRNA probes (Exiqon) for miR-27 and miR-1 following manufacturer's guidelines and processed as detailed in Supplementary material online.

2.5 DNA constructs

A 397-nucleotide sequence (spanning from nt 2968 to 3365; NM_002397) from the 3′untranslated region (3′UTR) of the Mef2c transcript encompassing the predicted miR-27-binding site was amplified by PCR and cloned into the *Xho*I–*Not*I site of pBluescript II SK vector (Stratagene), and miR-27b sites were mutated as described in the Supplementary material online.

2.6 Foetal cardiomyocyte primary cultures

Pregnant females (C57BL/6) were sacrificed and the embryonic hearts from 15 to 20 E16.5 mouse embryos were isolated, minced, and pooled. Tissue samples were trypsinized for 15 min rounds at 37°C, at least five times, and pre-plated for 1 h. The enriched cardiomyocyte fraction was subsequently collected and cultured in standard conditions as detailed in Supplementary material online.

2.7 Transfection assays

Sol8 skeletal myoblasts, HL-1 atrial myocardial cells, and mouse foetal (E16.5) and neonatal cardiomyocytes were used to assay microRNA-27 gain-of-function and loss-of-function experiments. Pre-miR-27, pre-miR-125b, pre-miR-219a (Ambion) and anti-miR-27b (Exiqon) were transfected with lipofectamine 2000 (Invitrogen) according to the manufacturer's guidelines, respectively. Negative controls included non-transfected cells as well as FAM-labelled pre-miR negative control transfected cells, which also allowed evaluation of the transfection efficiency. Cells were collected 24 h after transfection and processed for RNA isolation. qRT-PCR analyses were performed using standard conditions, as reported in Supplementary material online. Statistical analyses were performed using unpaired Student's *t*-significance level or *P*-values are stated on each corresponding figure legend.

2.8 Luciferase assay

Two hundred and ninety-three cells were maintained in DMEM medium supplemented with 10% foetal calf serum and antibiotics. Cells were co-transfected with 50 ng of the appropriate psicheck-2 luciferase reporter construct and double-stranded RNA oligonucleotides corresponding to miR-27b or a negative control oligonucleotide (Ambion). Culture conditions and luciferase assay were performed as described previously.[18]

3. Results

3.1 Comparative analysis of microRNA profiling during ventricular maturation

We have analysed the microRNA expression profile during mouse cardiac ventricular chamber formation by microarrays at three different developmental stages (E12.5, E15.5, and E18.5). Hierarchical clustering of the normalized microarray data of a total of 486 known mature microRNAs revealed a single set of microRNAs with increasing expression levels during the developmental window studied (cluster A; 55/486, 11%), as depicted in *Figure 1*. Although these microRNAs display increasing expression levels during cardiogenesis, they are also highly expressed at one or two developmental stages, but not at all three developmental stages (see Supplementary material online, *Table S1*), suggesting that microRNAs mainly display discrete

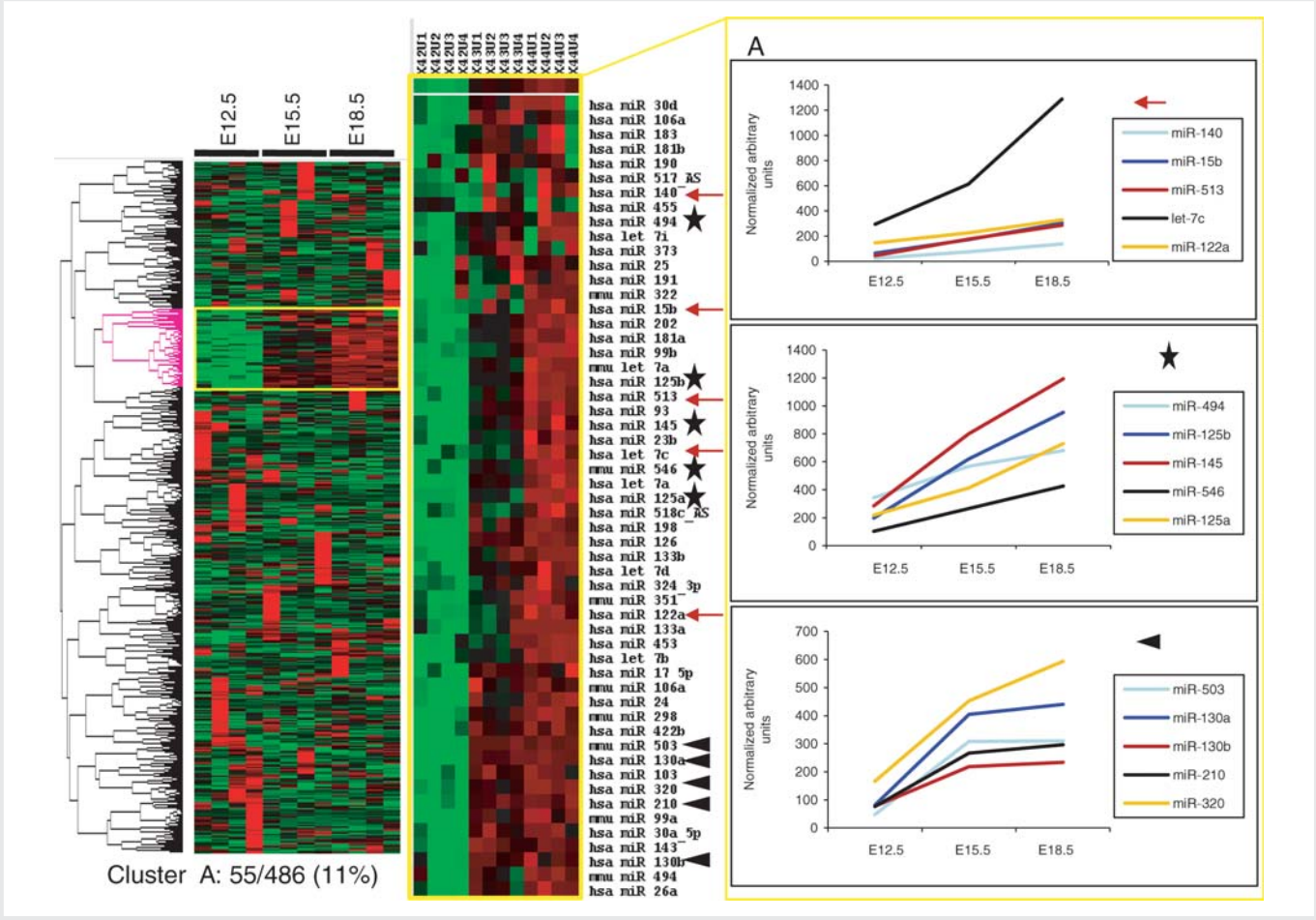

Figure 1 Hierarchical clustering of microRNA microarray expression profiles on murine E12.5, E15.5, and E18.5 ventricular chambers. The highlighted box (yellow) illustrates a microRNA set (cluster A) that displays increasing expression levels during ventricular maturation. The stars, arrows, and arrowheads illustrate the different trends of increasing expression profiles, as depicted in the corresponding adjacent graphs. Differentially expressed microRNAs always display increasing levels (55/486, ~11%). *Y*-axis represents the normalized arbitrary microarray hybridization signal.

peaks of expression within relatively short-time windows during ventricular development.

Hierarchical clustering of the microRNA expression data between E12.5 and E15.5 identified three sets of differentially expressed microRNA clusters as depicted in *Figure 2*. Clusters B and C display increased expression profiles between E12.5 and E15.5 (38/486, 7% and 28/486, 6%, respectively), whereas cluster D displays decreasing expression levels between E12.5 and E15.5 (24/486, 5%). Interestingly, of all distinctly expressed microRNAs between E12.5 and E15.5 (90/486, 21%), only a minority displayed statistical significance (Bonferroni's test; 10/486, 2%). Hierarchical clustering between E15.5 and E18.5 stages identifies a single cluster of distinctly expressed microRNAs as depicted in *Figure 2*. Within this cluster (cluster E; 41/486, 8%), an increasing trend is observed that reaches statistical significance for a subset of them (3/486, 0.5%). However, clustering microRNA expression profiles from E12.5 vs. E18.5 ventricular chambers revealed two clusters sets, displaying increasing (cluster F; 54/486, 11%) or decreasing (cluster G; 13/486, 2,6%) expression levels. As expected, a larger number of microRNAs display significant differences between these two groups (27/486, 5.5%; see Supplementary material online, *Table S1*) as illustrated in *Figure 3*. A comprehensive list of the differentially expressed microRNAs during ventricular chamber formation is provided in the Supplementary material online, *Tables S1* and *S2*. Several of these differentially expressed microRNAs have been validated by qRT-PCR as depicted in *Figure 4*. Thus, these data identify a highly relevant number of microRNAs that display differential expression during ventricular chamber development in the mouse heart (see Supplementary material online, *Figures S1–S4*), providing a framework to unravel the individual role of each microRNA during cardiogenesis.

MicroRNA microarray analyses also allowed us to identify the most prominently expressed microRNAs at each developmental stage. We have selected as the most abundantly expressed microRNAs those that display normalized expression levels more than one standard deviation above the mean. These microRNAs provide a fingerprint of highly abundant microRNAs for each developmental stage, as listed in Supplementary material online, *Table S3*. Among these highly abundant microRNAs, 17 were highly expressed at E12.5, 24 at E15.5, and 28 at E18.5. Consistent with our hierarchical clustering results, only a minority of stage-specific highly expressed microRNAs are commonly expressed between E12.5 and E15.5, whereas approximately half of them are highly expressed at both E15.5 and E18.5 (see Supplementary material online, *Figure S1*). Importantly, no microRNAs are maintained at high levels at all three time points, supporting the

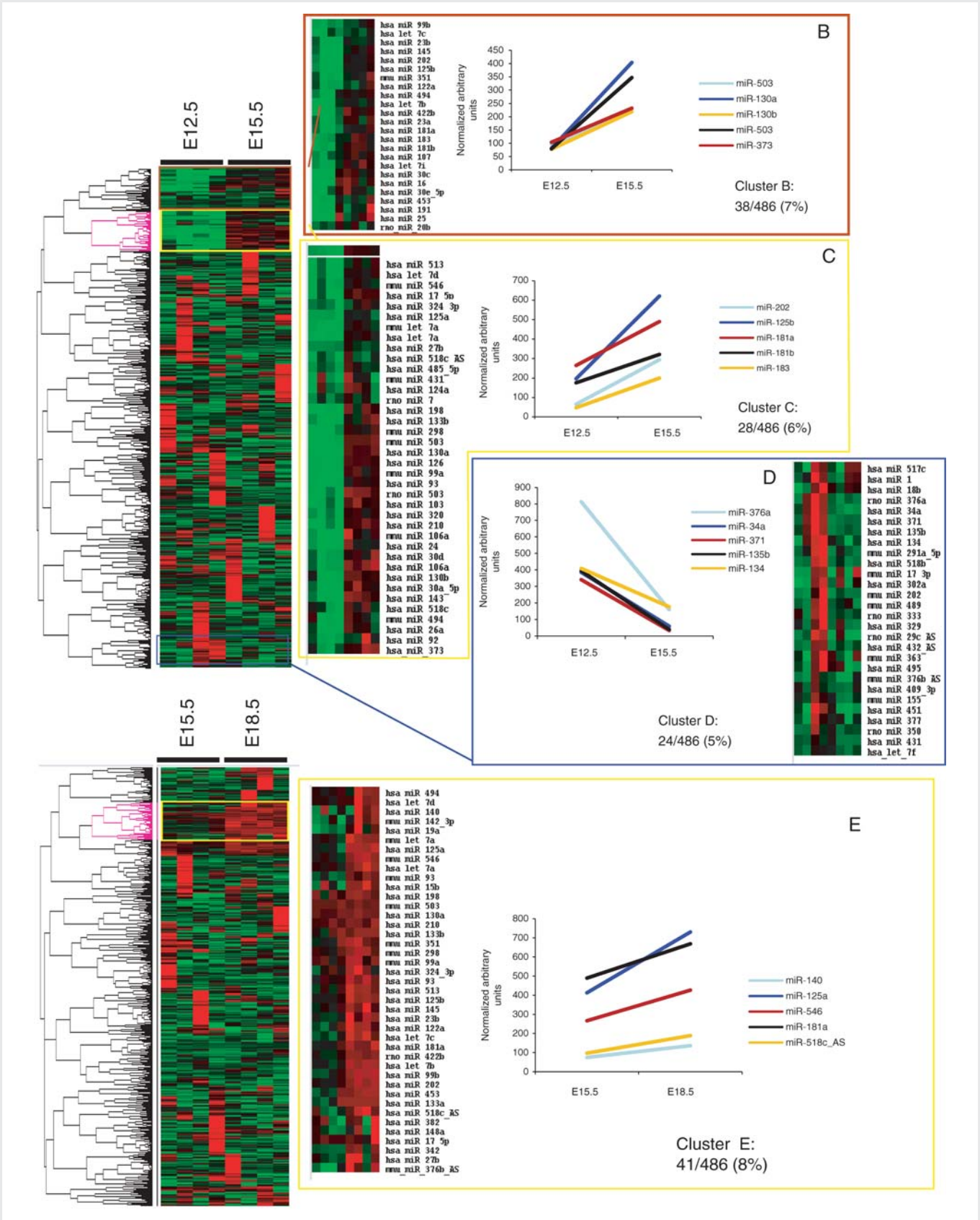

Figure 2 Hierarchical clustering of microRNA microarray expression profiles on murine E12.5 compared with E15.5 ventricular chambers (clusters B–D) as well as between E15.5 and E18.5 ventricular chambers (clusters E). The highlighted boxes in red (cluster B) and in yellow (cluster C) illustrate two distinct microRNA subsets that display increasing expression levels between E12.5 and E15.5. The highlighted box in blue (cluster D) illustrates microRNA subsets that display decreasing expression levels between E12.5 and E15.5. The highlighted box in yellow (cluster E) illustrates microRNA subsets that display increasing expression levels between E15.5 and E18.5. *Y*-axis represents the normalized arbitrary microarray hybridization signal.

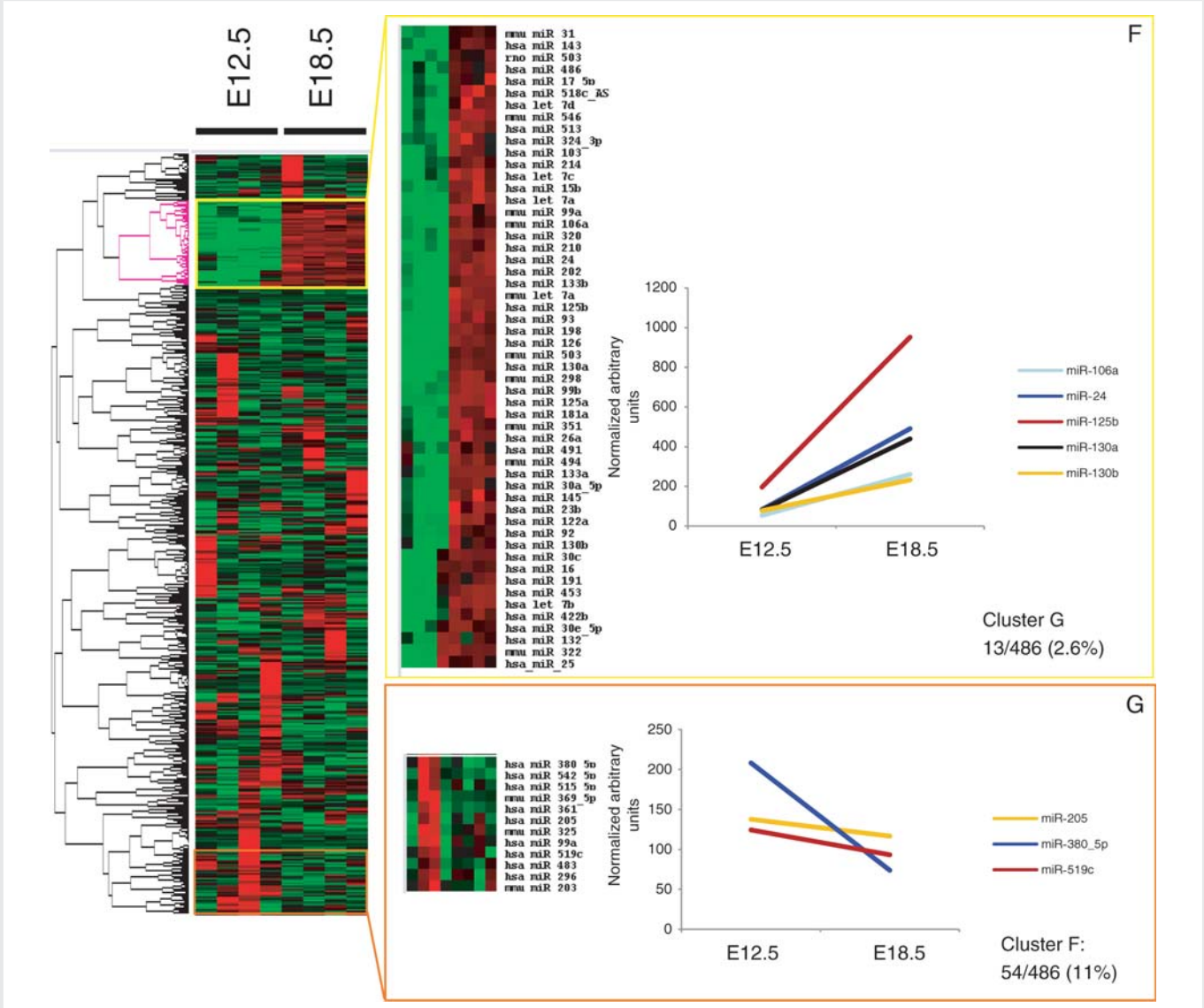

Figure 3 Hierarchical clustering of microRNA microarray expression profiles on murine E12.5 when compared with E18.5 ventricular chambers. The highlighted box in yellow (cluster F) illustrates microRNA subsets that display increasing expression levels during ventricular maturation. The highlighted box in red (cluster G) illustrates microRNA subsets that display decreasing expression levels during ventricular maturation. *Y*-axis represents the normalized arbitrary microarray hybridization signal.

notion of stage-specific roles for microRNAs. Graphical representation (see Supplementary material online, *Figure S1*) of the expression profiles of these highly expressed, stage-specific microRNAs reveals that only two microRNAs (miR-133a and miR-133b) display an overt and steadily increasing expression, in line with previous reports.[13] Interestingly, most microRNAs display either a plateau for two developmental stages, such as the E12.5- and E18.5-specific miRNAs (see Supplementary material online, *Figure S2*), or they peak and remain at a steady stage, such as those specifically up-regulated at E15.5 (see Supplementary material online, *Figure S2*).

3.2 Transcriptional control of microRNAs during ventricular development

The analysis of the genomic localization of the most abundantly and/or differentially expressed microRNAs during ventricular chamber development (see Supplementary material online, *Tables S2* and *S3*) demonstrates that a substantial number of these microRNAs are located in intergenic regions (47/78, ~60%), whereas the rest are either intronic (24/78, ~30%), exonic (6/78, ~7%), or located within 3′UTRs (1/78, ~1%). Transcriptional regulation of gene-embedded microRNAs is modulated in accordance with transcriptional regulation of the host gene, as recently reported for miR-208.[15] In this context, it is important to highlight that miR-23b and miR-27b display analogous expression profiles during cardiac development (*Figure 4H* and *J*) in line with the AC130827.3-201 host gene (*Figure 4M*). However, the transcriptional regulation of intergenic microRNAs remains largely unknown. It is interesting to realize that a relatively large number of these intergenic microRNAs are clustered in short genomic regions (between 200 and 1000 bp apart). It has been postulated that those microRNAs are transcribed as single polycistronic units (pri-miRNA transcript),[10] which are

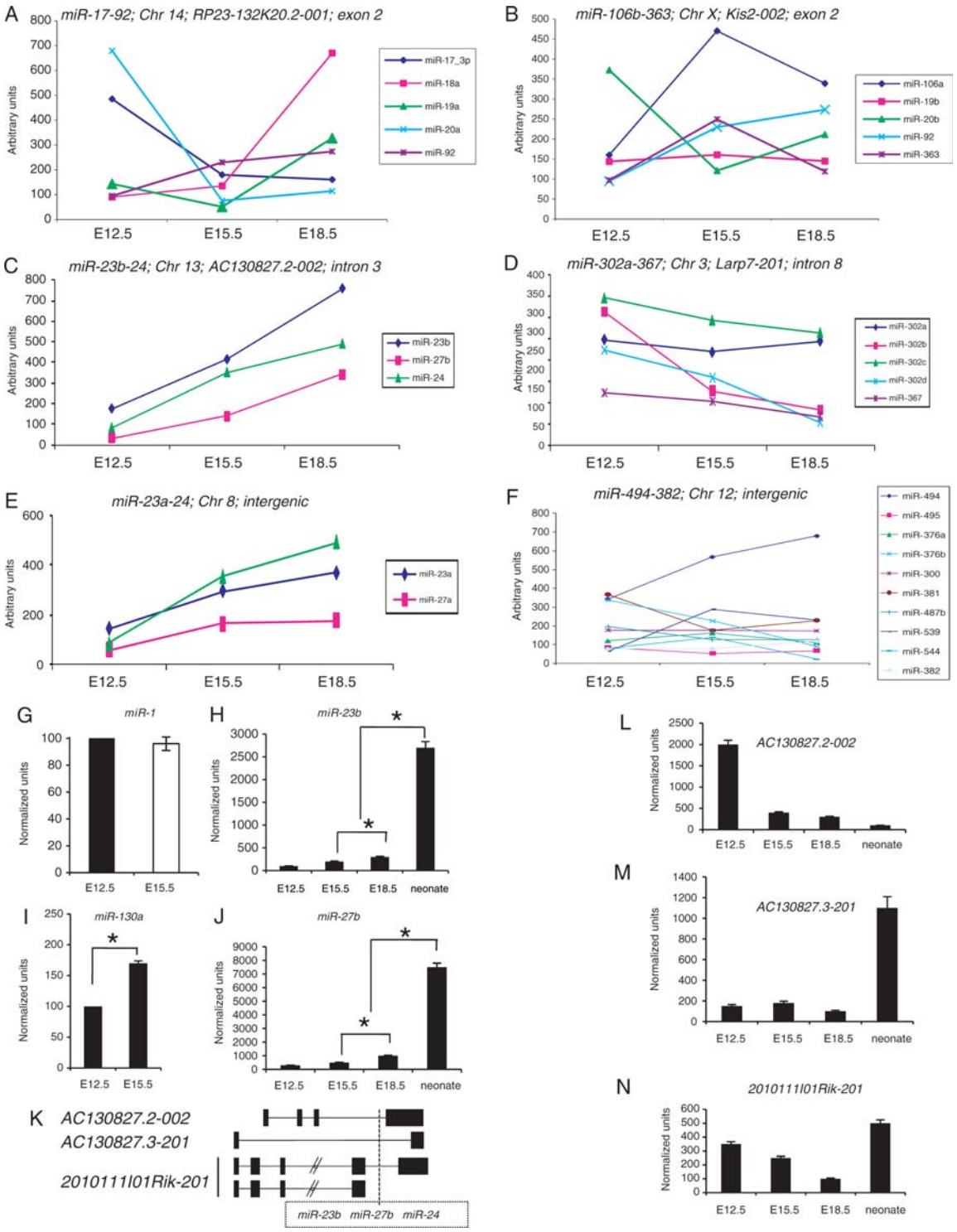

Figure 4 Graphical representation of normalized microRNA expression levels of exonic-clustered microRNAs (*A* and *B*), intronic-clustered microRNAs (*C* and *D*), and intergenic-clustered microRNAs (*E* and *F*). All microRNA clusters are <10 kb. Observe that expression levels of several of these closely linked microRNAs display divergent expression profiles during cardiac ventricular development. *Y*-axis represents the normalized arbitrary microarray hybridization signal. (*G–J*) qRT-PCR expression profiles of miR-1 (*G*), miR-130a (*I*), miR-23b (*H*), and miR-27b (*J*) during ventricular development. In line with microarray expression, miR-1 display no significant changes between E12.5 and E15.5, whereas miR-130a display increased expression levels at E15.5 when compared with E12.5. Similarly, miR-23b and miR-27b expression levels display no overt differential expression between E12.5 and E15.5, whereas it is highly increased at E18.5 and neonatal stages (*H* and *J*, respectively). (*K*) The genomic location of miR-23b/miR-27b/miR-24 which is embedded in three distinct introns, corresponding to three predicted genes as annotated in Ensembl (http://www.ensembl.org). (*L–N*) qRT-PCR expression profiles of *AC130827.2-002* (*L*), *AC130827.3.201* (*M*), and *2010111/01Rik-201* (*N*) during ventricular development. Note that only *AC130827.3.201* (*M*), but not *AC130827.2-002* (*L*) or *2010111/01Rik-201* (*N*) display a similar expression profile during cardiogenesis as miR-23b and miR-27b, suggesting therefore that transcriptional regulation of these clustered microRNAs is mainly mediated by *AC130827.3.201* (*M*) during ventricular development. *$P < 0.01$.

subsequently processed into single pre-miRNA molecules.[10] If this is the case, genome-clustered microRNAs are expected to display concordant expression levels at distinct developmental stages. *Figure 4* shows the expression profiles of those genome-clustered microRNAs with reported functional roles in the developing and/or adult heart, which also display high expression levels in our microarray analyses. Interestingly, both exonic-clustered microRNAs and intergenic-clustered microRNAs display divergent expression levels during ventricular development (*Figure 4A–C*). In contrast, expression levels are concordant for intronic-clustered microRNAs. Therefore, it seems plausible that intergenic- and exonic-clustered microRNAs are transcribed individually and/or as polycistrons, and if transcribed as polycistronic units, are subject to post-transcriptional regulation. On the other hand, intronic microRNA clusters might be transcribed as single polycistronic units and processed with little or no post-transcriptional regulation. Recent data in our lab, using bioinformatic analyses, support the notion that individual transcriptional units operate in several intergenic-clustered microRNAs.[19] Overall, these data support the notion that complex transcriptional and/or post-transcriptional modifications operate in all clustered microRNA loci.

3.3 Cardiac-specific expression of microRNAs; a role for miR-27 in the myocardium

In order to start dissecting the role of the distinct microRNAs differentially expressed during ventricular development, we decided to focus on those microRNAs that display early differential expression profiles (see Supplementary material online, *Figure S1* and *Table S2*). Among those microRNAs differentially expressed between E12.5 and E15.5, several have been already reported to play a role in vascular (miR-126[20]) and/or muscle development (miR-30a[21] and miR-27b[18]). miR-27b is differentially expressed from early stages of ventricular chamber formation, and recently, it has been reported to play a relevant role in skeletal muscle development.[18] Since skeletal and cardiac muscle development share common key transcriptional signalling pathways, we therefore centred our analyses on miR-27b. We validated the developmental expression of miR-27b during cardiogenesis by qRT-PCR (*Figure 4J*) as well as by *in situ* hybridization (ISH) (*Figure 5*). In order to conduct whole-mount ISH experiments with maximal penetrance, and therefore, reliably demonstrate the tissue specificity of miR-27 distribution, we first used mouse E10.5 embryos. miR-27b is widely expressed at E10.5 as revealed by whole-mount ISH, displaying high expression levels in the myotome, pharyngeal arches, limb buds, and developing heart (*Figure 5B* and *C*). Serial sections of these E10.5 embryos revealed a myocardial tissue-specific expression that is homogeneously observed in the developing myocardium. No expression is observed in the developing endocardial cushions or endocardial cells (*Figure 5D–E*). miR-27b remains highly expressed in the developing heart at later developmental stages (E12.5; *Figure 3F–G*), consistent with our microarray data.

3.4 Functional role of miR-27 during myogenesis

Bioinformatics approaches using complementary base pairing and orthologous conservation of the target site by TargetScan (http://www.targetscan.org) provide the means to identify possible functional targets of discrete microRNAs. We therefore sought to unravel the functional role of miR-27b during cardiomyogenesis and screened by TargetScan putative mRNA targets that might be relevant during cardiac development. Myocyte-enhancer factor 2c (Mef2c), a highly relevant transcription factor crucial for both skeletal and cardiac myogenesis[22,23] is predicted to be targeted by microRNA-27 (see Supplementary material online, *Figure S5*). We tested whether miR-27b is able to regulate *Mef2c* by generating luciferase reporter constructs with wild-type and miR-27 'seed' sequence-mutated Mef2c 3′UTRs. As illustrated in *Figure 6A*, miR-27b significantly decreased luciferase activity in the context of the wild-type Mef2c 3′UTR, whereas the mutation of miR-27b target sites (one predicted by TargetScan, one non-canonical seed sequence; see Supplementary material online, *Figure S5*) returned luciferase levels to that of the control oligonucleotide. Thus, these data demonstrate that miR-27b targets Mef2c transcripts. To further validate this, we tested whether miR-27b can regulate *Mef2c* in a cellular context. Transfection of miR-27 in Sol8 cells decreased *Mef2c* expression by ~40% (*Figure 6B*), whereas inhibition of miR-27b leads to a four-fold increase in *Mef2c* transcript expression. Transfection of miR-27 in HL-1 (*Figure 6C*) and primary culture of foetal cardiomyocytes (*Figure 6D*) elicit similar results on *Mef2c* transcripts, whereas *Scn5a* and *Scn1b* expression is not altered (*Figure 6C*). In addition, we tested whether other microRNAs, which have no predicted target site on the Mef2c 3′UTR, might affect *Mef2c*. Overexpression of miR-125a or miR-219 does not modify *Mef2c* transcript levels (*Figure 6E*). Thus, our data demonstrate a role for miR-27b regulating *Mef2c* expression levels in myogenic cells.

4. Discussion

During heart development, a series of transcriptional regulators are progressively activated to control cardiac morphogenesis.[24,25] However, to date, little insight has been gained as to the negative regulatory mechanisms that control the inhibition or extension of these transcriptional pathways.[26] MicroRNAs constitute a subset of single-stranded non-coding RNAs (19–25 nucleotides) that are capable of interfering with mRNA stability and/or protein translation.[10] It has been recently established that microRNAs play crucial roles during heart development, controlling both muscle and vascular development.[20] However, only a handful of microRNAs have been described so far to have relevant roles during cardiogenesis.[16,27,28] We therefore sought to investigate the microRNA profile during ventricular chamber maturation. We have compared the expression profile at three different developmental stages (E12.5, E15.5, and E18.5) by microarrays. To our knowledge, this is therefore the first study to report microRNA microarray analyses during cardiovascular development.

Bioinformatics analyses have demonstrated that only a relatively small proportion of microRNAs display differential expression patterns during ventricular maturation (29/486, ~6%). Strikingly, none of these microRNAs display statistically significant differences at all three developmental stages analysed, suggesting that the peak of microRNA expression is limited to short developmental periods. Nonetheless, a set of microRNAs can be distinguished by displaying increasing expression levels, whereas none display decreasing expression levels. Thus, these data support the notion that increasing microRNA expression levels provides a means to block and/or inhibit certain transcriptional programmes over time. In this regard, bioinformatics approaches aimed to dissect the role of differentially expressed

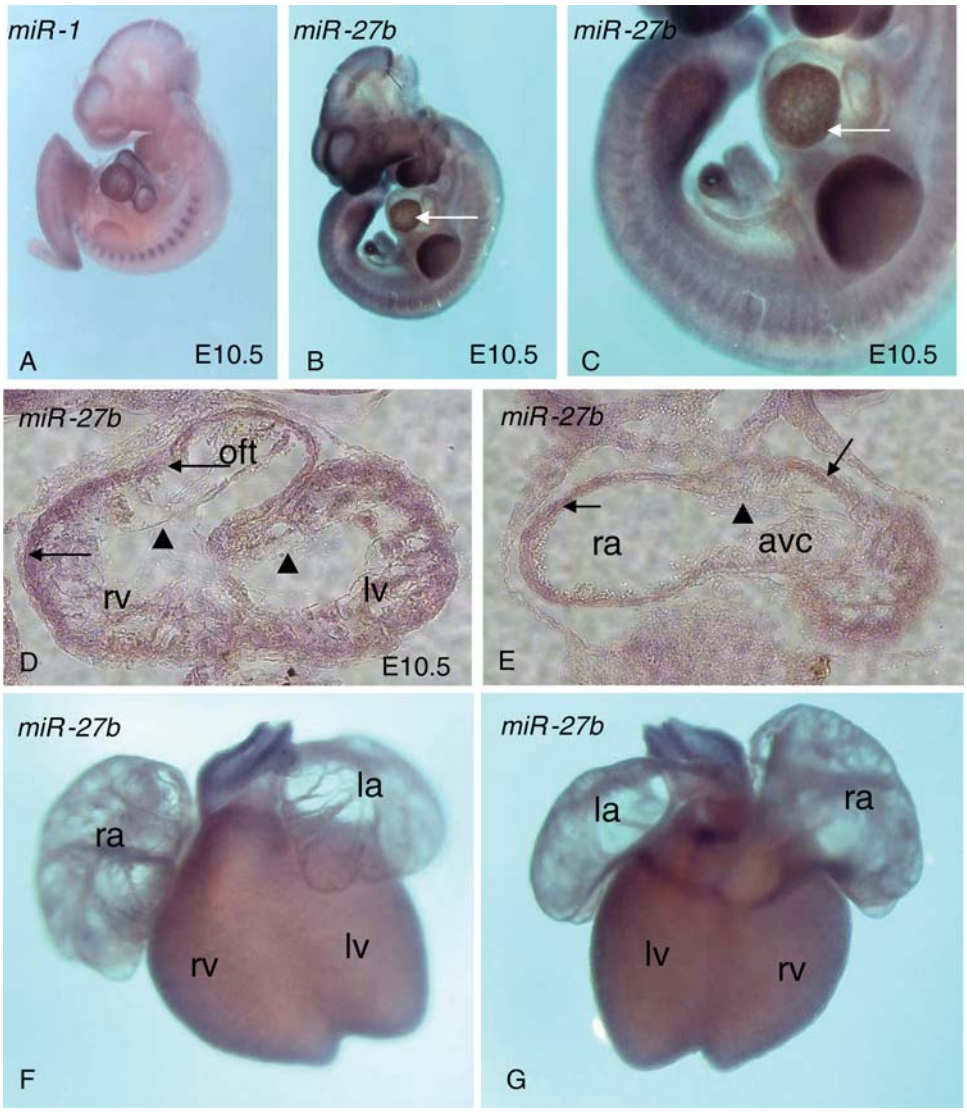

Figure 5 Whole-mount ISH analyses of miR-1 (control) and miR-27b expression during embryogenesis. (*A*) The expression profile of miR-1, which displays muscle-specific expression in the developing heart and skeletal muscle precursor cells consistent with previous reports.[34] (*B* and *C*) The expression profile of miR-27b at E10.5. miR-27b is highly expressed in the brain, pharyngeal arches, limb buds, and developing heart (arrows). (*D* and *E*) Serial sections of E10.5 mouse embryos hybridized to miR-27b. Note that expression is confined to the developing myocardium (arrows), whereas no expression is observed in the endocardium (arrowheads, *D*) or endocardial cushions (arrowhead, *E*). (*F* and *G*) miR-27b expression in E12.5 hearts. Note that expression of miR-27b is robust in the developing myocardium (*G*).

microRNAs during ventricular formation have revealed that these microRNAs seem to play a prominent role controlling MAPK and TGF-β signalling pathways.[29] In this context, it is important to note that differentially expressed microRNAs between E12.5 and E15.5 can putatively target all TGF-β receptors but not the downstream Smad proteins, whereas differentially expressed microRNA between E15.5 and E18.5 are predicted to target most of TGF-β receptors and downstream Smads transcription factors.[29] A similar result is obtained for the MAPK signalling pathway, sequentially targeting discrete MAPK cascades with relevant roles in cardiac function (i.e. p38 MAPK and ERK cascades).[30] Although experimental approaches are required to validate these models, these data support the hypothesis that increasing expression levels and temporally discrete expression of microRNAs during cardiovascular development progressively block discrete signalling pathways.

Side-by-side comparisons of distinct developmental stages reveal a larger differential expression of microRNAs between E12.5 and E15.5 compared with that between E15.5 and E18.5. This is interesting, since complex morphogenetic processes are occurring in the E12.5 mouse heart, when atrial and ventricular septation is underway and the endocardial cushions at the atrioventricular canal and outflow tract are initiating fusion,[2] compared with an E15.5 mouse heart when cardiac septation is complete. In fact, most morphogenetic processes are completed by E15.5 and are basically followed by a growth process from E15.5 to E18.5 in the ventricular chambers.[2] Thus, our data also support an important role for distinct microRNAs during cardiac septation, since a wide range of differential expression is observed during cardiac remodelling (E12.5 > E15.5), whereas only minor changes are observed during the subsequent growth stage (E15.5 > E18.5).

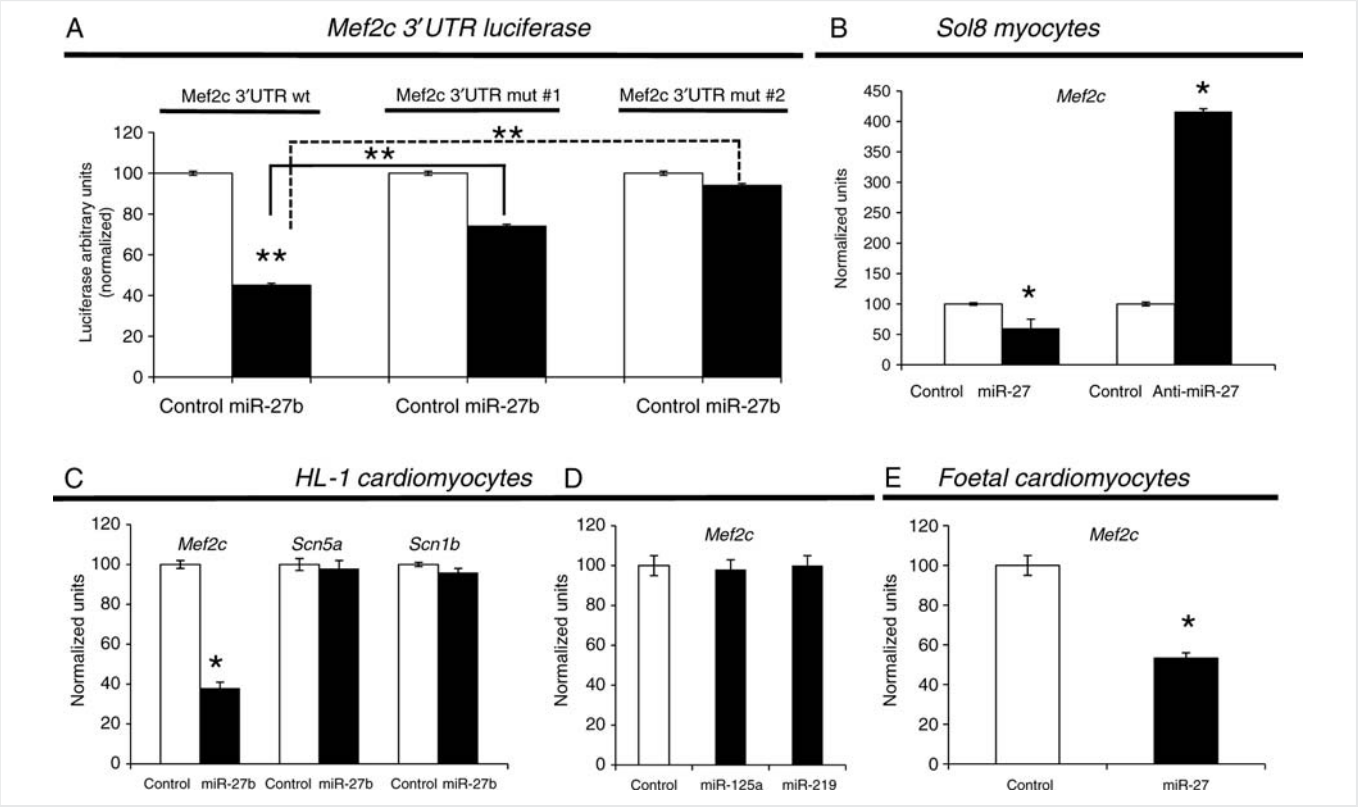

Figure 6 miR-27b functional assessment. (*A*) Luciferase reporter analysis of a Mef2c wild-type 3′UTR, as well as one TargetScan miR-27 site mutated (Mef2c 3′UTR mut#1) and also combined with a second non-canonical seed site mutated (Mef2c 3′UTR mut#2); as depicted in Supplementary material online, *Figure S5*. (*B*) *Mef2c* expression levels as measured by qRT-PCR of miR-27b gain-of-function (miR-27) and loss-of-function (anti-miR-27) approaches in the Sol8 cell line. Overexpression of miR-27 leads to decreased *Mef2c* transcripts, whereas miR-27b inhibition leads to increased *Mef2c* transcripts. (*C*) Overexpression of miR-27 in HL-1 cardiomyocytes, similarly as in skeletal muscle myoblasts, leads to decreased Mef2c transcript levels, while not affecting *Scn5a* and/or *Scn1b* levels. (*D*) *Mef2c* expression levels as measured by qRT-PCR of miR-125a and miR-219 gain-of-function approaches in HL-1 cell line, respectively. Neither overexpression of miR-125a nor miR-219 leads to significant changes of *Mef2c* transcript levels. (*E*) Overexpression of miR-27b in primary culture of mouse foetal cardiomyocytes, which leads to ~50% reduction of Mef2c transcripts. Standard deviations are from three independent experiments. $*P < 0.01$ and $**P < 0.001$.

Our microRNA microarray approach identifies for the first time a set of microRNAs that display differential expression during ventricular maturation. Although a direct implication of microRNAs in ventricular development remains to be demonstrated, the expression profiling of microRNAs in distinct cardiac settings supports our currently described microRNA expression data. Several microarray studies have been performed on microRNA expression profiles in the adult cardiovascular system. Wang *et al.*[31] have identified highly expressed microRNAs in the adult rat lung and heart, supporting our data, i.e. high expression of miR-126, miR-143, miR-145, miR-23b, miR-24, and miR-27b in the developing heart. Cheng *et al.*[32] reported several highly expressed microRNAs in the normal adult mouse heart, some of which are also highly expressed in our microarray approach (miR-133b, miR-125a, miR-143, miR-99a, miR-424, miR-126, miR-27b, miR-26a, miR-145, and miR-181a). Thus, these data identify a subset of microRNAs that are highly expressed in both the developing and adult heart, of which some display cardiac tissue-specific expression.

However, only a small number of these newly identified microRNAs in the developing ventricular chambers have been functionally studied. Several of them have been reported to play essential roles in the developing cardiovascular system, such as miR-30 in cardiac fibrosis homeostasis and miR-126 in angiogenesis/vasculogenesis,[20,21] whereas others, such as miR-133 and miR-1, play pivotal roles in cardiac physiopathological conditions such cardiac hypertrophy and arrhythmias.[14,33] Nonetheless, for most differentially and/or abundantly expressed microRNAs in the cardiac ventricular chambers, a role in cardiac development and/or disease remains to be established.

4.1 Insights into the transcriptional regulation of microRNAs during cardiogenesis

The distribution of microRNAs in the genome is highly diverse since they can be located between distinct genes (intergenic) or within intronic or exonic gene sequences. Our understanding of transcriptional regulation of microRNAs is yet limited. Several seminal papers have provided evidence that Mef2c regulates the expression of miR-1-2 and miR-131-1,[34] and myogenic regulatory factors are capable of transcriptionally controlling the expression of miR-1, miR-133, and miR-206 in the skeletal muscle.[35,34] At present, it is believed that microRNAs embedded in introns and exons are co-regulated with their cognate host gene, as has been reported for miR-208.[15] However, intergenic transcriptional regulation of

microRNAs is highly unexplored. It is remarkable that a great proportion of highly and/or differentially expressed microRNAs during ventricular development display intergenic genomic localization. Furthermore, in some cases, several microRNAs are clustered in small genomic areas and it is believed that these clustered microRNAs are transcribed as single polycistronic units (pri-miR), which are later processed into single microRNA containing units (pre-miR).[10] If this is the case, clustered microRNAs are expected to display concordant expression profiles at distinct developmental stages. Our data demonstrate that such a linear relationship is not always maintained, suggesting that either each microRNA is transcribed individually, not forming thus part of a polycistronic unit, or post-transcriptional regulation of individual microRNAs occur at distinct developmental stages during ventricular development. Bioinformatic analyses provide additional evidence that discrete transcriptional units might exist.[29] Thus, our data support the existence of additional complexity in the production of microRNAs during cardiogenesis.

4.2 A role for miR-27 in cardiac development

Most microRNAs identified in our study as displaying differential and/or abundant expression levels in the developing heart remain to be functionally explored. Therefore, we screened their expression profiles using online resources (Geisha database; http://geisha.arizona.edu/geisha/index.jsp) as well as performing whole-mount ISH experiments in mouse embryo hearts. Among those screened, we centred our attention on miR-27b; first, because it has been recently reported to play a pivotal role in skeletal muscle development,[18] and secondly, because it displays a robust cardiac expression within the myocardium. Moreover, miR-27b has been previously reported to be highly expressed in the adult heart[30,36] by microarrays. Thus, these data support a role of miR-27b in cardiac muscle development, further supported by our results using microarrays, qRT-PCR, and ISH during ventricular development.

Interestingly, miR-27b is predicted to target *Mef2c*, a muscle-specific transcription factor which plays essential roles during cardiac and skeletal myogenesis.[25] *Mef2c* expression is initiated early in the development,[22] at the cardiac crescent stage, and soon thereafter in the developing myotomes. Thus, *Mef2c* expression is highly similar to that of miR-27b. Furthermore, *Mef2c* plays an essential role in the establishment of cardiac hypertrophy.[37] At present, the functional role of miR-27b in the developing and/or adult heart has not been investigated. We provide herein the first evidence that miR-27b activity targets *Mef2c* 3′UTR, and thus, transcript levels of *Mef2c* are decreased without altering the expression of other cardiac genes, such as *Scn5a* and *Scn1b*. Thus, these data indicate a specific role for miR-27b in the regulation of *Mef2c*, which might be of great therapeutic interest for physiopathological conditions such as cardiac hypertrophy.

In summary, in this manuscript, we provide a comprehensive view of microRNA expression profiling during ventricular development, supporting a relevant role of these non-coding RNAs in the modulation of key signalling and developmental pathways. We also provide evidence for the cardiac and skeletal muscle-enriched expression of miR-27b, and furthermore, we demonstrate that this microRNA regulates *Mef2c*, suggesting potential therapeutical usage.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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