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Article

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Abstract: A large diversity of epigenetic factors, such as microRNAs and histones modifications, are known to be capable of regulating gene expression without altering DNA sequence itself. In particular, miR-1 is considered the first essential microRNA in cardiac development. In this study, miR-1 potential role in early cardiac chamber differentiation is analyzed through specific signaling pathways. For this, we performed in chick embryos functional experiments by means of miR-1 microinjections into the posterior cardiac precursors -of both primitive endocardial tubes- committed to sinoatrial region fates. Subsequently, embryos were subjected to whole mount in situ hybridization, immunohistochemistry and RT-qPCR analysis. As a relevant novelty, our results revealed that miR-1 increases *Tbx5*, *Gata4* and *AMHC1*, while this microRNA diminishes *Mef2c* expression, during early differentiation of cardiac sinoatrial region. Furthermore, we observed in this developmental context that miR-1 upregulates *CRABPII* and *RARß*, and downregulates *CRABPI*, which are three crucial factors in retinoic acid signaling pathway. Interestingly, we also noticed that miR-1 directly interacts with *HDAC4* and *Calmodulin*, as well as with *Erk2/MAPK1*, which are three key factors actively involved in *Mef2c* regulation. Our study shows, for the first time, a key role of miR-1 as an epigenetic regulator in early differentiation of cardiac sinoatrial region through orchestrating opposite actions between retinoic acid and *Mef2c*, fundamental to properly assign cardiac cells to their respective heart chambers. A better understanding of those molecular mechanisms modulated by miR-1 will definitely help in fields applied to therapy and cardiac regeneration and repair.

Keywords: miR-1; epigenetic regulator; cardiac sinoatrial region; molecular mechanisms; HDAC4; Calmodulin; pErk2/MAPK1; Mef2c activity; retinoic acid signaling; cardiac development

1. Introduction

During early embryogenesis, the cardiogenic mesoderm organizes -at both sides of the embryo- the primitive endocardial tubes, which fuse to form the primitive heart tube in the midline [1–4]. Subsequently, the posterior cardiac tube segment differentiates into the cardiac sinoatrial region, including *atrium* and inflow tract/*sinus venosus* [5,6]. Concomitantly, the anterior cardiac tube segment contributes to ventricular and outflow tract differentiation [7,8]. Previous studies have revealed that this segmentation process is regulated by several cardiac transcription factors, including *Tbx5*, *Gata4* and *Mef2c*, among others, which activate the expression of early cardiac structural genes, such as myosin heavy chain (*MHC*) and myosin light chain (*MLC*) [9–13]. It is also

well known that phenotype of cardiac sinoatrial region is influenced by retinoic acid (RA) signaling, mediating the expression pattern of early molecular markers such as *Tbx5*, *Gata4* and *AMHC1* (atrial myosin heavy chain), which together determine the posterior heart tube segment leading to a venous and atrial cell fate [14–16]. In fact, exclusion of RA from ventricular precursors is essential for correct specification of the ventricles [17]. Furthermore, RA synthesis is largely controlled by retinaldehyde dehydrogenase 2 (*Raldh2/Aldh1a2*) [18,19], in such a way that *Raldh2$^{-/-}$* knockout mice present reduced *Tbx5* and *Gata4* expression and severely impaired cardiac sinoatrial region development, while *Mef2c* expression, which induces ventricular cardiomyocyte differentiation [20], is unaltered [21]. Along with the above, *Mef2c$^{-/-}$* knockout mice display ectopic *Tbx5* expression, suggesting that the mutant ventricle acquires atrial-specific characteristics and, therefore, *Mef2c* could act directly on cells and make them nonresponsive to RA inflow differentiation signaling [22].

Several authors have also reported that a large diversity of epigenetic processes, including the expression of non-coding RNAs (especially microRNAs), histones modifications and DNA methylation, are capable of regulating gene expression, by influencing transcription or inhibiting translation through several functional overlapping and cross-talking mechanisms [23,24]. In particular, microRNAs are considered as a post-transcriptional type of epigenetic regulatory processes, since the degree of complementarity microRNA/target mRNA determines its final outcome, either by degradation or translational inhibition [24,25]. Specifically, miR-1 has been the first essential microRNA identified in cardiac development, with a specific expression pattern in cardiac muscle [26–31]. Interestingly, experimental overexpression of miR-1 during heart development results in defective ventricular myocyte proliferation, accompanied by hypoplasia of the cardiac ventricular conduction system [32]. To date, the specific effects of miR-1 during early differentiation on cardiac sinoatrial region are unknown. The previous study that analysed the functional role of miR-1, which was performed in murine embryonic stem cells, was focused on the development and function of sinoatrial cells, corresponding specifically to the sinoatrial node, which determines the cardiac pacemaker [33].

Furthermore, it has been proposed that miR-1 modulates cardiomyocyte growth responses by negatively regulating *Calmodulin*, a miR-1 target gene [34]. In addition, miR-1 regulates human cardiomyocyte progenitor cell differentiation via repression of histone deacetylase 4 (*HDAC4*), also identified as a miR-1 target gene, altering histone-DNA-binding activity [35]. Nevertheless, *HDAC4* can also interact with non-histones by covering the promoter region of a gene, thus inhibiting its expression [36–38]. For instance, *HDAC4* can interact with *Mef2c* by repressing its transcriptional activity, which is required for muscle cell differentiation [39]. Additionally, as described in cardiac hypertrophy, it has been reported that the activation of *Mef2c* transcription is mediated both by Calmodulin and Erk1/2 -extracellular signal regulated kinases 1 and 2, a signal transducer in the family of mitogen activated protein kinase, MAPK- [40–44]. In this sense, it has been recently reported, in a zebrafish model, that Erk1/2 signaling is required to promote ventricular chamber differentiation [45].

Given the complexity of those molecular mechanisms involved in cardiac chamber formation, this experimental study aims to analyse miR-1 potential functions in early differentiation of the cardiac sinoatrial region. By means of gain- and loss-of-function experiments, we show that miR-1 is required for an adequate expression of RA-dependent molecular markers *Tbx5*, *Gata4* and *AMHC1*, and RA signaling pathway genes *RARβ*, *CRABPII* and *CRABPI*. Significantly, our results reveal that miR-1 suppresses *Mef2c* by directly repressing *Mef2c* regulators *HDAC4*, *Calmodulin* and *Erk2/MAPK1*. Additionally, after miR-1 overexpression, we observed morphological alterations, characterized by an enlargement of the heart posterior domain and a reduction of the ventricular region. Altogether, our data support a model in which miR-1 plays a dual role by modulating both *Mef2c* activity and RA signaling pathway, placed as a key epigenetic regulator of appropriate cardiac cell specification, thus promoting the initial differentiation of the cardiac sinoatrial region.

2. Results

Our results show, as a novel approach, that miR-1 administration enhances the expression of *Tbx5*, *Gata4* and *AMHC1*, while it diminishes *Mef2c* expression during early differentiation of the cardiac sinoatrial region. Another original contribution of this work includes the analysis of miR-1 expression pattern, as well as HDAC4, Calmodulin and phospho-Erk2/MAPK1 (pErk2) distributions, from the primitive endocardial tubes to the initial cardiac looping stages. Noticeably, we also observed -for the first time in this developmental context- that miR-1 directly downregulates *HDAC4* and *Calmodulin*, as well as *Erk2/MAPK1*, which are required for *Mef2c* expression. Moreover, this microRNA enhances CRABPII and RARß expressions, while it downregulates CRABPI -three essential factors in RA signaling pathway. Supporting the above, after antimiR-1 administration, we obtained the opposite results.

2.1. miR-1 Regulates Specific Gene Expressions during Early Differentiation of Cardiac Sinoatrial Region

In this research work, we analyse the effect of miR-1 on *Tbx5*, *Gata4*, *AMHC1* and *Mef2c* expression during early differentiation of cardiac sinoatrial region (Figure 1 and Supplementary Figure S1). For this purpose, we carried out miR-1 gain- and loss-of-function experiments by means of respective microinjections of premiR-1 and antimiR-1 into the posterior cardiac precursors -in primitive endocardial tubes- committed to sinoatrial region fates (Figure 1A). Additionally, in an experimental group, cardiac *asa* were dissected and subsequently subjected to RNA extraction and RT-qPCR analysis, under each experimental condition (Supplementary Figure S2). Our results show that miR-1 leads to increased *Tbx5* (Figure 1B), *Gata4* (Figure 1C) and *AMHC1* (Figure 1D) expressions, and RT-qPCR analyses support these findings (Figure 1B-D). Interestingly, miR-1 inhibits mRNA and protein levels of *Mef2c* gene in the cardiac sinoatrial region (Figure 1E). Supporting these data, our miR-1 loss-of-function experiments show an opposite effect (Figure 1, right column, and RT-qPCRs).

Noticeably, in experimental embryos we observed morphological alterations based on an expansion of the posterior domain of the heart after miR-1 administration, concomitantly with a reduced ventricle region (Figure 1 and Supplementary Figure S2A,B).

Additionally, we have observed that microinjections of RA -into the same areas above mentioned- mediate positively the expression of *Gata4* (Supplementary Figure S3), and also *Tbx5* and *AMHC1* as we previously reported [46]. All the above mentioned findings reveal that miR-1 mimics the effects observed with highlighted RA levels.
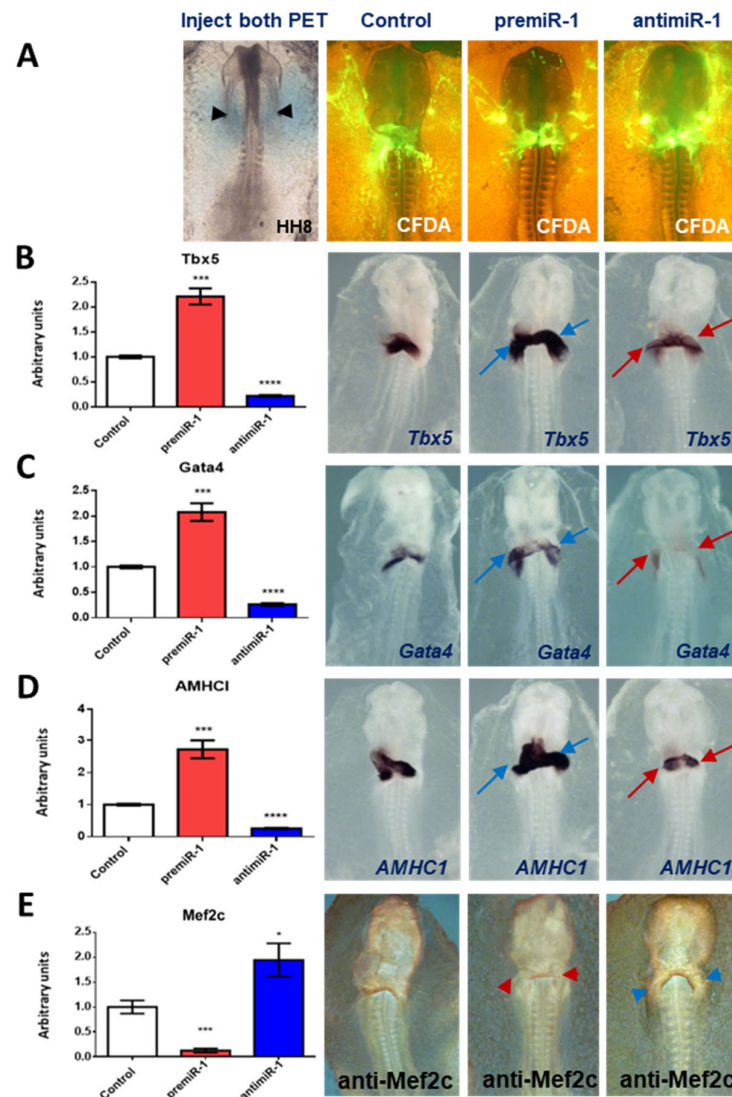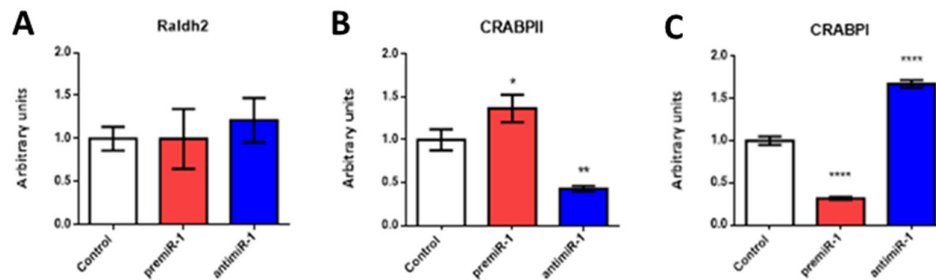
Figure 1. Effects of miR-1 gain- and loss-of-function experiments during early differentiation of cardiac sinoatrial region. Embryos microinjected with CFDA (control), premiR-1 or antimiR-1, at the level of the posterior cardiac precursors of both primitive endocardial tubes, and visualization of CFDA (**A**). Whole-mount ISH for *Tbx5* (**B**) *Gata4* (**C**), *AMHC1* (**D**) and IMH for Mef2c (**E**). Note the increased and expanded expression of *Tbx5*, *Gata4* and *AMHC1* after premiR-1 treatment (blue arrows), accompanied by diminished protein levels of Mef2c (red arrowheads). The posterior diminished expressions of *Tbx5*, *Gata4* and *AMHC1* in the heart tube after antimiR-1 treatment is indicated by red arrows, whereas Mef2c is increased (blue arrowheads). Left side illustrates RT-qPCR of RNA from dissected cardiac *asa* of embryos microinjected either with CFDA, premiR-1 or antimiR-1. High level of miR-1 leads to increased Tbx5, Gata4 and AMHC1 transcripts, whereas miR-1 inhibition leads to Mef2c increased transcripts. Standard deviations are from three independent experiments. Student's *t*-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, **** $p < 0.001$ with respect to control (CFDA) embryos.

2.2. miR-1 Modulates RA Signaling Pathway during Early Differentiation of Cardiac Sinoatrial Region

Since miR-1 modulates RA target gene expression during differentiation of cardiac sinoatrial region, through RT-qPCR we analysed RNAs from dissected cardiac *asa* of embryos microinjected

either with premiR-1 or antimiR-1, in order to explore the relationship between miR-1 and those factors involved in the RA signaling pathway (Figure 2).
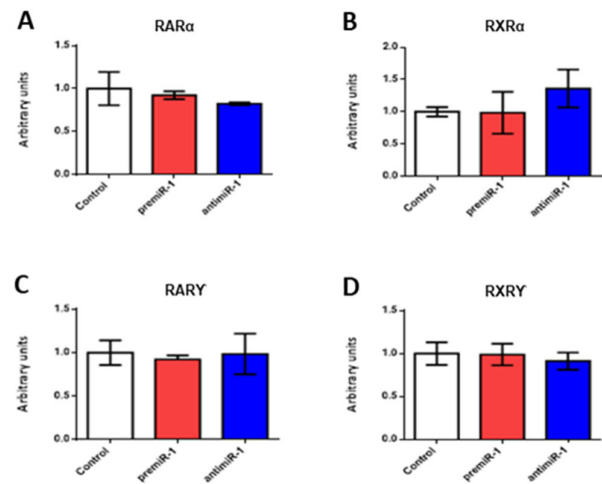

Figure 2. Effects of miR-1 gain- and loss-of-function experiments on RA pathway during posterior differentiation of cardiac tube. RT-qPCR of RNA from dissected cardiac *asa* of embryos microinjected either with CFDA, premiR-1 or antimiR-1. High level of miR-1 leads to increased expression of *CRABPII* and decreased expression of *CRABPI*. The opposite effect is observed with anti-miR1 treatment. Note that nonsignificant expression of *Raldh2* is observed after miR-1 overexpression or miR-1 inhibitor treatment, as compared to control (CFDA). Standard deviations are from three independent experiments. Student's *t*-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, **** $p < 0.001$ with respect to control (CFDA) embryos.

Our findings reveal that miR-1 does not modify the expression levels of *Raldh2* (Figure 2A), a RA synthesis modulator [47]. We also observed that miR-1 is able to increase the endogenous expression levels of *CRABPII* (Figure 2B), a RA target gene that regulates RA delivery to its nuclear receptors (RARs) [48,49]. In addition, our results show that miR-1 leads to reduced levels of *CRABPI* (Figure 2C), which functions as a sequester of RA to facilitate its catabolism [50], thus supporting that miR-1 may upregulate RA signaling. In line with the above results, we obtained the opposite effects through antimiR-1 administration (Figure 2).

With respect to the nuclear retinoic acid receptors, it is to note that *Rarß* is a RA target gene essential for cardiovascular development [51–54]. In this study we include a detailed analysis of this cardiac gene (Figure 3). By using immunohistochemistry (IMH) in a control group of embryos, we identified *Rarß* distribution starting in both primitive endocardial tubes, following in the primitive cardiac tube and continuing in the early cardiac looping formation, presenting a location in sinoatrial region (Figure 3A). Our miR-1 gain-of-function experiments led to an increased expression of *Rarß* and its protein levels (Figure 3B). Supporting these results, we obtained the opposite effect after our miR-1 loss-of-function experiments (Figure 3, right column, and RT-qPCRs). On the other hand, expression modifications of nuclear retinoic acid receptors RARα, RXRα, RARγ and RXRγ were not detected after miR-1 experimental assays (Figure 4).

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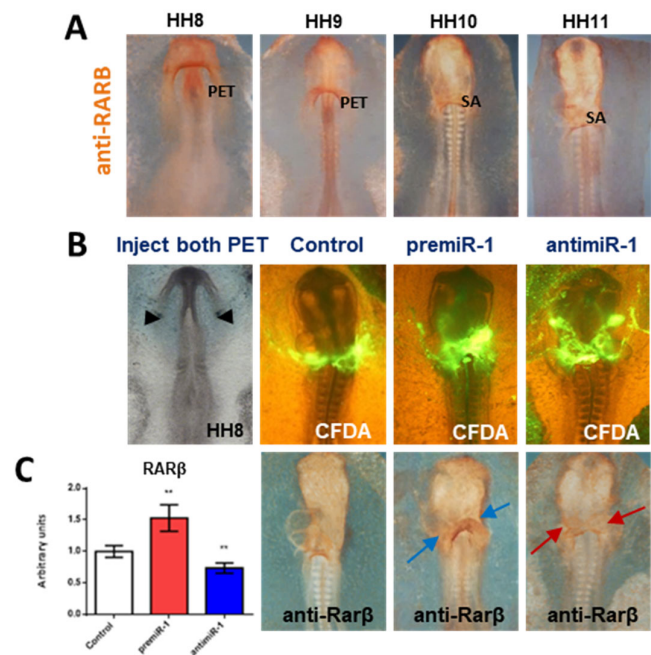
Figure 3. Embryos subjected to *Rarß* study. **A**: Whole-mount IMH for *Rarß* during early chick cardiac development, from HH8 through HH11, in control embryos. Note *Rarß* distribution in both primitive endocardial tubes (PET), being observable in sinoatrial (SA) region at later stages. **B**: Experimental embryos microinjected with CFDA (control), premiR-1 or antimiR-1, at the level of the posterior cardiac precursors of both primitive endocardial tubes (arrowheads), and visualization of CFDA. **C:** Whole-mount IMH illustrate *Rarß* markedly increased after miR-1 administration (blue arrows). Note *Rarß* diminished after antimiR-1 administration (red arrows). RT-qPCR of RNA from dissected cardiac *asa* (left side) show the levels of *Rarß* transcripts. Standard deviations are from three independent experiments. Student's *t*-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, **** $p < 0.001$ with respect to control (CFDA) embryos.

Figure 4. Effects of miR-1 gain- and loss-of-function experiments on RA pathway during posterior differentiation of cardiac tube. RT-qPCR of RNA from dissected cardiac *asa* of embryos microinjected either with CFDA, premiR-1 or antimiR-1. Note that nonsignificant expression of RARα, RXRα, RARY, RXRY are observed after miR-1 overexpression, or miR-1 inhibitor treatment as compared to control (CFDA). Standard deviations are from three independent experiments.

2.3. miR-1 Modulates HDAC4, Calmodulin and Erk2/MAPK1 during Early Differentiation of Cardiac Sinoatrial Region

Given that our experiments revealed that miR-1 decreases both mRNA and protein levels of *Mef2c*, which is not a miR-1 target gene, we searched for pivotal miR-1 targets genes as potential *Mef2c* regulators during differentiation of cardiac sinoatrial region. By using bioinformatics analyses through Target-Scan software, we identified *HDAC4*, *Calmodulin* and *Erk2/MAPK1* as putative targets of miR-1 (Supplementary Figure S4), suggesting that it might exert control on their post-transcriptional regulation. Our luciferase assays show that luciferase signals from plasmids harbouring 3′UTRs of each gene are reduced with respect to control, proving that miR-1 recognizes and directly binds to *HDAC4*, *Calmodulin* and *Erk2/MAPK1* 3′UTRs, thus triggering their mRNA degradation (Figure 5).
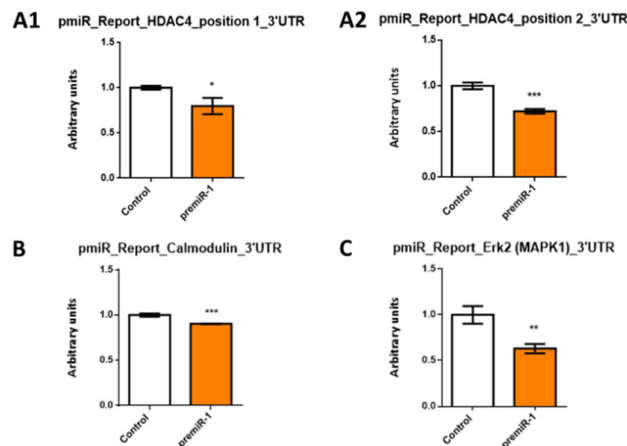
Figure 5. Representative data of *HDAC4* (**A1**: position 1; **A2**: position 2), *Calmodulin* (**B**) and *Erk2/MAPK1* (**C**) 3′UTR luciferase assays after premiR-1 overexpression in 3T3 fibroblasts. Luciferase activity was compared to non-transfected controls. Each luciferase assay was carried out in triplicate. Student's *t*-test: $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

As illustrated in Figure 6, through in situ hybridization (ISH), we report miR-1 expression pattern, starting in both primitive endocardial tubes, following in the primitive cardiac tube and continuing in the early cardiac looping formation. By means of IMH, we observed a similar distribution of HDAC4, Calmodulin, and pErk2 in sinoatrial region during the stages under analyses. Interestingly, our comparative analysis demonstrates a complementary expression pattern among them, suggesting a plausible regulatory link during early posterior cardiac tube differentiation.
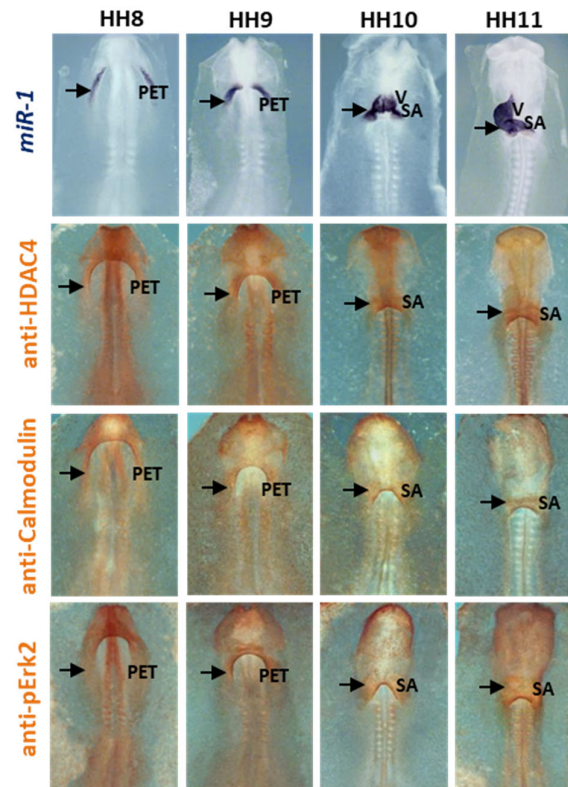
Figure 6. Whole-mount ISH for miR-1 and whole-mount IMH for HDAC4, Calmodulin and phospho-Erk2 (pErk2) during early chick cardiac development, from HH8 through HH11, in control embryos. Note miR-1 expression pattern in both primitive endocardial tubes (PET), being observable in sinoatrial (SA) region and ventricle (V) at later stages. Note the location of HDAC4, Calmodulin and pErk2 in the PET and, subsequently, in the SA region. Arrows: positive stains.

With respect to the miR-1 functional role on *Mef2c* regulators, our gain-of-function experiments show that miR-1 represses both mRNA and protein levels of *HDAC4*, *Calmodulin* and *Erk2* in the sinoatrial region (Figure 7). In agreement with these results, the opposite effects were observed after antimiR-1 administration (Figure 7, right column, and RT-qPCRs). Therefore, our findings indicate that miR-1 modulates *Mef2c* expression through the repression of *HDAC4*, *Calmodulin* and *Erk2* during early differentiation of cardiac sinoatrial region.
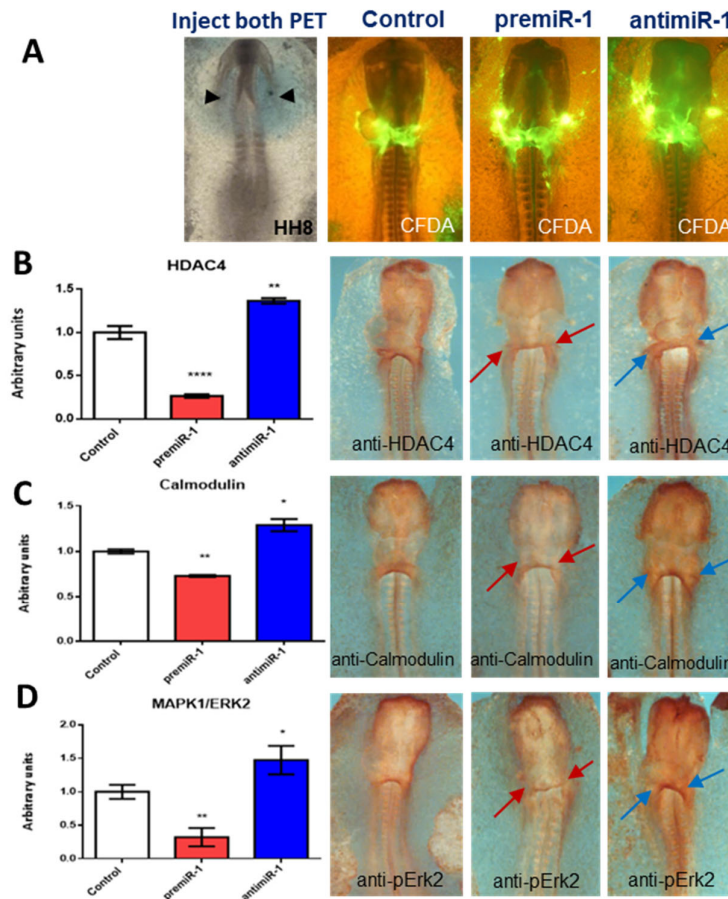
Figure 7. Embryos microinjected with CFDA (control), premiR-1 or antimiR-1, at the level of the posterior cardiac precursors of both primitive endocardial tubes (arrowheads), and visualization of CFDA (**A**). Effects of miR-1 gain- and loss-of-function on target genes HDAC4, Calmodulin and phospho-Erk2/MAPK1 (pErk2) during posterior differentiation of cardiac tube (**B-D**). Whole-mount IMH reveal that HDAC4 (**B**), Calmodulin (**C**) and pErk2 (**D**) are dramatically reduced in the sinoatrial region (red arrows) after premiR-1 administration, whereas they are markedly increased after miR-1 inhibition (blue arrows). RT-qPCR of RNA from dissected cardiac *asa* (left side) in embryos microinjected either with CFDA, premiR-1 or antimiR-1, show that miR-1 leads to decreased *HDAC4*, *Calmodulin* and *Erk2/MAPK1* transcripts, whereas miR-1 inhibition leads to increased transcripts. Standard deviations are from three independent experiments. Student's *t*-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, **** $p < 0.001$ with respect to control (CFDA) embryos.

3. Discussion

In this work, we have obtained original findings about numerous molecular factors involved in the posterior cardiac tube segment differentiation. In this sense, this is the first time there is clear evidence about the function of miR-1 modulating Mef2c activity and RA signaling pathway during early cardiac chamber differentiation. Our results reveal (Figure 8) that miR-1 administration into the posterior cardiac precursors in the primitive endocardial tubes give rise to high expression levels of *Tbx5*, *Gata4* and *AMHC1*, which have previously demonstrated to be RA target genes, essential for early differentiation of cardiac sinoatrial region [16,46,55]. Significantly, the expression of *Mef2c*, a ventricular cardiomyocyte inductor [20,22], was diminished after miR-1 administration. Additionally, morphological alterations were detected after miR-1 overexpression, characterized by an enlarged posterior domain of the heart and a reduced ventricle region. All the above results show that miR-1 mimics the effects previously associated to highlighted RA levels [14–19,21], indicating

that miR-1 actively interferes with one or more factors involved in RA signaling pathway. In this line, our results reveal that miR-1 upregulates *CRABPII* and *RARß*, while it downregulates *CRABPI* -three crucial factors in RA signaling pathway. Interestingly, this microRNA also mimics the results obtained after deletion of *Mef2c* [20,22], indicating that miR-1 clearly modulates *Mef2c* transcription regulators. In addition to the above data, our results show that: i) miR-1 recognizes and directly binds to *Erk2/MAPK1*, *HDAC4* and *Calmodulin*, the last two also previously reported as miR-1 target genes in culture cardiac cells [34,35]; ii) this microRNA presents a complementary expression pattern with these three target genes during early posterior cardiac tube differentiation; and iii) it represses both mRNA and protein levels of *HDAC4*, *Calmodulin* and *Erk2/MAPK1* in the sinoatrial region. Our findings support the fact that miR-1 actively interacts with these three potential molecular factors, and also that these factors are involved both in *Mef2c* regulation and RA signaling pathway during early differentiation of cardiac sinoatrial region (Figures 8 and 9).
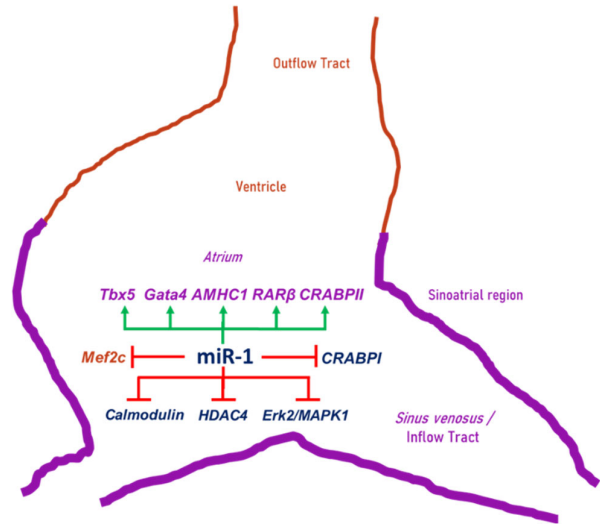
Figure 8. Schematic drawing that summarizes the results of this work. miR-1 induces *Tbx5*, *Gata4*, *AMHC1*, *RARβ* and *CRABPII*, while it suppresses *CRABPI*, *Calmodulin*, *HDAC4* and *Erk2/MAPK1*, as well as *Mef2c*, thus modulating early differentiation of cardiac sinoatrial region.
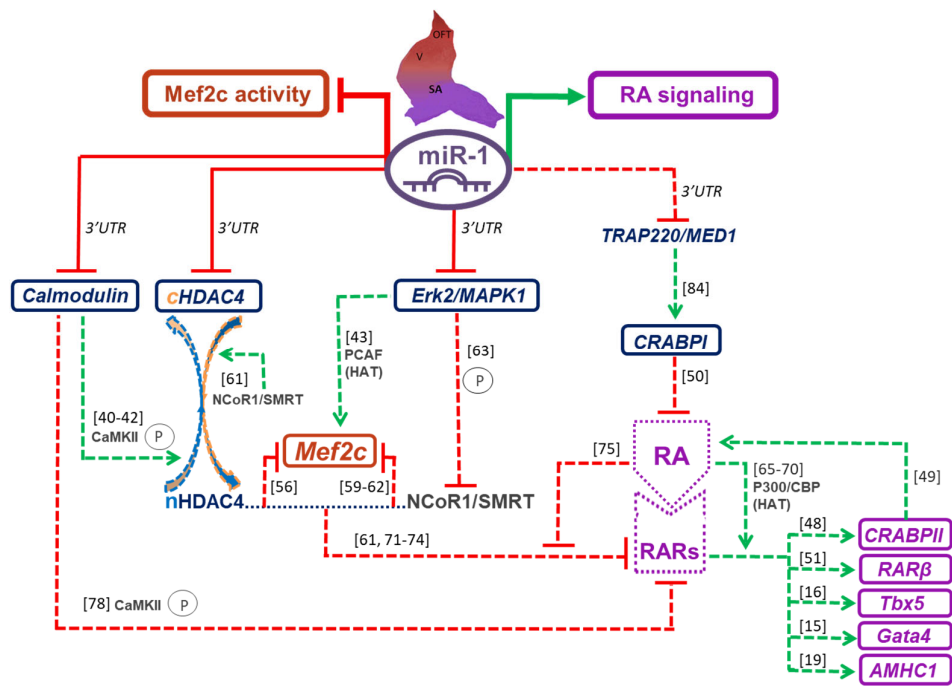
Figure 9. Proposed model illustrating the network of molecular mechanisms governed by miR-1 during early differentiation of cardiac sinoatrial region. Solid lines indicate our results from this work. Dashed lines correspond to previous studies in different research fields referred in the "Discussion" section. Our model indicates that miR-1 plays a crucial role repressing *Mef2c* through modulation of *HDAC4*, *Calmodulin* and *Erk2/MAPK1*. In these molecular mechanisms, miR-1 modulates opposite effects between *Calmodulin* and NCoR1/SMRT on nuclear HDAC4 (nHDAC4), thus increasing *Mef2c* interaction with nHDAC4, which inhibits *Mef2c* expression. Also, miR-1 suppresses *Erk2/MAPK1* and, consequently, diminishes Mef2c activity. Additionally, our model indicates that RA function is modulated by miR-1, thus promoting the expression of RA target genes *Tbx5*, *Gata4*, *AMHC1* and *RARβ*. Moreover, miR-1 increases *CRABPII* and supresses *CRABPI*, thus increasing RA activity. The presence of RA disrupts the interaction of nHDAC4-NCoR1/SMRT with RARs, thus allowing transcription of RARs target genes. Also miR-1 represses *Calmodulin* capability to inhibit RARs activity, thus enhancing the expression of RA target genes. In our model miR-1 modulates the opposite actions between RA and *Mef2c*, promoting RA signaling pathway and suppressing Mef2c activity to allow properly assign cells to their cardiac chamber. OFT: outflow tract, V: ventricular region, SA: sinoatrial region, P: phosphorylate, [cites listed in the "References" section].

3.1. miR-1 Modulates Mef2c Activity

Our results show that miR-1 diminishes both mRNA and protein levels of *Mef2c* in the posterior domain of the developing heart. It has been proposed that *Mef2c* promotes outflow-specific differentiation and directs cells in a nonresponsive manner to the inflow-specifying actions of RA [22]. Since *Mef2c* is not a miR-1 target gene, we analysed the effects of miR-1 on *Mef2c* modulators and observed that this microRNA represses both mRNA and protein levels of citoplasmatic *HDAC4* (*cHDAC4*), *Calmodulin* and *Erk2/MAPK1* (Figure 9). In this context, it is particularly known that nuclear HDAC4 (nHDAC4) directly binds to *Mef2c* and blocks its activity [36,39,56]. Through pharmacological inhibition of HDAC4 activity by trichostatin A (TSA) [57], our experimental model (Supplementary Figure S5) revealed that M*ef2c* expression presented a dramatic increase, proving the importance of histone deacetylation in this transcription factor regulation. It is also known that Calmodulin -through CaMKII induction- leads to phosphorylation of nHDAC4 and its translocation to the cytoplasm, thus promoting *Mef2c* activation [40–42,44,58]. Therefore, in our model (Figure 9), given that low levels of Calmodulin -miR-1 induced- do not allow the export of nHDAC4 from the nucleus to the cytoplasm, miR-1 would increase *Mef2c* interaction with nHDAC4, which inhibits *Mef2c* expression. It has also been reported that nHDAC4 together with the nuclear corepressor NCoR1/SMRT synergize to inhibit *Mef2c* activity [59,60]. Furthermore, NCoR1/SMRT actively translocates cHDAC4 from the cytoplasm into the nucleus and prevents its nuclear export [60–62]. The above data support the fact that there are opposite effects of Calmodulin and NCoR1/SMRT on nHDAC4 modulated by miR-1.

On the other hand, it is known that NCoR1/SMRT phosphorylation by Erk2 may destabilize the association between *Mef2c* and nHDAC4, thus increasing *Mef2c* expression [60,63,64]. It has also been reported that *Mef2c* may be enhanced by Erk1/2 through upregulation of coactivator P300/CBP-associated factor (PCAF), which inherently presents histone acetyltransferases (HAT) activity, thus increasing both expression and function of *Mef2c* [43]. Our results show that miR-1 directly recognizes and represses *Erk2/MAPK1* expression (Figure 9), and consequently this microRNA diminishes *Mef2c* activity, results supported by the above data. In this line, it has been observed during zebrafish development that excessive levels of Erk1/2 in atrial location cause ectopic expression of ventricular specific genes [45], effect that also supports our model, proposing miR-1 as a key regulator of atrial differentiation by repressing Erk1/2 expression.

Based on all the molecular mechanisms mentioned above, we propose in our model (Figure 9) that, in these early stages of cardiac development, miR-1 plays a crucial role to repress *Mef2c* through *Erk2/MAPK1*, *HDAC4* and *Calmodulin* modulation.

3.2. miR-1 Modulates RA Signaling Pathway

With respect to miR-1 inductive role on RA signaling pathway during early differentiation of cardiac sinoatrial region (Figure 8), we found that this microRNA increases expression levels of RA target genes *Tbx5*, *Gata4* and *AMHC1*. It is known that RA establishes an epigenetic switch for histone acetylation, mediated by p300/CBP, which inherently presents histone acetyltransferases (HAT) activity to allow transcription of RARs target genes [65–70]. We also observed that miR-1 represses epigenetic repressor HDAC4, which has been reported as a negative regulator of RA target genes [71]. In line with the above, we show in this work that *Tbx5*, *Gata4* and *AMHC1* expressions increase with HDAC4 inhibitor TSA (Supplementary Figure S5), confirming that RA regulates these cardiac genes through direct modulation of histone deacetylation. Furthermore, previous studies have reported that nHDAC4 is recruited and retained in the nucleus by NCoR1/SMRT, which is associated to RARs in absence of RA [61,72–74]. As a matter of fact, it is known that the presence of RA disrupts the interaction of NCoR1/SMRT with RARs [64,75], thus allowing transcription of RARs target genes (Figure 9). Other studies have described that Erk2/MAPK1 phosphorylates nuclear corepressors N-CoR1/SMRT, reducing the interaction between N-CoR1/SMRT and RARα [63], which would allow us to suggest an increase of RARs target genes transcription. However, our results showed that miR-1 directly supresses *Erk2/MAPK1* but it also increases RA target genes. Based on these results, we propose that *Erk2/MAPK1* is not sufficient to modulate atrial gene expressions, so that RA signal is necessary to disrupt the interaction between N-CoR1/SMRT and RARs [72]. Keeping all the above in mind, our results show that miR-1 upregulates RA function, thus promoting the expression of its target genes.

Interestingly, we found in our study that miR-1 increases expression levels of *CRABPII*, a RA target gene -with RA response element (RARE)- involved in delivering RA to its nuclear receptors RARs [48,49,76,77]. In addition, we observed that miR-1 increases both mRNA and proteins of RARβ, a previously defined RA target gene with RARE located in its promoter [51,54,68,75,77]. Moreover, by means of IMH, we carried out a detailed dynamic analysis of RARβ distribution, which showed a specific location in the cardiac sinoatrial region. The above findings, together with the fact that RARβ repression is linked to cardiac abnormalities [52], support miR-1 active role on RA signaling pathway through *RARβ*. Nevertheless, the levels of nuclear retinoic acid receptors RARα, RXRα, RARγ and RXRγ did not show significant changes after our miR-1 experimental assays, proving that these receptors are not targeted either by miR-1 or RA. It is noteworthy, based on our results, that miR-1 directly suppresses Calmodulin -a protein that also acts as an inhibitor of RARα activity by means of CaMKII induction, which mediates RARα phosphorylation and enhances the interaction between RARα and N-CoR1/SMRT, and subsequently supresses RARα target genes transcription [78]. In our proposed model (Figure 9), miR-1 represses Calmodulin capability to inhibit RARs activity, thus promoting the expression of RA target genes. Supporting these results, previous studies showed that the blockage of RARα function diminishes *Gata4* transcripts, specifically interfering with the inflow tract formation [79,80].

Additionally, our results show that miR-1 overexpression does not generate significant changes in the expression level of *Raldh2*, a RA synthesis modulator [47,74,81] involved in cardiac sinoatrial region development [18,19]. Supporting our results, previous studies [82] have shown that *Raldh2* expression is not altered in a nuclear corepressor mutant mouse model –SMRTmRID- which is characterized by enhanced transcription of RARs targets.

Noticeably, we observed that miR-1 diminishes the expression levels of *CRABPI*, a protein described as a sequester of RA, facilitating its catabolism, limiting RA concentration and regulating the amount of RA that is accessible to nuclear receptors RARs [50,83]. This supports the fact that miR-1 enhances RA activity. Given that *CRABPI* is not a miR-1 target gene, our model (Figure 9) proposes that miR-1 inhibits TRAP220/MED1 (a miR-1 target gene identified through in silico analysis), which is a *CRABPI* coactivator associated with RA [84]. Therefore, miR-1 would be able to promote RA signals.

Taking into account all the above, our novel experimental model integrates a network of molecular mechanisms modulated by miR-1, which plays a crucial role during early stages of cardiac chamber formation by promoting atrial differentiation together with complementary suppression of

ventricular formation. In conclusion, our study reveals, for the first time, a key role of miR-1 as an epigenetic factor modulating RA and *Mef2c* in their opposite actions, which are required to properly assign cells to their respective cardiac chambers. Since miR-1 has also been identified in cardiomyopathy processes [85–90], further and deeper understanding of this microRNA as a modulator of molecular mechanisms governing specific signaling pathways could be helpful in therapy and cardiac regeneration and repair.

4. Materials and Methods

Experimental protocols with animals were performed in agreement with the Spanish law in application of the EU Guidelines for animal research, and conformed to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication no.85-23). Approval by the University of Extremadura bioethics board was obtained prior to the initiation of the study.

4.1. Early Chick Whole Embryo Culture

Fertilized eggs (Granja Santa Isabel, Córdoba, Spain) were incubated at 37°C in forced draft humidified incubators. Embryos were staged (HH stages) [91,92] and subjected to early chick (EC) embryo culture [93].

4.2. Embryo Microinjections into the Posterior Cardiac Precursors of Both Primitive Endocardial Tubes

Stage HH 8 (3-4 somites) cultured embryos were microinjected (using an Inject + Matic micro injector system) in both primitive endocardial tubes into the posterior cardiac precursors committed to sinoatrial region fates [19,46].

For gain-of-function experiments, two different groups of embryos were microinjected with premiR-1 and all-trans retinoic acid (RA), respectively. Taking into account that premiR is a precursor miRNA that contains single stem-looped structure to be further processed into mature miRNA, which takes place in the miRNA-induced silencing complex; this, based on the sequence complementary between target mRNA and miRNA, gives rise to translation repression or target RNA degradation [94,95].

Likewise, for loss-of-function experiments, three different group of embryos were microinjected with antimiR-1 (that inhibits the binding of miRNA with its target mRNA); with an inhibitor of RA synthesis, Citral (3,7-dimethyl-2,6-octadienal); and with an inhibitor of HDAC, trichostatin A (TSA). A working solution containing 2.5 mM CFDA (Molecular Probes) and 1/10 volume of 0.5% (wt/vol) fast green FCF was prepared both experimental and control (CFDA) embryos. In the experimental embryos the working solution additionally contained a final concentration of 1μM for microRNAs premiR-1 (Ambion) or antimiR-1 (Ambion); of 10 μg/mL for RA (Sigma), of 10 mmol/L for Citral (Sigma); and as well as of 50 nmol/L for TSA (Sigma).

After 10-12h of additional incubation, embryos were photographed under bright and fluorescent light (Nikon digital, SIGHT DS-U1), and were selected according to the location and extent of the injection. The selected embryos were either fixed in 4% PFA—processed for gene expression (ISH) or immunochemistry (IMH) analysis- or cardiac loops were collected for RNA isolation as previously described [46].

4.3. Whole-Mount In Situ Hybridization (ISH)

Two different ISH-procedures were performed, following our previous procedures [96]. One group of control embryos was processed [97] for LNA-ISH using miR-1 LNA-labelled microRNA probe (miRCURY LNA™ Detection probe 5'-DIG and 3'-DIG labelled, Exiqon), while another group of premiR-1, antimiR-1, RA, Citral and control (CFDA) embryos were processed for ISH following standard procedures [98] using antisense-Tbx5, -Gata4 and -AMHC1 labelled probes [55].

4.4. Whole-Mount Immunohistochemestry (IMH)

Control, experimental and control (CFDA) embryos were subjected to whole mount IMH performed as we previously described [96,99], using polyclonal antibodies rabbit for HDAC4 (1:100, Invitrogen PA5-29103), phospho-Erk1/Erk2 (1:20, Invitrogen 44-680G), RARß (1:100, Invitrogen PA5-33016) and Mef2c (1:50, Proteintech 18290-1-AP), followed by Goat anti-rabbit IgG-HRP antibody (1:1000, Upstate, 12-348) and monoclonal antibody mouse for Calmodulin (1:20, Invitrogen MA3-918), followed by Goat anti-mouse Ig-HRP antibody (1:200, Jackson Immunoresearch Laboratories, West Grove, PA).

4.5. RNA Isolation and RT-qPCR

Cardiac loops isolated from experimental and control (CFDA) embryos (Supplementary Figure S1) were subjected to RT-qPCR analysis following MIQE guidelines [100–102]. RNA was extracted and purified by using ReliaPrep RNA Cell Miniprep System Kit (Promega) according to the manufacturer's instructions. For mRNA expression measurements, 0,5 μg 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 performed with 2μL of cDNA, GoTaq qPCR Master Mix (Promega) and corresponding primer sets (Supplementary Table STI). For miR-1 expression analyses, 20ng of total RNA was used for retro-transcription with Universal cDNA Synthesis Kit II (Exiqon) and the resulting cDNA was diluted 1/80. Real time PCR experiments were performed with 1 μL of diluted cDNA, Go Taq qPCR Master Mix (Promega) as well. All RT-qPCRs were performed using CFX384TM thermocycler (Bio-Rad) following the manufacture's recommendations. The relative expression of each gene was calculated by using *Gusb* and *Gapdh* as internal controls for mRNA expression analyses and *5S* and *6U* for miR-1 expression analyses, respectively [103]. Each PCR reaction was carried out in triplicate and repeated in at least three distinct biological samples to obtain representative means.

4.6. Analysis In Silico

Target-Scan 8 software (https://www.targetscan.org/vert_80/) was used to perform bioinformatics analysis of the binding sites of miR-1 at 3'UTRs of predicted targets as described before [104].

4.7. Luciferase Assays and 3T3 Transfection

HDAC4, Calmodulin and Erk2 (MAPK1) 3'UTR constructs (Supplementary Table STII) were PCR amplified and cloned into the pMIR_REPORT vector respectively. 3T3 fibroblasts (ATCC) were co-transfected with 100ng of different luciferase-based constructs and 300ng of pcLux vector control for internal normalization, respectively. Luciferase activity (Pierce™ Gaussia Luciferase Flash Assay Kit) was normalized to pcLux vector control (Pierce™ Cypridina Luciferase Flash Assay Kit) and compared to non-transfected controls. Each luciferase assay was carried out in triplicates and repeated in at least three distinct biological samples to obtain representative assays as described previously.

4.8. Statistical Analyses

For statistical analyses of datasets, unpaired Student's t-tests were used as previously reported [105,106]. Significance levels or p-values are stated in each corresponding figure legend. A $p < 0.05$ was considered statistically significant.

and analysed and discussed the results. All authors have read and agreed to the published version of the manuscript.

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