To Reviewers

We thank the reviewers for their helpful comments and supportive remarks indicating the manuscript is interesting and new. The reviewers asked a number of questions and concerns that have been addressed through additional experiments. In addition, the sections of Introduction, Method and Mterials and Discussion have been updated with adding new information. The references has been accordingly updated. The English language has been edited through ordering a professional editing service.

Overall, all of the specific questions raised by the four reviewers have been fully addressed. The response to reviewers in point-by-point is enclosed. The revised manuscript is now submitted for further review. Thank you.

To reviewer #1 (03471268)

We appreciate the reviewer's important suggestions which have been followed. All questions have been addressed by additional experiments.

Q1: 1. The authors found that there were no changes in the formation of early embryoid bodies and stem cell marker expression by IF staining between miR-301a and control groups. There are some other assays such as stemness gene expression detected by real-time PCR, AP staining and teratoma formation which are usually used to evaluate the stemness maintenance of ES cells. How about the results in these assays? Are there any changes between miR-301a and control groups?

Following the reviewer's suggestion, real-time PCR and AP staining assays were applied to validate the stemness maintenance of mES cells with or without overexpression of miR-301a. There were no changes observed bewteen miR-301a and control groups as shown in Figure 2D and Supplemental Figure S2, further demosntrating the very limited effect of miR-301a on the stemness maintenance of mES cells and formation of early EBs.

Q2. It is showed that overexpression of miR-301a in H9C2 cells promoted cellular survival against ISO-induced apoptosis. However, it seems that this part has no close relationship with the topic which is cardiomyocytes differentiation from ES cells. There is also no related discussion.

Thank the reviewer for the very important point. We totally agree that H9C2 cells are different from the ES cell-differentiated cardiomyocytes. Although a paragraph of discussion about the relationship between cardiac cellular injury protection and cardiomyocytes differentiation from ES cells was given, it is still somehow out of the range of the main topic of this article. Following the reviewer and editor's suggestion, we removed that part of results in Figure 1 about "miR-301a protected H9C2 cells against ISO-induced cell apoptosis" from the revised Figures. The related text in Result, Method & Materials, Discussion and Figure Legends were updated accordingly. As such, the revision is now focused on one topic that miR-301a induces cardiomyocytes differentiation from mES cells.

Q3. The study also proved that miR-301a is capable of inducing the expression of cardiac transcription factors. Figure 3 showed there were significant improvements for some TFs such as GATA4, TBX5, MHC and MLC on Day 4. Is there any difference in the expression of these factors between two groups before differentiation (undifferentiated ES cells)?.

In order to address this question, real-time PCR assay was performed to analyze the expression of MEF2C, GATA4, TBX5, MHC and MLC in mES cells with or without overexpression of miR-301a. Meanwhile, EBs undergoing differentiation at day 8 were used as a positive control. As shown in Supplemental Figure S3, there are very low or undetectable expression of these cardiac markers in mES cells. Their expression in mES cells did not show influence by miR-301a.

Q4. The Schematic representation of the procedure to induce cardiomyocytes differentiation from ES cells (Figure 2A) is not clear. What does "suspension culture" mean? Does this step refer to "~1000 cells in 20μl medium were hung from the bottom of the culturing plates without coating with gelatin for 2 days forming embryoid bodies"? If so, what about "spheroid structured embryoid bodies (EBs) were formed from day 2 to day 4 at the beginning of mES cell differentiation"? When does the step of "adherent cell culture with differentiation medium" begin? The authors need to clarify and mark several critical timepoints in the flow chart to help readers to understand clearly.

Thank you for the important comment. In order to address this question, detailed information about "Embryoid bodies formation and cardiomyocytes differentiation" has been

added in the section of Materails and Methods. Additional labels were added to the flow chart in Figure 2A to clearly indicate the cell culture condition at each timepoints.

Q5. About the formation of EB, do you use single ES cells for EB formation in the normal culturing plates and culture them for 2 days before differentiation? Could you please give more detailed description and more figures involved in EB formation?

As described in Materails and Methods, single cell suspension (5×10^4 mES cells/ml) in differentiation medium was split to cell droplets with ~1000 cells in 20μl for each drop, hung from the bottom of bacterial-grade dishes upside down to culture for 2 days, followed by suspension culture for two more days in 10ml differentiation medium using bacterial-grade dishes on a shaking platform at 40 rpm. This step is for maturation of EBs. After that, the EBs were moved to 0.1% gelatin-coated plates for adherent culture for additional 8 days. This step is for cardiomyocytes differentiation.

Q6. The scale bar in Fig. 2C is 200 μm and that in Fig. 2D is 20 μm, indicating the diameter of EB is about 30 μm. Is it an error?

We apologize for the typo error. The scale bar should be 200 μm. The average diameter of EBs is about 300 μm as shown in Figure 2F.

Q7. Do the authors use FBS to coat the plate for mES culturing?.

As described in cell culture of Materails and Methods, the mES cell culture plates were coated with FBS. However, bacterial-grade dishes were used for early EB formation, and then move to 0.1% gelatin-coated plates for cardiomyocytes differentiation.

To reviewer #2 (02567328)

We thank the reviewer for the helpful suggestions and supportive remarks indicating "The topic is interesting". All the suggestions have been followed and all the questions have been addressed in the revised manuscript.

Q1: The authors do not explain convincingly why they chose to evaluate the role of miR 301. In the work of Rangrez et al (MicroRNA miR-301a is a novel cardiac regulator of Cofilin-2. Rangrez AY, Hoppe P, Kuhn C, Zille E, Frank J, Frey N, Frank D. PLoS One. 2017 Sep 8;12(9):e0183901) the effect of miR301 is well explained. miR301 has been associated strongly with many human cancer including prostate cancer, malignant melanoma, osteosarcoma etc. miR301 targets Cfl2 a major regulator of actin dynamics.

Cfl2 increases the RhoA mediated SRF activation, transcription factors involved in transcription of myofibroblast genes. When miR301 targets Cfl2 in cardiomyocytes, the activation of SRF signalling is inhibited. This is the state of art. Why the authors have choosen miR301 considering its negative role? Their results support a role for miR301 that promotes differentiation, but how do they explain the difference with the literature?

In order to address this question, a paragraph of description about the background of miR-301a and the reason why we chose miR-301a to perform this study was added in Introduction and Discussion. In addition, a comparison between the current study and literature, the tissue-specific function of miR-301a, and the translational application prospect of miR-301a in cardiomyocytes differentiation were also discussed. The special expression pattern of miR-301a in the heart of embryos makes it to be a potential molecule regulating cardiomyocyte differentiation and heart development. As such we performed the current study to determine the effect of miR-301a on cardiomyocyte differentiation from mES cells. We found the early activation of cardiac TFs and induction of cardiomyocyte differentiation by miR-301a, demonstrating the potential of miR-301a in treatment of heart disease using ES cell-based cell therapy. Although miR-301a has been reported to be oncogenic in multiple tissues, we suggested to develop a stem cell-specific gene expression system or a cardiomyocyte-targeted local delivery system for miR-301a, which will avoid the risk of oncogenic side effect of miR-301a in other tissues.

Q2: In paragraph" miR-301a activated mTOR-Stat3 signaling by targeting Pten" the authors suggest that Pten is a potential target of miR-301. Why did they choose the pTen signaling pathway? A link between miR-1 and PTEN has been demonstrated in the literature to increase the differentiation of cardiomyocytes from EC mouse (MicroRNA-1 transfected embryonic stem cells enhance cardiac myocyte differentiation and inhibit apoptosis by modulating the PTEN/Akt pathway in the infarcted heart. Glass C, Singla DK. Am J Physiol Heart Circ Physiol. 2011 Nov;301(5):H2038-49) .

In order to address this question, a paragraph of discussion about miRNA-PTEN interaction and PTEN-AKT signaling pathway was added in the section of Discussion to explain why the PTEN signaling pathway was chosen for mechanism study. Details as shown below.

"The activation of mTOR-Stat3 signaling by the PI3K-AKT pathway has been validated in cardiomyocytes [33, 34]. Stat3 is essential for cardiomyocyte differentiation, directly promoting the expression of cardiac markers, including TBX5, NKX2.5 and GATA4 [35]. Pten dephosphorylates PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) to PIP2 (phosphatidylinositol (4,5)-bisphosphate), thereby inhibiting the PI3K/AKT pathway and regulating cell proliferation and cell differentiation in cardiomyocytes [36]. Pten is a key regulator of the PI3K/AKT pathway. Pten/PI3K/AKT signaling-mediated miRNA regulation of ES cell differentiation to cardiomyocytes has been reported for miR-1 [37]. Target interactions between miR-301a and Pten have been demonstrated in cervical cancer [38] and pancreatic cancer [39]. In the current study, we demonstrated that miR-301a activates the PI3K-AKT-mTOR-Stat3 signaling pathway, promoting cardiomyocyte differentiation from mES cells, which was mediated by the target interaction between miR-301a and Pten. These findings further demonstrated the importance of miRNAs and AKT signaling in regulating mES cell differentiation to cardiomyocytes".

Q3: In Figure 4B AKT activation and Pten inhibition are very slight. Up regulation of mTor is evident but not for pStat3.

In order to address this question, Figure 4B was updated with additional western blot results to total AKT, total mTOR and total Stat3. A semiquantitative analysis to the images was applied by normalization on GAPDH. As shown in Figure 4B and Figure 4C, the upregulation of p-AKT, p-mTOR and p-Stat3 was associated with downregulation of PTEN in the miR-301a-mES-derived cardiomyocytes at day 12 after differentiation, while total AKT, total mTOR and total STAT3 did not show difference between miR-301a group and control.

Q4. Discussion is a summary of introduction and redults. Please modify.

In the revised manuscript, the section of Discussion has been modified by discussing literature about miR-301a study, a comparison between literature and the current study, and tissue-specific function of miR-301a. The translational prospect of miR-301a in cardiomyocyte differentiation was also discussed. We found the early activation of cardiac TFs and induction of cardiomyocyte differentiation by miR-301a, demonstrating the potential of miR-301a in treatment of heart disease using ES cell-based cell therapy. Although miR-301a has been reported to be oncogenic in multiple tissues, we suggested to develop a stem

cell-specific gene expression system or a cardiomyocyte-targeted local delivery system for miR-301a, which will avoid the risk of oncogenic side effect of miR-301a in other tissues.

To reviewer #3 (02446041)

We thank the reviewer for the important questions. Additional experiments as well as discussions have been applied to address the reviewer's questions. The new data has strengthened the conclusion and improved the quality of the manuscript.

Q1: Although "miR-301a/PTEN/mTOR-Stat3" signaling pathway is known, the Manuscript NO: 49708 that attempted to address in vitro miR-301a induced cardiac differentiation of embryonic stem cells is of interest. It is lack of clarity, however, for the transition from "Overexpression of miR-301a in H9C2 cells promoted cellular survival against isoproterenol-induced apoptosis" to "Overexpression of miR-301a significantly induced the expression of cardiac transcription factors in mES cells, thereby promoting cardiomyocyte differentiation and beating cardiomyocyte cloning formation."

Thank the reviewer for the very important point. Although a paragraph of discussion about the relationship between cardiac cellular injury protection and cardiomyocytes differentiation from ES cells was given, it is still somehow out of the range of the main topic of this article. Following the reviewers and editor's suggestion, we removed that part of results in Figure 1 about "miR-301a protected H9C2 cells against ISO-induced cell apoptosis" from the revised Figures. The related text in Result, Method & Materials, Discussion and Figure Legends were updated accordingly. As such, the revision is now focused on one topic that miR-301a induces cardiomyocytes differentiation from mES cells.

Q2:) Page 3: "Cardiovascular disease is becoming the leading cause of death all over the world." Check the fact with citation: not becoming but is.

It has been revised following the reviewer's suggestion. A relevant reference was cited.

Q3: Page 3: "heart injury" – specify the nature of "heart injury" – some beyond repair – neither stem cells nor any means can amend.

It has been revised to use term “myocardial infarction (MI)” instead of “heart injury” in the revised manuscript.

Q4: Page 3: “Although the cell proliferative potential of cardiomyocytes in adult is occasionally reported [3], the regenerated cells are far from enough for function recovery of the injured heart.” They mix up with different concepts: proliferation is not equal to regeneration.

It has been revised to “Although the cell proliferative potential of cardiomyocytes in adults is occasionally reported [4], this ability cannot produce enough cardiomyocytes for functional recovery of the injured heart.”

Q5: Page 3: “pathological injury” – define specifically.

In the revised manuscript, it has been defined as “pathological injury in the heart including that after MI”.

Q6: Page 4: “mouse ES cells to induce cardiomyocyte differentiation in vitro to determine the differentiation efficiency and therapeutic potential for heart failure.” What do they mean using “the differentiation efficiency” or “therapeutic potential for heart failure?”

It has been revised to “mouse ES cells were applied for the induction of cardiomyocyte differentiation in vitro, determining the therapeutic potential of ES cell-based cell transplantation in the treatment of heart failure” in the revised manuscript.

Q7: Fig. 3A, miR-301a expression should have been included as a control.

In order to address this important question, real-time PCR analysis was applied to determine the miR-301a levels at different timepoints of cardiomyocyte differentiation from mES cells. As shown in Supplemental Figurre S1, ~5-fold increase of miR-301a was observed in EBs at both day 8 and day 12, compared to day 4.

Q8: Fig. 4, they should show the total proteins for all signaling molecules, not just phosphorylation of these proteins.

Thank you for the important point. In the revised manuscript, Figure 4B has been updated with additional western blot analysis to total AKT, total mTOR and total Stat3. A semiquantitative analysis to the images was applied by normalization on GAPDH. As shown in Figure 4B and Figure 4C, the upregulation of p-AKT, p-mTOR and p-Stat3 was associated with downregulation of PTEN in the miR-301a-mES-derived cardiomyocytes at day 12 after

differentiation, while total AKT, total mTOR and total STAT3 did not show difference between miR-301a group and control.

Q9: In section Introduction, they did not mention much-relevant publications on miR301a. How do they integrate and reconcile on a possible detrimental effect of miR-301a? For example, "the feedback loop between miR-301a and JAK/STAT3 pathway" works opposite the direction of the current manuscript. (Refer to Carcinogenesis. 2019 Jun 22. pii: bgz121. doi: 10.1093/carcin/bgz121., which found that "miR-301a is an oncogenic miRNA whose recognized conduce to NF-κB activation in pancreatic cancer" with the conclusion: "MicoRNA-301a Promotes Pancreatic cancer Invasion and Metastasis through the JAK/STAT3 Signaling Pathway by Targeting SOCS5." Such cancer-related impacts include cervical cancer (Innate Immun. 2019 May;25(4):217-223. doi: 10.1177/1753425919840702), colorectal cancer (J Chin Med Assoc. 2019 Mar;82(3):215-220. doi: 10.1097/JCMA.0000000000000031), lung cancer (Mol Cancer. 2019 May 23;18(1):99. doi: 10.1186/s12943-019-1024-0.), liver cancer (J Exp Clin Cancer Res. 2019 Apr 10;38(1):153. doi: 10.1186/s13046-019-1128-9.), pancreatic cancer (Cancer Chemother Pharmacol. 2019 May;83(5):975-991. doi: 10.1007/s00280-019-03807-4), prostate cancer (Urol Oncol. 2018 Nov;36(11):503.e9-503.e15. doi: 10.1016/j.urolonc.2018.07.014), and breast cancer (Aging (Albany NY). 2019 May 6;11(9):2628-2652. doi: 10.18632/aging.101934.).

Thank the reviewer for the important comment. The Introduction and Discussion sections have been modified by adding description about the background of miR-301a and the reason why we chose miR-301a to perform this study. In addition, a comparison between the current study and literature, the tissue-specific function of miR-301a, and the translational application prospect of miR-301a in cardiomyocytes differentiation were also discussed. The special expression pattern of miR-301a in the heart of embryos makes it to be a potential molecule regulating cardiomyocyte differentiation and heart development. As such we performed the current study to determine the effect of miR-301a on cardiomyocyte differentiation from mES cells. We found the early activation of cardiac TFs and induction of cardiomyocyte differentiation by miR-301a, demonstrating the potential of miR-301a in treatment of heart disease using ES cell-based cell therapy. Although miR-301a has been reported to be oncogenic in multiple tissues, we suggested to develop a stem cell-specific gene expression system or a cardiomyocyte-targeted local delivery system for miR-301a, which will avoid the risk of oncogenic side effect of miR-301a in other tissues.

"The enrichment of miR-301a in active cardiomyocytes was originally determined from our miRNA screening study in the hearts of neonatal rodents [26], which was recently confirmed by Rangrez AY, et al., who detected much higher expression of miR-301a in isolated cardiomyocytes than fibroblasts [24]. The study also found that miR-301a negatively regulates SRF signaling through inhibiting the expression of the target gene Cofilin-2 in cardiomyocytes, suggesting the therapeutic potential of miR-301a in the treatment of cardiac disorders caused by the deregulation of Cofilin-2 [24]. Here, we first showed that miR-301a has a high level in the hearts of late-stage embryos, while it is low in undifferentiated ES cells and cardiomyocytes in early-stage embryos. Subsequent functional assays demonstrated the induction of cardiomyocyte differentiation from mES cells by miR-301a, suggesting a cell-type specific function for this miRNA. Our findings add to knowledge of miR-301a in the treatment of heart disease by ES cell-based strategies. Notably, the development of a stem cell-specific gene expression system or a cardiomyocyte-targeted local delivery system for miR-301a will be required given the possible oncogenic side effects of miR-301a".

Q10: They did not discuss two tightly related papers (J Am Heart Assoc. 2018 Feb 25;7(5). pii: e008472. doi: 10.1161/JAHA.117.008472.) and (PLoS One. 2017 Sep 8;12(9):e0183901. doi: 10.1371/journal.pone.0183901.).

The relevant references have been added and discussed in the revised manuscript about the relationship between their discovery and our findings in the current study.

Q11: Page 5: "miR-301a in H9C2 cells" – why this line? "Embryonic rat heart tissue-derived cell line H9C2" – the nature? Biomarkers? Citation?

Thank the reviewer for the very important point. We agree that H9C2 cells are different from the ES cell-differentiated cardiomyocytes. Following the reviewer and editor's suggestion, we removed the data in Figure 1 about "miR-301a protected H9C2 cells against ISO-induced cell apoptosis" from the revised Figures. The related text in Result, Method & Materials, Discussion and Figure Legends were updated accordingly. As such, there is no H9C2 related data and description included in the revised manuscript.

Q12: Page 5: why "isoproterenol-induced apoptosis" – literature support to choose?

Please refer answer above to Q11.

Q13: Fig. 1: "miR-301a overexpression in H9C2 cells transfected with miR-301a mimic or negative control (NC)." Define "NC" for?

Although we removed data about H9C2 cells in the revised manuscript, transfection with miR-301a mimic or negative control (NC) are still applicable to ES cells. In the revision, NC has been defined as "a scrambled small RNA sequence was used as negative control (NC) for miR-301a mimic" in the Figure Legends.

Q14: Fig.1C, D: How did they calculate "quantitative analysis of apoptotic cell percentage?" Bar graph showing percentages of Q2+Q4?

In the revision, that part of results has been removed from the revision following the reviewer and editor's suggestion due to different cell type between H9C2 and mES cells.

Q15: In the section "Materials and Methods" – nothing was mentioned about the animal study, but surprisingly, Fig 1 with mouse hearts. Why? How those dosages determined? Why 24 hours?

Thank the reviewer for the important comment. The information about animal study has been added into the section of "Materials and Methods" by stating "Animal studies were approved by the Institutional Animal Care and Use Committee of the Tongji University School of Medicine. C57BL/6J male mice were purchased from Silaike Animal Company (Shanghai, China). The hearts were collected from mouse embryos at E11.5, 13.5, 15.5, 17.5, and 19.5 and from neonatal and adult mice and placed into TRIzol for total RNA isolation using a Tissue Homogenizer".

For the information about dose and hours for ISO treatment, that part of results has been removed from the revision following the reviewer and editor's suggestion due to different cell type between H9C2 and mES cells.

Q16: How did Fig. 1 mouse embryo stages correspond to "mES cell differentiation" process stages concerning biomarker expression?

This question has been addredd in the revised manuscript by stating "we assessed several cardiac-specific markers and cardiac-specific transcription factors at day 4 (EB formation, corresponding to ~E7.5 mouse embryos), day 8 (cardiac differentiation, corresponding to ~E10.5-E16.5 mouse embryos) and day 12 (formation of immature cardiomyocytes, corresponding to ~E17.5 and thereafter mouse embryos) after mES cell differentiation" .

Q17: Grammatical errors and choices of style should be consistent with standard English: e.g., "from mice embryos from days 11.5, 13.5, 15.5, 17.5, and 3-day-old postnatal and 6-week-old adult mice" preferred: mouse embryos at days 11, 13, etc." Another example, "3) Page 3: "Although the cell proliferative potential of cardiomyocytes in adult is occasionally reported [3], the regenerated cells are far from enough for function recovery of the injured heart." – should be "functional recovery."

Thank the reviewer for the language help. We have made corrections accordingly in the revision. In addition, a language editing service has been ordered to check all grammatical and spelling errors throughout the manuscript.

Q18: Fig. 4 and related discussion: "miR-301a targeted Pten and activated mTOR-STAT3 signaling pathway" (Innate Immun. 2019 May;25(4):217-223. doi: 10.1177/1753425919840702) (Cancer Res. 2018 Aug 15;78(16):4586-4598. doi: 10.1158/0008-5472.CAN-17-3841.)

A paragraph subtitled "mTOR-Stat3 signaling regulates cardiomyocyte differentiation" has been updated by adding relevant references and discussion in the revised manuscript.

Q19: Page 15: "miR-301a-Pten target interaction and PI3K-AKT-mTOR-Stat3 signaling pathway (Figure 4C)." – They need to elaborate on how they could manipulate the dual actions of the molecule for benefits. Specifically, given the literature on miR-301a-mediated cancer, how could they propose to reconcile on the convergence such as "Convergence of normal stem cell and cancer stem cell developmental stage: Implication for differential therapies" (World J Stem Cells. 2011 Sep 26;3(9):83-8. doi: 10.4252/wjsc.v3.i9.83.). They canNOT simply ignore that side effects of the story as they did in the current draft in the sections of Introduction and Discussion, simply opt out of the knowledge.

Thank the reviewer for the important point. In order to address ths question, the dual actions of miR-301a and miR-301a administration for potential clinial application have been discussed in the revised manuscript. In addition, a comparison between the current study and literature and the tissue-specific function of miR-301were also discussed. The special expression pattern of miR-301a in the heart of embryos makes it to be a potential molecule regulating cardiomyocyte differentiation and heart development. As such we performed the current study to determine the effect of miR-301a on cardiomyocyte differentiation from mES cells. We found the early activation of cardiac TFs and induction of cardiomyocyte

differentiation by miR-301a, demonstrating the potential of miR-301a in treatment of heart disease using ES cell-based cell therapy. Although miR-301a has been reported to be oncogenic in multiple tissues, we suggested to develop a stem cell-specific gene expression system or a cardiomyocyte-targeted local delivery system for miR-301a, which will avoid the risk of oncogenic side effect of miR-301a in other tissues.

In the section of Discussion, we made a statement that "Subsequent functional assays demonstrated the induction of cardiomyocyte differentiation from mES cells by miR-301a, suggesting a cell-type specific function for this miRNA. Our findings add to knowledge of miR-301a in the treatment of heart disease by ES cell-based strategies. Notably, the development of a stem cell-specific gene expression system or a cardiomyocyte-targeted local delivery system for miR-301a will be required given the possible oncogenic side effects of miR-301a" .

"We demonstrated that miR-301a promoted transcriptional activation of the cardiomyocyte-driving genes during mES cell differentiation to cardiomyocytes, which may be mediated by the miR-301a-Pten target interaction and PI3K-AKT-mTOR-Stat3 signaling pathway (Figure 4D). As discussed above, application of a heart-specific local delivery system for miR-301a administration will be required to avoid potential side effects of miR-301a."

To reviewer #4 (00609434)

We thank the reviewer for the helpful comments and supportive remark indicating "This manuscript is interesting and new". All the questions have been addressed in the revised manuscript.

Q1: Please check English grammar and synthax carefully.

Following the reviewer's suggestion, we have checked English grammar and synthax carefully throughout the manuscript. In addition, a language editing service has been ordered to double check and correct all grammatical and spelling errors.

Q2: There is a little bit of confusion in reference numbering in the text. Materials and Methods starts with reference number 27 while the Introduction ends with reference number 16. Please check.

Thank you for the comment. The references and reference numbering have been updated in the revised manuscript.

Q3: In the Introduction (page 4 last line) the sentence ending with "… and so on." has no mention to the literature, please add the opportune references.

Thank you for the comment. The relevant references have been added in the revised manuscript.

Q4: In the Materials and Methods a lot of information is lacking on the procedures and the sources of the materials used, here is a non comprehensive list of information needed: -In the cell experiments, both for the cardiomyocyte cell line and the ES cell line, please give details on the number of cell used in each experiments, how many replicates for each treatment, where cells were cultured, how many days after miR transfection the apoptosis assay was performed, where does the Annexin V kit come from, as well as how many days after transfection the embryoid body experiments were performed. -In the quantitative PCR analyses, please describe the method of RNA purification used since this is essential for the yield of miRNAs obtained in the extracted total RNA, please give sequences of all set of primers used in a separate table (not in the text) indicating the Accession number of each investigated gene and length of the PCR product for each primer set. Please check the dilution used in the qPCR analysis, are you sure is 1:1000? That means as low as 100 pg of total cDNA template was used for the amplification, is the method used sensitive enough for its detection? -Since in Figure 1 there is a quantification of miR310a expression in mouse embryos and newborns please give all details in the materials and methods of the experimental animal procedure and RNA extraction from tissues, mouse strain used, housing facility, ethical committee approval etc…

We really appreciate the reviewer's helpful comments. The section of Materials and Methods has been carefully checked and modified by describing all related information as much detail as possible. All question raised by the reviewer has been addressed. The modified methods and materials include but not limited to cell culture condition, cell numbers used in each experiment, replicate number for each treatment, dose and time for reagent treatment, purchase source for all materials, all primer information for RT-PCR, claim for animal experiments, method for EB formation, method for RNA extraction, et al. For RT-PCR of

miRNA, 500ng of total RNA was used for reverase transcripton, 1:1000 dilution of cDNA was applied for PCR reaction.

Q5 Also the Results section and Figures and Legends need improvement for a better understating of the findings in this work: -At page 9, in the description of Figure 1A the authors should give in the text the percentages of gene expression of the various tissues respect to the calibrator sample (mature heart tissue?), and also in the Figure itself in the Y-axis it should be stated respect to what calibrator sample the percentage of gene expression is calculated. This observation applies to all graphs reporting the qPCR results in the whole manuscript. -In Figure 1B it is not really an expression of miR301a that is measured but the miR transfection efficiency, or if you prefer the miR cell loading or its internalization, please choose another term to describe this quantification. At Page 9 in the description of Figure 2B the authors should give in the text the percentage of gene expression upregulation of CM(AD) respect to the calibrator sample (ES(BD)??). In the description of Figure 2E, please explain how the statistics was performed, how many bodies were measured to derive the graph, at which day? -At Page 11 in the description of Figure 3A and 3B please add the percentages of upregulation of the genes and proteins in the text (for 3B a semiquantitative analysis of protein expression normalized on GAPDH protein can be easily performed), this may help understand the strength of miR301a in promoting cardiac differentiation. Again, in Figure 3A the calibrator sample is not mentioned (mRNA levels respect to what?). In the description of Figure 3C, please explain how the statistics was performed, how many bodies or microscope fields were measured to derive the graph? -At Page 12 in the description of Figure 4B (and in the figure itself), also here it would be useful to add a semiquantitative analysis of protein expression in the two samples (control and treated) to better appreciate miR301a contribute to feed the signal of the transduction pathway described.

Thank you for the helpful and constructive comments. Following the reviewer's suggestion, all relevant figure labelings, figure legends and results description have been modified to match the findings in this study. The modification inludes but not limited to description about the fold change of gene expression in various tissues respect to the adult heart tissue in Figure 1A, Y-axis labeling in all graphs of gene expression, method for calculating fold change of miR-301a expression in Figure 2B, number and stage of EBs counted for qiantitative analysis in Figure 2F, semi-quantitative analysis of protein levels

normalized on GAPDH in Figure 3B and Figure 4B, number of EBs counted at each timepoint for calculating the percentage of beating EBs in Figure 3C.