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Posted Date: 12 January 2024

doi: 10.20944/preprints202401.0999.v1

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miR-223, but Not miR-195, Modulates Myocardial-Induced Proepicardial Cell Migration

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Abstract: Cardiac development is a complex developmental process that results in the formation of the four-chambered organ from a single linear heart tube. In the early stages of development, the linear heart tube is composed of only two tissue layers, an external myocardium that is internally lined by the endocardium. Subsequently, the proepicardium emerges at the *septum transversum* and soon thereafter proepicardial cells will migrate into the naked myocardium, leading to the formation of the embryonic epicardium. As cardiac development proceeds, epicardial-derived cells migrate into the subepicardial space and subsequently invade the developing ventricular chambers leading to the contribution of distinct cardiovascular cell types such as the cardiac fibroskeleton and different components of coronary vasculature. At present, the molecular mechanisms that regulate the transitional process from the proepicardium to the embryonic myocardium are largely unexplored. In this study we have implemented an *ex vivo* proepicardium/septum transversum (PE/ST)-embryonic myocardium explant model and we demonstrated that miR-223, but not miR-195, is capable of modulating PE/ST migration, a process that seems to be mediated by *Slug* expression. Thus, our study demonstrates for the first time the implication of distinct microRNAs in the PE/ST to embryonic myocardium transition in chicken embryonic hearts.

Keywords: microRNAs; proepicardium; cell migration

1. Introduction

Cardiac development is a complex developmental process leading to the formation of four-chambered organ from a single linear heart tube [1]. In the early stages of development, the linear heart tube is composed of only two tissue layers, an external myocardium that is internally lined by the endocardium. With subsequent development, a rightward looping invariably occurs and the atrial and ventricular chambers are progressively formed [2,3]. At this stage, the proepicardium (PE) emerges at the *septum transversum* [4–6] and soon thereafter proepicardial cells will migrate into the naked myocardium, giving rise to the formation of the embryonic epicardium [7–9]. As cardiac development proceeds, compact and trabecular compartments are distinguished in the developing ventricular chambers and epicardial-derived cells (EPDCs) migrate into the subepicardial space [10] and subsequently invade the developing ventricular chambers [7–9], leading to the contribution of distinct cardiovascular cell types. In chicken embryos, EPDCs particularly contribute to the cardiac fibroskeleton and coronary vasculature [11]. Importantly, experimental evidence consistently demonstrated that impaired formation of the proepicardium and/or subsequently of the embryonic epicardium hampers ventricular compact layer formation as well as the coronary vasculature development [12]. Thus, these data demonstrate an epicardial-myocardial crosstalk during cardiac development.

It is important to realize in this context that the mode of transition from the proepicardium to the embryonic epicardium is distinctly achieved in different species. While in chicken, the proepicardium/*septum transversum* (PE/ST) cells directly contact with the naked embryonic myocardium, providing a physical bridge that facilitates cell migration to colonize and extend along

 doi:10.20944/preprints202401.0999.v1

the entire myocardium [7–9,12], in other species, such as zebrafish and mice, small cellular vesicles are delivered to the pericardial fluid that subsequently favours the transportation of these cistae toward to embryonic myocardium [13]. At present, the molecular mechanisms that regulate the transitional process from the proepicardium to the embryonic myocardium are largely unexplored.

Non-coding RNAs are emerging as novel transcriptional and post-transcriptional regulators in multiple biological processes, including embryonic development. Currently, non-coding RNAs are subdivided into two distinct categories according to their length, i.e. small (<200 nt) and long (>200 nt) non-coding RNAs [14]. Within the small non-coding RNAs, microRNAs represent the most abundant and well-studied subclass [14]. microRNAs are 22-24 nt length, they are nuclearly encoded and they modulate post-transcriptional regulatory mechanisms by anchoring to target transcripts through base-pair complementarity [14]. Over the last decades we have witnessed an increasing number of evidences on the pivotal roles of microRNAs in cardiovascular development and diseases [15–22]. Furthermore, microRNAs are essential for epicardial formation as reported by Singh et al. [23], since conditional deletion of *Dicer*, a key microRNA processing exonuclease, in the developing epicardium is essential for correct development of the coronary vessels in mice. In addition, Brønnum et al. [24] identified miR-21 as a key microRNA regulating *Pdcd4* and *Spry1* and thus controlling fibrogenic epithelial-to-mesenchymal transition (EMT) while Pontemezzo et al. [25] reported that Tgf-β1 induced EMT resulted in miR-200c inhibition that, in turn, modulated *Fstl1* impacting thus on mouse epicardial cell transition.

We have recently reported that miR-195 and miR-223 can modulate PE/ST cell fate, increasing the cardiomyogenic lineage commitment [26]. Particularly, miR-195 promotes such enhancement by modulating Smurf1 and Smad3 [26]. However, it remains to be elucidated whether these microRNAs also affect the PE/ST to embryonic epicardium transition. In this study we have implemented an *ex vivo* PE/ST-embryonic myocardium interactive explant model and we demonstrated that miR-223, but not miR-195, is capable of modulating PE/ST migration, a process that seems to be mediated by *Slug* expression. Thus, our study demonstrate for the first time the implication of distinct microRNAs in the PE/ST to embryonic myocardium transition in chicken embryonic hearts.

2. Materials & Methods

2.1. Chicken Embryos and Tissue Collection

Fertilized Leghorn White chicken eggs (Granja Santa Isabel, Córdoba, Spain) were incubated at 37°C and 50% humidity until HH17 stage (~72h) [27]. Embryos at this stage were used for PE/ST and ventricles isolation. The embryos were removed from the egg by cutting the blastocyst margin with iridectomy scissors and placing them into Phosphate Buffered Saline (PBS) supplemented with P/S 1x (SIGMA). Subsequently, HH17 embryonic hearts and PE/STs, respectively, were isolated individually and preserved for *in vitro* explant cultures.

2.2. Tissue Cultures and miRNA Transfections

HH17 naked ventricles were cultured into collagen gels as previously described Bonet et al. [20], and incubated for 24 or 48 hrs at 37°C, respectively. PE/ST transfection was carried out with Lipofectamine 2000 (Invitrogen) where 50 nM of pre-miRNAs (microRNA precursors) or 75 nM anti-miRNAs (microRNAs inhibitors) were applied in hanging drops for 24 or 48 hrs, respectively. Negative controls, HH17 PE/ST explants treated only with Lipofectamine, were run in parallel.

2.3. PE/ST Migration Assays and Time-Lapse Confocal Image Analyses

After 24h or 48h of transfection, PE/ST were treated with Vybrant CFDA SE Cell Tracer Kit (V12883 – Invitrogen) following the manufacturer's recommendations. This labeling method enables *in vivo* tracing of the stained PE/ST by fluorescence microscopy. The PE/ST were settled next to the HH17 naked ventricles cultured previously into collagen gels. Different conditions were analyzed based on the initial distance between the PE/ST and the naked ventricle; i.e. <300 μM, 300-600 μM and >600 μM. Transfections with corresponding pre-miRNAs, anti-miRNAs and negative controls,

 doi:10.20944/preprints202401.0999.v1

respectively, were immediately placed into the culture chamber of the time-lapse laser confocal microscopy (Leica TCS SP5) maintaining suitable cell tissue culture conditions. Time-lapse analysis was performed over 24h, with images captured every 10 minutes.

2.4. Quantitative Analyses of Time-Lapse Migration

Quantification of the recorded time-series images of PE/ST migration towards the naked ventricle was analyzed using the annotation tool within the Leica LAS AF Lite software. Data are represented as the percentage of migration in relation to the initial seeding distance between the embryonic myocardium and the PE/ST explant.

2.5. RNA Isolation and qPCR

RNA samples from the transfected PE/ST were used. All RT-qPCR experiments followed MIQE guidelines [28] and were conducted similarly to previously reports [20,29]. Briefly, RNA was extracted and purified by using Arcturus™ PicoPure™ RNA Isolation Kit (Thermo 12204-01) according to the manufacturer's instructions. For microRNA expression analyses, 10 ng of total RNA was used for retro-transcription with miRCURY™ LNA™ RT kit (Qiagen 229340) and the resulting cDNA was diluted 1/80, following manufacture´s guidelines. Real time PCR experiments were performed with 4 µL of the diluted cDNA, GoTaq qPCR Master mix (Promega A6002) and corresponding primer sets. For mRNA expression measurements, 100 ng of total RNA was used for retro-transcription with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). Real time PCR experiments were conducted with 2 µL of the diluted cDNA, GoTaq qPCR Master mix (Promega A6002) and corresponding primer sets. All qPCRs were performed using a CFX384™ thermocycler (Bio-Rad) following the manufacturer's recommendations. The relative expression levels of each gene was calculated as described by Livak & Schmittgen [30] using *Gapdh* and *Gusb* as internal control for mRNA expression analyses and *5S* and *6U* for microRNA expression analyses, respectively. Each PCR reaction was carried out in triplicate and repeated in at least three distinct biological samples to obtain representative means.

2.6. Statistical Analyses

For statistical analyses of datasets, one way ANOVA and unpaired Student's t-tests were used, as required. Significance levels or P values are stated in each corresponding figure legend. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Establishment of an Ex Vivo Model of Myocardial-Epicardial Cell Migration

In order to dissect the functional role of the embryonic myocardium in inducting the migration and subsequent lining of proepicardial cells onto the naked embryonic myocardium, we established an *ex vivo* model of proepicardial-myocardial interaction. HH17 embryonic PE/ST and hearts were dissected, and PE/ST were labelled with CFDA vital marker to assess their migratory behavior. Both HH17 PE/ST labelled and embryonic hearts were co-cultured on collagen gels at distinct distances between each other ranging from 50 to 800 microns for 24h and observed using by time-lapse confocal scanner laser microscopy (**Figure 1A–C**). Comparative analyses of the distance between the PE/ST and the embryonic heart after 24h of culture demonstrate that PE/ST cell migration is enhanced at closer distances (0-300 microns) while it is significantly diminished at larger distances (>600 microns), displaying an intermediate range those located between 300 to 600 microns (**Figure 1D**). Overall these data demonstrate that myocardial induction of PE/ST cell migration is dependent on the proximity of both tissues.

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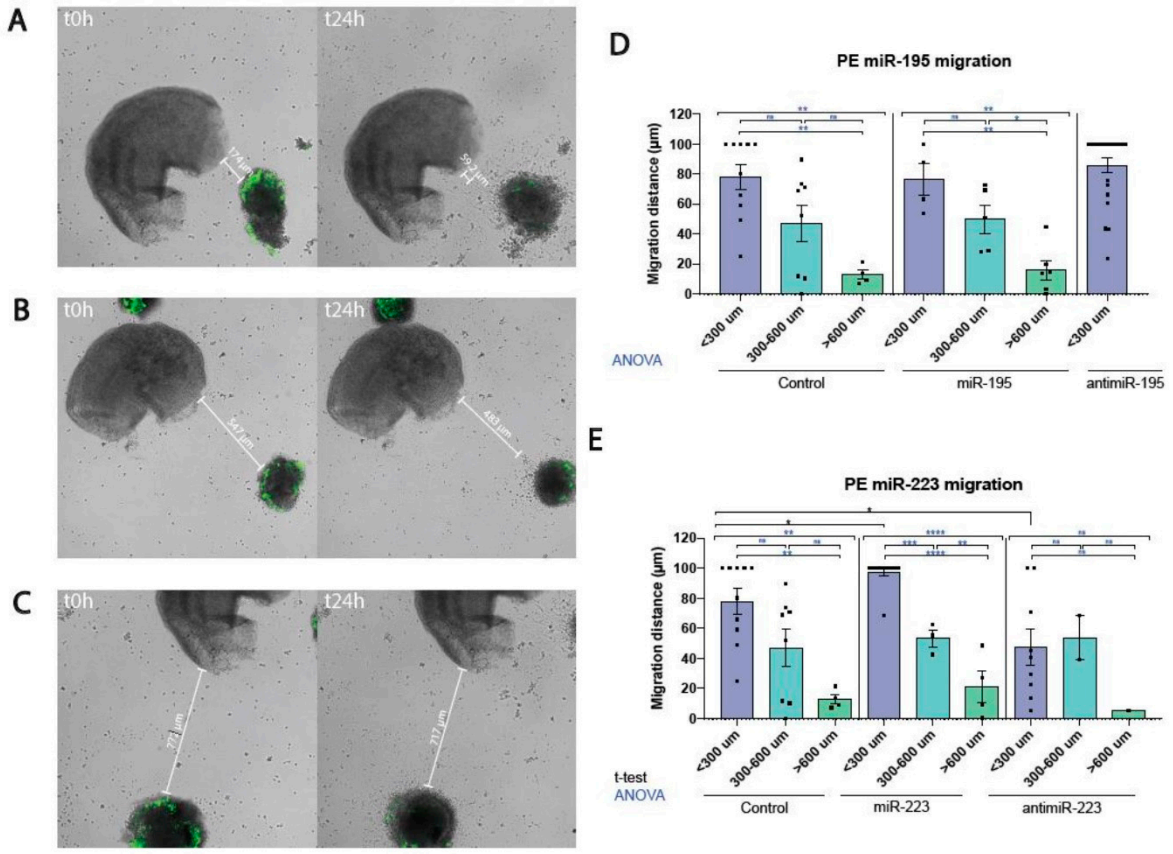

Figure 1. Representative images of the *ex vivo* model of myocardial-PE/ST cell migration assay at three distinct distances (panel A <300 µm, panel B, 300-600 µm and panel C >600 µm) at t=0 and at t=24 h, respectively. Panel D displays the quantitative analyses of the migratory behavior of PE/ST cells in controls, miR-195 overexpression as well as in miR-195 inhibition conditions, as the percentage of migration in relation to the initial seeding distance. Panel E displays the quantitative analyses of the migratory behavior of PE/ST cells in controls, miR-223 overexpression as well as miR-223 inhibition conditions, as the percentage of migration in relation to the initial seeding distance. Significant differences, as revealed by ANOVA analyses, are obtained by comparing three distinct distances in controls, miR-195 and miR-223 treated conditions. Furthermore, observe the significant differences for miR-223 gain- and loss-of-function, leading to enhanced and decreased migration, respectively, as revealed by unpaired Student's t-tests. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

3.2. The Role of miR-195 Modulating PE/ST Cell Migration

We have previously documented that miR-195 plays a fundamental role in promoting cell lineage determination during chicken PE/ST development [26]. We now elaborated on whether miR-195 can influence PE/ST cell migration. Taking advantage of the above mentioned model of PE-embryonic myocardial migration assay, we developed both miR-195 gain- and loss-of-function strategies by pre-incubating the isolated PE/ST with pre-miR-195 mimics (24 hours) and anti-miR-195 (48 hours) prior to seeding in close apposition to the HH17 embryonic hearts. Analyses of the migratory behavior of these PE/ST under these different conditions demonstrated that neither miR-195 gain-of-function nor miR-195 loss-of-function significantly altered PE/ST cell migration in any of the three distinct ranges analyzed (<300 microns, 300-600 microns and >600 microns; for miR-195 loss-of-function only <300 microns is available) (**Figure 1D**). Importantly, as in controls, a significant difference was observed between these conditions, in such a way that PE/ST cell migration is enhanced at closer distances (0-300 microns) while it is significantly diminished at larger distances (>600 microns),

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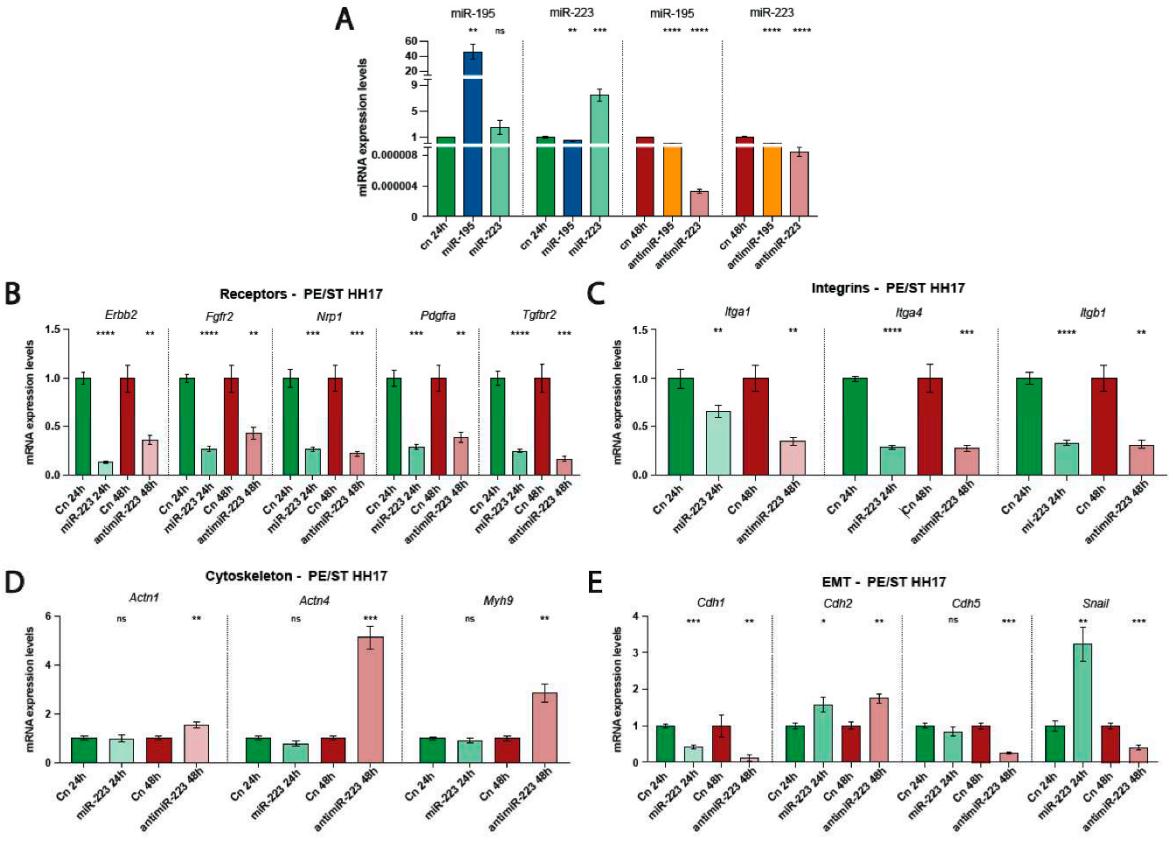
3.3. The Role of miR-223 Modulating PE/ST Cell Migration

Similarly, previous studies demonstrated a pivotal role for miR-223 in chicken PE/ST development [26] and thus we analyzed in this study its plausible contribution to PE/ST migration. Gain- and loss-of-function strategies by pre-incubating the isolated PE/ST with pre-miR-223 mimics (24 hours) and anti-miR-223 (48 hours) prior to seeding in close apposition to the HH17 embryonic hearts. Analyses of the migratory behavior of these PE/ST conditions demonstrated that miR-223 gain-of-function significantly increased PE/ST cell migration at close distances (<300 microns), while no significant differences were observed at intermediate and large distances (300-600 microns and >600 microns). Importantly, miR-223 loss-of-function significantly decreased PE/ST cell migration at close distances (<300 microns), while no significant differences were observed at intermediate and large distances (300-600 microns and >600 microns) (**Figure 1E**). Thus, these data demonstrate that miR-223 plays an essential role in regulating PE/ST cell migration, particularly in short PE/ST *vs.* embryonic heart distances. Furthermore, these data also reinforce the notion that such mechanisms are only regulated in a limited range of distance between both tissues.

3.4. Co-Regulation of miR-195 and miR-223 Expression in PE/ST Explants

Validation of miRNA gain- and loss-of-function assays were done in the corresponding treated PE/ST. As expected, miR195 mimic administration leads to upregulation of miR-195 while no significant differences were observed in miR-223 expression. On the contrary, miR-223 mimic administration, while increased miR-223 expression as expected, miR-195 expression was significantly down-regulated (**Figure 2A**). We subsequently tested miR-195 inhibition after anti-miR-195 treatment, that resulted in significant downregulation of miR-195 as expected but surprisingly miR-223 was also significantly downregulated. Furthermore, miR-223 inhibition by anti-miR-223 administration lead to significant downregulation of both miR-223 as well as miR-195 (**Figure 2A**). Thus these data demonstrate a molecular crosstalk between these microRNAs, suggesting that coordinated regulation of these microRNAs might be required as proepicardium /embryonic epicardium development proceeds.



 doi:10.20944/preprints202401.0999.v1

Figure 2. RT-qPCR analyses of microRNAs and candidate target mRNAs. Panel A RT-qPCR analyses of miR-195 and miR-223 after gain- and loss-of-function assays. Observe that over-expression of miR-195 and miR-223 leads to increased expression of the corresponding miRNA, as expected, but surprisingly, the miR-195 expression is down-regulated after miR-223 overexpression. Similarly, miR-195 and miR-223 inhibition, led to decreased expression of the corresponding miRNA, but similarly, the other microRNA expression was also significantly downregulated. Panels B-E. RT-qPCR analyses of candidate target mRNAs corresponding to distinct receptors (panel B), integrins (panel C), cytoskeletal (panel D) and EMT (panel E) markers. Observe that miR-223 gain-and loss-of-function assays can distinct modulate several of these markers, but only *Slug* displays increased and decreased expression after miR-223 gain-and loss-of-function, respectively. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

3.5. Dissecting the Molecular Mechanisms Driving miR-223-Mediated PE/ST Cell Migration

Since miR-223 gain and loss-of-function displayed complementary behavioral patterns on PE/ST cell migration, we tested whether distinct molecular markers of cell signaling pathways, cytoskeleton and/or EMT involved in cell migration are distinctly modulated by miR-223. microRNAs have the potential to modulate the expression of hundreds of target mRNAs [31,32] as revealed by predictive tools such as TargetScan, MirWalk and RNA22 among others. Taking into account such predictions and considering that miR-223 administration might primarily acts on membrane receptors that can modulate myocardial instructive signals, we selected a discrete number of receptors involved in cell signaling such as *Nrp1, Erbb2, Tgfb2, Fgfr2* and *Pdgra.* RT-qPCR analyses of these receptors were tested in miR-223 gain- and loss-of-function PE/ST conditions. Curiously all selected receptors were significantly down-regulated in both miR-233 gain- and loss-of-function assays (**Figure 2B**). Similarly, we also tested cell-extracellular matrix interacting molecules such as integrins. *Itgb1*, *Itga1* and *Itga4* expression was also down-regulated in both miR-233 conditions, supporting the notion that these signaling pathways are not involved in PE/ST cell migration upon miR-223 administration (**Figure 2C**). On the other hand, RT-qPCR analyses of actin cytoskeleton proteins, such as *Actn1, Acnt4* and *Mhy9* were not significantly modulated by miR-223 mimics administration while all of them were significantly increased after miR-223 loss-of-function (**Figure 2D**). These data suggest that cytoskeleton modulation might be required for cell migration inhibition but not progression, although additional experiments are required to further support this hypothesis.

Importantly, EMT marker *Slug* was significantly upregulated after miR-223 mimics administration and significantly downregulated after miR-223 loss-of-function, supporting the notion that *Slug* modulation might be the triggering partner associated with miR-223 modulation of PE/ST cell migration (**Figure 2E**). Curiously, cadherin expression was distinctly altered on these two distinct conditions; *Cdh1* was significantly downregulated while *Cdh2* was significantly upregulated in both conditions and additionally, *Cdh5* was significantly downregulated after miR-223 inhibition but not altered by miR-223 mimic administration (**Figure 2E**). Overall these data support the notion that although miR-223 can selectively modulate distinct cadherin expression, such modulation is not directly related to the increase or decrease of PE/ST cell migration observed after miR-223 over-expression and inhibition, respectively.

4. Discussion

It has been previously demonstrated that PE/ST cells can attach to the naked embryonic myocardium, primarily at the interventricular sulcus, providing an anchor for subsequent expansion along the surface of the ventricular and atrial myocardium in chicken embryos [12]. Mechanistically, little insights have been gained on the molecular determinants of this process in chicken embryos [32,33] while some piece of evidence are reported in other species, particularly in zebrafish [13,34–37]. Importantly, the role of microRNAs in the development of the epicardium was reported by the generation of *Dicer*-specific mutant mice [23], yet the contribution of discrete microRNAs is less documented [24–26]. We have therefore established an *ex vivo* experimental model of chicken PE/ST-myocardium interaction to decipher the functional role of distinct microRNAs in this process. We

 doi:10.20944/preprints202401.0999.v1

have previously documented that over-expression of miR-195, and to a lesser extent miR-223, in chicken PE/ST explants enhances cardiomyogenic lineage specification [26]. However, the role of these microRNAs in PE/ST to embryonic epicardial transition remains unexplored.

The functional role of miR-195 has been reported in multiple cardiovascular contexts [38–40]; i.e. is upregulated in heart failure [41], regulates cardiac metabolism [42], and promotes fibrosis in myocardial infarction [43,44] as well as cardiac hypertrophy [45]. Additionally, it has been reported that miR-195 can modulate cell migration, particularly in cancer [46–50], but also in other biological contexts [51,52]. We therefore tested whether miR-195 gain- or loss-of-function in PE/ST explants could modulate PE/ST myocardial-induced cell migration. Analysis of the PE/ST migratory behavior resulted in no significant differences as compared to controls supporting the notion that miR-195 does not play a role in PE/ST cell migration.

Similarly, the role of miR-223 has also been reported in several cardiovascular contexts [53–59] as well as in promoting cell migration, particularly in oncogenic processes [60–65]. Interestingly, in contrast to miR-195, miR-223 gain- and loss-of-function assays significantly modulate PE/ST cell migration. We therefore sought to investigate the plausible molecular mechanisms underlying this miR-223-driven PE/ST cellular behavior. We reasoned that since PE/ST behavior was dependent on the PE/ST to myocardial explant distance, a myocardial emanating signal might be promoting PE/ST cell migration and thus miR-223 mediated modulation of PE/ST cell migration might be due to membrane receptor targeting. We therefore scrutinized several signaling pathway receptors involved in cell migration such as *Erbb2* [66,67], *Fgfr2* [68,69], *Nrp1* [70,71], *Pdgfra* [68] and *Tgfbr2* [72,73]. Unexpectedly, none of these receptors were differential modulated by miR-223 gain- and loss-of-function assays, supporting the notion that they do not play a role in miR-223 mediated modulation of PE/ST cell migration. We subsequently tested whether such modulation might be mediated by cell-extracellular matrix receptors, i.e. integrins, given their pivotal role in cell migration in distinct cardiovascular contexts [74–76]. Similarly, *Itga1, Itga4* and *Itgb1* were not differential modulated by miR-223 gain- and loss-of-function assays. Curiously, in both cases, cell signaling and cell-extracellular matrix receptors were significantly downregulated in both conditions, i.e. miR-223 gain- and loss-of-function assays and therefore further analyses are required to fully understand these observations.

Thus, we decided to investigate if changes in cytoskeletal proteins were modulated by miR-223 gain- and loss-of-function, given their prominent role in cell migration [77]. Interestingly, significant up-regulation was demonstrated for *Actn1*, *Acnt4* and *Mhy9* after miR-223 loss-of-function, while no significant differences were observed in PE/ST miR-223 gain-of-function assays. Therefore, these results indicate that miR-223 mediated cytoskeletal re-arrangement might be required for cell migration inhibition, but dispensable for cell migration enhancement, providing that cohesive cell migration is occurring. Finally, we also analyzed different key players of EMT [78,79]. Importantly, we demonstrated that *Slug* is significantly up-regulated after miR-223 over-expression and down-regulated after miR-223 inhibition, supporting the notion of a key role for *Slug* promoting miR-223 mediated cell migration. Curiously, cadherin expression is not similarly modulated, supporting the notion that miR-223/*Slug* might mediate cell migration without promoting cell-cell detachment, i.e. cohesive cell migration, as recently reported in distinct oncogenic contexts [80]. It is importantly to highlight that we detected a co-regulatory expression modulation between miR-195 and miR-223 in PE/ST explants. Co-regulation of microRNAs and transcription factors or target genes has been recently reported [81,82], yet to the best of our knowledge this is the first report of miRNA-miRNA co-regulation. While the nature of such co-regulatory mechanisms remains enigmatic, our data support the notion that miR-223 modulation of PE/ST cell migration is not disturbed by miR-195 expression. These observations further indicate that co-regulation is necessary for proper proepicardium development, raising the possibility that miR-223 is required for cell migration in early stages, as reported herein, while miR-195 is needed for subsequent cell lineage differentiation, as previously reported [26], and thus down-regulation is compulsory at early stages of proepicardium development. However, additional experiments will be mandatory to further dissect such miRNA-miRNA co-regulatory modules, as recently proposed *in silico* [83–85].

In sum, our study that PE/ST migration towards the embryonic myocardium is distance dependent and that such PE/ST migratory behavior can be modulated by miR-223, but not miR-195, in such a way that high miR-223 levels enhance migration while low miR-223 expression halted it, a process that seems to be *Slug* dependent.

Acknowledgments: This work is supported by grants of the Spanish Government to D.F and A.A. (PID2022-138163OB-C32) and by grant-in-aid (CTS-446) of the Junta de Andalucia Regional Council.

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