

*Communication*

## **miR-27b and miR-23b Modulate Cardiomyocyte Differentiation from Mouse Embryonic Stem Cells**

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**Abstract:** Diverse types of stem cells represent a potentially attractive source of cardiac cells for the treatment of cardiovascular diseases. However, most of the functional benefits reported for stem cell have been modest and mainly due to paracrine effects rather than differentiation into cardiomyocytes of the applied cells. Therefore, new tools need to be developed in order to improve the efficiency of stem cell differentiation towards specific cardiovascular lineages. Here we show that microRNAs that display early differential expression during ventricular maturation, such as miR-27b, inhibits cardiac differentiation from mouse embryonic stem cells whereas miRNAs that display late differential expression, such as miR-23b, regulates the beating phenotype during *in vitro* cardiac differentiation from Embryonic Stem Cells (ESCs). This study could have an impact on regenerative medicine since we showed that miR-27b and miR-23b overexpression differentially modify the ESC cell fate towards the cardiac lineage.

**Keywords:** miR-27b; miR-23b; embryonic stem cells

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## 1. Introduction

Heart development takes place through a complex series of steps, including the induction of cardiac mesoderm, formation of the cardiovascular progenitor cells and the commitment of cells to the cardiovascular lineage. Each step is precisely regulated at various levels, including transcriptional, posttranscriptional and posttranslational mechanisms [1].

microRNAs (miRNAs) have been implicated as critical regulators in cardiovascular development and pathological conditions at posttranscriptional level. The essential role of miRNA in early heart development was first revealed by a knockout study of Dicer, a miRNA-processing enzyme. The early lethality in Dicer mutant indicated the crucial role of miRNAs in early heart development [2]. Moreover, conditional Dicer deletion in the adult mouse heart causes various cardiac defects and postnatal lethality [3].

Additional studies led to the discovery of functional miRNAs such as miR-1, miR-15, miR-133, miR-138, miR-218 in regulating different step of vertebrate cardiogenesis [4–8]. Therefore, miRNAs now constitute an important component of the regulatory circuits that govern heart development and this knowledge has revolutionized our understanding of the diverse roles of non-protein-coding RNA in cardiac biology. However, with the human and mouse genome encoding over 4000 miRNAs, only a handful of which have been studied in the heart, much remains to be learned about this new class of regulatory RNA.

Recently we have reported a comprehensive profile of microRNA expression during cardiac maturation [9]. This analysis showed that only a relatively small set of microRNAs display increased expression levels, whereas none display decreased expression levels, supporting the notion that increasing microRNA expression levels provides a means to block and/or inhibit certain transcriptional programs over time. Here we show that microRNAs that display early differential expression profiles during cardiac maturation, such as miR-27b, inhibits cardiac differentiation from mouse embryonic stem cells whereas miRNAs that display late differential expression profiles, such as miR-23b, regulates the beating phenotype during *in vitro* cardiac differentiation from ESCs. These data support the notion that increasing expression levels of microRNAs in discrete temporal developmental windows during *in vitro* cardiac differentiation progressively modulate particular signaling pathways, which can alter the cardiogenic lineage differentiation.

## 2. Material and Methods

### 2.1. ESC Culture and microRNA Transfection Assays

R1-ESC line, derived from the 129 S1/SvImJ strain, was kindly provided by Andras Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada). R1-ESCs were cultured and differentiated as previously described [10]. We designated day 0 as when embryonic bodies (EBs), obtained from hanging drops, were plated. In general, we identified contracting regions within the EBs between 3–4 days after plating (10–11 total days).

microRNA transfection assays were performed when EBs were plated (day 0) as previously described [10]. Pre-miR-27b and pre-miR-23b and negative controls (a scrambled oligonucleotide, scrambled-pre-miR) were obtained from Ambion (Life Technologies). In order to maintain miRNA

over-expression levels during EB culture, miRNA-transfections were repeatedly carried out every two days of culturing, as the EBs-differentiation medium was changed. EBs or dissected beating areas respectively were collected at 7 days of transfection.

## 2.2. RNA Isolation, Reverse Transcriptase Reaction and Quantitative Real Time PCR

Because spontaneously contracting CMs constitute only a small fraction of EBs, parts of EBs containing beating areas were dissected under a binocular microscope. EBs as well as dissected specimens were used to perform reverse transcriptase reactions (RT). Total RNA isolation, RT reactions and quantitative real time PCR was performed as previously described [10]. Specific primers for each gene analyzed, annealing temperature and amplicon size are shown in the Table 1.

**Table 1.** Gene-specific primers, annealing temperatures, and amplicon sizes for quantitative Real-Time PCR.

Primers	Sequence	Annealing Temperature	Amplicon size
<i>Wnt3a</i>	Sense: 5'-TTCTGCAGGA ACTACGTGGA-3' Antisense: 5'-AGATAGCAGCTGATCCCTCTG-3'	60 °C	179 bp
<i>Brachyury</i>	Sense: 5'-CGACCACAAAGATGTAATGGAG-3' Antisense: 5'-AACTGAGGGTGGGAGCTG-3'	59.5 °C	120 bp
<i>Kcnk3</i>	Sense: 5'-TGTGCACCTTCACCTACCTG-3' Antisense: 5'-GGTGATGGCGAAGTAGAAGG-3'	59.7 °C	203 bp
<i>Kcnh1</i>	Sense: 5'-ACGCCCTTCAGAAAGTGCTA-3' Antisense: 5'-GTGGTCAGGAGGCAGGATAA-3'	62 °C	182 bp
<i>c-Troponin T</i>	Sense: 5'-TTCGACCTGCAGGAAAAGTT-3' Antisense: 5'-GCACAGCTTTGACGAGAACA-3'	64 °C	133bp
<i>Hcn4</i>	Sense: 5'-CAGCGTCAGAGCGGATACTT-3' Antisense: 5'-TGTGGAGGAGGATGGAGTTC-3'	60 °C	158bp
<i>Cx40</i>	Sense: 5'-CAGAGCCTGAAGAAGCCAAC-3' Antisense: 5'-ATGCGGAAAATGAACAGGAC-3'	60 °C	178bp
<i>Cx43</i>	Sense: 5'-GAGAGCCCGAACTCTCCTTT-3' Antisense 5'-TGGAGTAGGCTTGGACCTTG-3'	60 °C	158bp
<i>miR 27b</i>	5'-UUCACAGUGGCUAAGUUCUGC-3'	60 °C	21 bp
<i>miR 23b</i>	5'-AUCACAUUGCCAGGGAUUACC-3'	60 °C	21 bp

### 3. Results

#### 3.1. miR-27b Inhibits *in Vitro* Cardiac Differentiation by Modulating Wnt-Beta Catenin Canonical Pathway

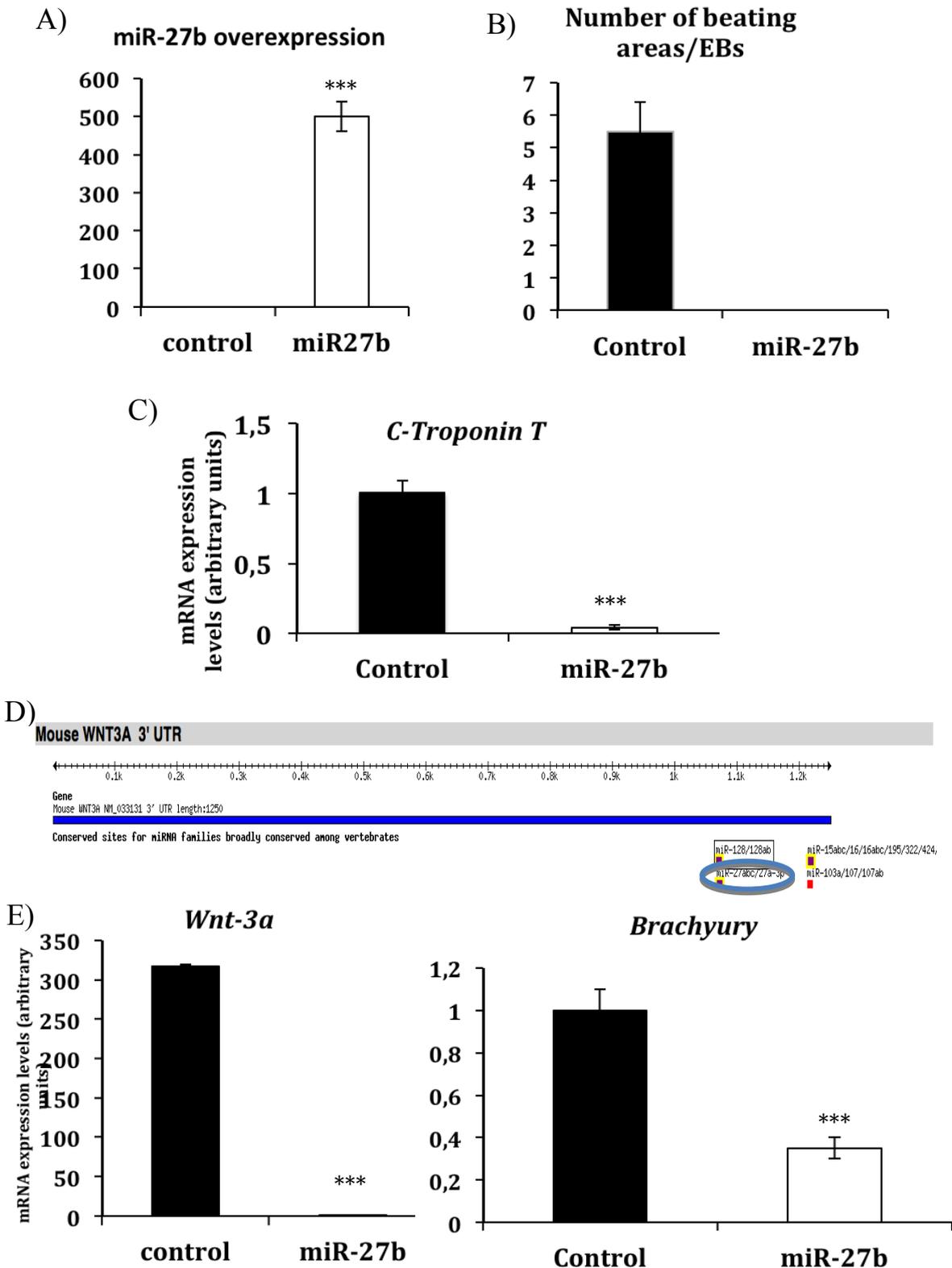
Our previous microarray analyses of the microRNA expression profile during mouse cardiac chamber formation at three different developmental stages (E12.5, E15.5, and E18.5) revealed that only a cluster, representing the 11% of the analyzed miRNAs, display increasing expression levels during this developmental window [9]. To further explore the role of this cluster of miRNAs on cardiomyocyte differentiation we have used the *in vitro* system to cardiomyocyte differentiation from murine embryonic stem cells. We decided to analyze first the effects of those microRNAs that display early differential expression profiles, such as miR-27b. For this purpose, embryos bodies formed from mouse embryonic stem cell R1 cell line were transfected when plated with pre-miR-27b and cultured for 7 days on differentiating medium. Cardiac phenotype was analyzed at this step. Isolated beating areas and embryos bodies were collected for RT-PCR analyses. Surprisingly we observed that miR-27b-transfected EBs fail to form beating areas and the expression of cardiac markers such as c-Troponin T was clearly diminished, indicating that cardiac differentiation was inhibited in the EBs overexpressing miR-27b (Figure 1A–C, Supplementary Material Figures 1 and 2).

We have previously demonstrated that miR-27b regulates Mef2c in differentiated myogenic cells [9]. However, Mef2 transcription factor family members are dispensable for cardiac lineage specification and determination [11]. Therefore, we have searched for novel miR27 targets, which might mediate early cardiac specification. Using bioinformatics analyses (Targetscan software) we identify *Wnt3a* as a miR-27b predicted target (Figure 1C). RT-PCR analyses showed that *Wnt3a* is clearly down regulated on EBs-overexpressing miR-27b (Figure 1D) indicating that this effective activator of the Wnt canonical pathway is regulated by miR27b in our system. Given that in murine ES cells, the early inhibition of the canonical *Wnt3a* pathway blocks the expression of mesoderm specific marker genes such as Brachyury [12], we have looked for Brachyury expression in our system. As showed in Figure 1D, the expression levels of Brachyury was clearly decreased on EBs-overexpressing miR-27b indicating that the lost of mesoderm induction mediated by Brachyury may underlay the inhibition of cardiac differentiation in this system. These results point out a role of miR-27b regulating mesoderm specification and as a consequence cardiac differentiation from cultured EBs.

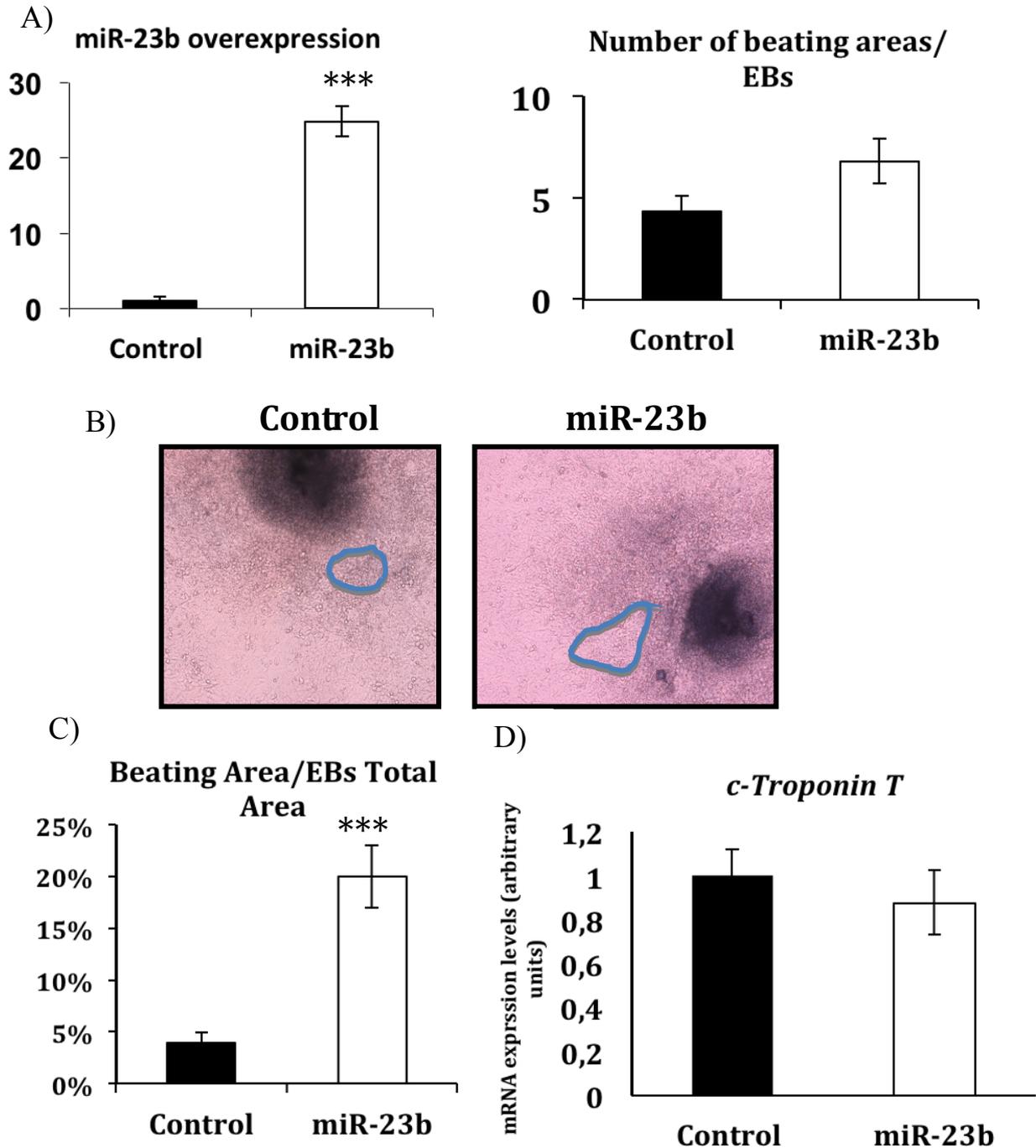
#### 3.2. miR-23b Regulates the Beating Phenotype During *in Vitro* Cardiac Differentiation from ESCs

Next, we tested the effects on *in vitro* cardiomyocyte differentiation of the microRNAs that display late differential expression profiles during cardiac maturation, such as miR-23b. In contrast to miR-27b, EBs overexpressing miR-23b normally develop beating areas in a similar ratio as to control EBs, indicating that miR-23b overexpression does not alter beating areas formation (Figure 2A). However the size of beating areas from EBs overexpressing miR-23b was significantly larger than the size of control beating areas (Figure 2B,C) whereas no changes in the expression levels for terminally differentiated genes such as c-troponin T were detected (Figure 2D). Moreover, we found that the number of beats per minute was significantly higher in beating areas-overexpressing miR-23b (Figure 3A, Supplementary Material Figures 2 and 3), suggesting that miR-23b overexpression instead to increase cardiac differentiation, might be modifying the beating activity of cardiomyocytes derived from ESCs.

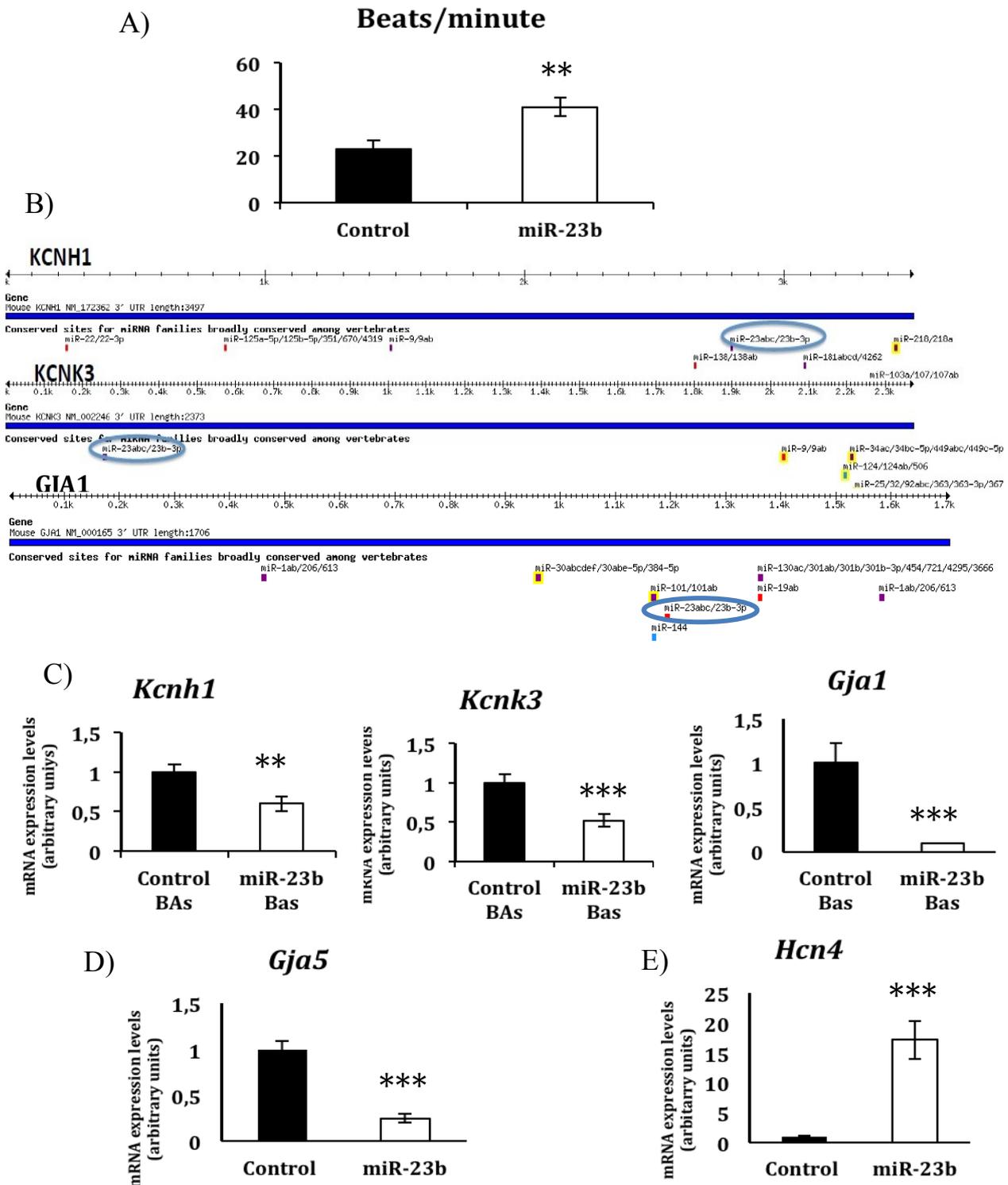
**Figure 1.** (A) Overexpression of miR-27b in embryonic bodies (EBs) derived from murine Embryonic Stem Cells (ESCs) analyzed by RT-PCR. (B) EBs-overexpressing miR-27b fail to form beating areas (n = 22). (C) *c-Troponin T* expression profiles on EBs overexpressing miR-27b. (D) Schematic representation of the putative microRNA binding sites in *Wnt3a* 3'-UTR region as revealed by TargetScan algorithm (www.targetscan.org). (E) *Wnt3a* and *Brachyury* expression profiles on EBs overexpressing miR-27b.



**Figure 2.** (A) Overexpression of miR-23b in EBs derived from murine ESCs analyzed by RT-PCR and number of beating areas in EBs overexpressing miR-27b respect to control. (B) Representative image showing the size of beating areas in EBs overexpressing miR-23b respect to control. (C) Percentage of the size of beating areas respect to total area of EBs (n = 20). (D) *c-Troponin T* expression profiles on EBs overexpressing miR-27b.



**Figure 3.** (A) Quantification of the number of beats per minute in beating areas from EBs overexpressing miR-23b (n = 15) in comparison with beating areas derived from EBs control (n = 13). (B) Schematic representation of the putative microRNA binding sites in *Hcnh1* and *Kcnk3* 3'-UTR regions as revealed by TargetScan algorithm (www.targetscan.org). (C) *Hcnh1*, *Kcnk3* and *Gja1* expression profiles on EBs overexpressing miR-27b. (D) *Gja5* expression profile on EBs overexpressing miR-27b. (E) *Hcn4* expression profile on EBs overexpressing miR-27b.



Interestingly, using bioinformatics algorithms as previously mentioned, we identified *Kcnh1*, *Kcnk3* and *Gjal* as predicted targets for miR-23b (Figure 3B). *Kcnh1* and *Kcnk3*, encode different potassium channels, which distinctly modify the action potential of excitable cells [13,14] and *Gjal* encodes for connexin 43 (Cx43), one of the fast conductance gap junctions more extensively distributed in the myocardium [15]. RT-PCR analysis for *Kcnh1*, *Kcnk3* and *Gjal* revealed that *Kcnh1*, *Kcnk3* and more significantly *Gjal* were down-regulated in beating areas overexpressing miR-23b (Figure 3C). Additionally, connexin 40 (Cx40, *Gja5*); which together with Cx43 is abundantly expressed in the myocardium and the conduction system but sparsely expressed in the sinoatrial and atrio-ventricular nodes [15], was found to be also down regulated (Figure 3D). However, an up-regulation for the pacemaker specific marker *Hcn4* was detected in beating areas overexpressing miR-23b (Figure 3E). Taken together, these findings reveal that miR-23b plays a role in modulating the electrophysiological features of differentiating cardiomyocytes from embryonic stem cells.

#### 4. Discussion

Since the functional benefits reported for stem cell have been modest and mainly due to paracrine effects rather than differentiation of the applied cells into cardiomyocytes [16]; new tools are necessary in order to improve stem cell differentiation towards specific cardiovascular lineages. In the last decade microRNAs have been found to play important roles in the regulation of multiple biologic functions, including the control of stem cell and tissue differentiation [16]. Here, we report the role of miR-27b and miR-23b in cardiomyogenesis using an overexpression strategy in murine embryonic stem cells.

Early in development cardiac progenitors cells are specified in the mesodermal germ layer and many studies have shown that the formation of mesoderm is dependent on canonical Wnt signaling. In fact, loss of Wnt3a function is accompanied by the absence of mesoderm-specific marker genes [12,17]. Here we have shown that miR-27b regulates cardiac differentiation from murine embryonic stem cells by modulating Wnt canonical pathway leading to down-regulation of mesoderm-specific genes such as *Brachyury*. These finding reveal previous unknown functions of miR-27b on early cardiac specification.

Cardiomyocyte maturation from embryonic stem cells requires the acquisition of specific electrophysiological properties that lead to the formation of specific cardiac phenotype such as atrial, nodal or ventricular cardiomyocytes [18] and it has been previously reported that miRNAs such as miR-1 and miR-133 can post-transcriptionally regulate the electrophysiological characteristics on cardiomyocytes [19]. Here we report that miR-23b overexpression modify the beating during *in vitro* cardiac differentiation from ESCs. We have shown that miR-23b overexpression down-regulates the expression of its putative targets *Kcnh1*, *Kcnk3* and *Gjal*. The *Kcnh1* gene encodes a voltage-gated potassium channel highly expressed in neurons and is involved in tumor cell proliferation [20]; however, its physiological on the heart remain largely unclear. *Kcnk3* encodes for a member of the two-pore-domain potassium channel family, the TASK-1 channels which has been considered to have a role in determining the action potential duration [14]. *Gjal* (Cx43) and *Gja5* (Cx40) down-regulation together with *Hcn4* up-regulation has been recently reported to be crucial for activation of the pacemaker gene program, and suppression of the working myocardial gene program in EBs [21]. Besides *Gjal* down-regulation we have found that the expression levels for *Gja5* were lower and the

expression levels for *Hcn4* higher in beating areas overexpressing miR-23b suggesting that miR-23b overexpression leads to a more defined pacemaker phenotype. Therefore, our findings point out a role of miR-23b defining the electrophysiological characteristics of cardiomyocytes derived from ESCs.

## 5. Conclusions

In conclusion, this study could have a relevant impact in regenerative medicine since we showed that miR-27b and miR-23b overexpression modify the cell fate through cardiac lineage from ESCs. Additional experiments and further analyses could lead us to propose these miRNAs as excellent tools to obtain specific cardiac cell types from ESCs.

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## Author Contributions

Jose Manel Vilches: Collection and/or assembly of data; Antonio Pulido: Collection and/or assembly of data; Francisco Hernández-Torres: Collection and/or assembly of data; Diego Franco: Data analysis and interpretation; Manuscript writing; Amelia Aránega: Conception and design; Data analysis and interpretation, Financial support, Manuscript writing.

## Conflicts of Interest

The authors declare no conflict of interest.

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