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PEER-REVIEW REPORT

Name of journal: World Journal of Stem Cells

Manuscript NO: 49708

Title: miR-301a promotes embryonic stem cell differentiation to cardiomyocytes

Reviewer's code: 03471268
Position: Editorial Board
Academic degree: PhD
Professional title: Associate Professor
Reviewer's country: Japan

Science editor: Ying Dou

Reviewer accepted review: 2019-06-19 09:45

Reviewer performed review: 2019-06-22 06:37

Review time: 2 Days and 20 Hours

SCIENTIFIC QUALITY	LANGUAGE QUALITY	CONCLUSION	PEER-REVIEWER STATEMENTS
[ ] Grade A: Excellent	[ ] Grade A: Priority publishing	[ ] Accept (High priority)	Peer-Review:
[ ] Grade B: Very good	[Y] Grade B: Minor language polishing	[ ] Accept (General priority)	[Y] Anonymous
[Y] Grade C: Good	[ ] Grade C: A great deal of language polishing	[ ] Minor revision	[ ] Onymous
[ ] Grade D: Fair	[ ] Grade D: Rejection	[Y] Major revision	Peer-reviewer's expertise on the topic of the manuscript:
[ ] Grade E: Do not publish		[ ] Rejection	[ ] Advanced
			[Y] General
			[ ] No expertise
			Conflicts-of-Interest:
			[ ] Yes
			[Y] No

SPECIFIC COMMENTS TO AUTHORS

In this study, the authors found that overexpression of miR-301a in mES cells significantly induced the expression of cardiac transcription factors, thereby promoting

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cardiomyocyte differentiation and beating cardiomyocyte cloning formation, which may be mediated by the miR-301a-Pten interaction and activated mTOR-Stat3 signaling pathway. Here are the comments: 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? 2. 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. 3. 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)? 4. 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. 5. 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

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figures involved in EB formation? 6. 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? 7. Do the authors use FBS to coat the plate for mES culturing?

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PEER-REVIEW REPORT

Name of journal: World Journal of Stem Cells

Manuscript NO: 49708

Title: miR-301a promotes embryonic stem cell differentiation to cardiomyocytes

Reviewer's code: 02567328
Position: Editorial Board
Academic degree: PhD
Professional title: Assistant Professor
Reviewer's country: Italy

Science editor: Ying Dou

Reviewer accepted review: 2019-06-20 13:59

Reviewer performed review: 2019-06-26 11:11

Review time: 5 Days and 21 Hours

SCIENTIFIC QUALITY	LANGUAGE QUALITY	CONCLUSION	PEER-REVIEWER STATEMENTS
[ ] Grade A: Excellent	[ ] Grade A: Priority publishing	[ ] Accept (High priority)	Peer-Review:
[ ] Grade B: Very good	[Y] Grade B: Minor language polishing	[ ] Accept (General priority)	[Y] Anonymous
[ ] Grade C: Good	[ ] Grade C: A great deal of language polishing	[ ] Minor revision	[ ] Onymous
[Y] Grade D: Fair	[ ] Grade D: Rejection	[Y] Major revision	Peer-reviewer's expertise on the topic of the manuscript:
[ ] Grade E: Do not publish		[ ] Rejection	[Y] Advanced
			[ ] General
			[ ] No expertise
			Conflicts-of-Interest:
			[ ] Yes
			[Y] No

SPECIFIC COMMENTS TO AUTHORS

In this manuscript the authors evaluate the role of miR-301a in embryonic stem cell differentiation to cardiomyocytes. The topic is interesting but there are some criticisms: -

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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 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 Figure 4B AKT activation and Pten inhibition are very slight. Up regulation of mTor is evident but not for pStat3. - Discussion is a summary of introduction and redults. Please modify

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PEER-REVIEW REPORT

Name of journal: World Journal of Stem Cells

Manuscript NO: 49708

Title: miR-301a promotes embryonic stem cell differentiation to cardiomyocytes

Reviewer's code: 02446041
Position: Editor-in-Chief
Academic degree: PhD
Professional title: Adjunct Professor,Research Scientist,Senior Research Fellow
Reviewer's country: United States

Science editor: Ying Dou

Reviewer accepted review: 2019-06-19 17:43

Reviewer performed review: 2019-06-26 22:08

Review time: 7 Days and 4 Hours

SCIENTIFIC QUALITY	LANGUAGE QUALITY	CONCLUSION	PEER-REVIEWER STATEMENTS
[ ] Grade A: Excellent	[ ] Grade A: Priority publishing	[ ] Accept (High priority)	Peer-Review:
[Y] Grade B: Very good	[Y] Grade B: Minor language polishing	[ ] Accept (General priority)	[ ] Anonymous
[ ] Grade C: Good	[ ] Grade C: A great deal of language polishing	[ ] Minor revision	[Y] Onymous
[ ] Grade D: Fair	[ ] Grade D: Rejection	[Y] Major revision	Peer-reviewer's expertise on the topic of the manuscript:
[ ] Grade E: Do not publish		[ ] Rejection	[Y] Advanced
			[ ] General
			[ ] No expertise
			Conflicts-of-Interest:
			[ ] Yes
			[Y] No

SPECIFIC COMMENTS TO AUTHORS

Comment: (778 words) Although "miR-301a/PTEN/mTOR-Stat3" signaling pathway is known, the Manuscript NO: 49708 that attempted to address in vitro miR-301a induced

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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." Thus, they need to address the following 18 specifics for coherence and logic. (refer to Authors: Lixiao Zhen1,2, Yuying Gu1, Qian Zhao1,2, Huifang Zhu1, Jinhui Lü1,2, Shujun Li1,2, Zhen Xu3, Li Li1, and Zuoren Yu1*). Specific comments: 1) Page 3: "Cardiovascular disease is becoming the leading cause of death all over the world." Check the fact with citation: not becoming but is. 2) Page 3: "heart injury" – specify the nature of "heart injury" – some beyond repair – neither stem cells nor any means can amend. 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." They mix up with different concepts: proliferation is not equal to regeneration. 4) Page 3: "pathological injury" – define specifically. 5) 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?" 6) Fig. 3A, miR-301a expression should have been included as a control. 7) Fig. 4, they should show the total proteins for all signaling molecules, not just phosphorylation of these proteins. 8) 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

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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.) 9) 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.). 10) Page 5: "miR-301a in H9C2 cells" – why this line? "Embryonic rat heart tissue-derived cell line H9C2" – the nature? Biomarkers? Citation? 11) Page 5: why "isoproterenol-induced apoptosis" – literature support to choose? 12) Fig. 1: "miR-301a overexpression in H9C2 cells transfected with miR-301a mimic or negative control (NC)." Define "NC" for? 13) Fig.1C, D: How did they calculate "quantitative analysis of apoptotic cell percentage?" Bar graph showing percentages of Q2+Q4? 14) 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? 15) How did Fig. 1 mouse embryo stages correspond to "mES cell differentiation" process stages concerning biomarker expression? 16) 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

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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." 17) 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.) 18) 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.

INITIAL REVIEW OF THE MANUSCRIPT

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PEER-REVIEW REPORT

Name of journal: World Journal of Stem Cells

Manuscript NO: 49708

Title: miR-301a promotes embryonic stem cell differentiation to cardiomyocytes

Reviewer's code: 00609434
Position: Editorial Board
Academic degree: PhD
Professional title: Associate Professor
Reviewer's country: Italy

Science editor: Ying Dou

Reviewer accepted review: 2019-06-19 13:08

Reviewer performed review: 2019-07-03 11:07

Review time: 13 Days and 21 Hours

SCIENTIFIC QUALITY	LANGUAGE QUALITY	CONCLUSION	PEER-REVIEWER STATEMENTS
[ ] Grade A: Excellent	[ ] Grade A: Priority publishing	[ ] Accept (High priority)	Peer-Review:
[ ] Grade B: Very good	[Y] Grade B: Minor language polishing	[ ] Accept (General priority)	[Y] Anonymous
[Y] Grade C: Good	[ ] Grade C: A great deal of language polishing	[Y] Minor revision	[ ] Onymous
[ ] Grade D: Fair	[ ] Grade D: Rejection	[ ] Major revision	Peer-reviewer's expertise on the topic of the manuscript:
[ ] Grade E: Do not publish		[ ] Rejection	[ ] Advanced
			[Y] General
			[ ] No expertise
			Conflicts-of-Interest:
			[ ] Yes
			[Y] No

SPECIFIC COMMENTS TO AUTHORS

The manuscript from Zhen et al. reports the upregulated expression of miR-301a in embryonic and neonatal cardiomyocytes and its role in the regulation of embrionyc stem

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cell differentiation into mature cardiomyocytes in vitro. This manuscript is interesting and new but presentation of results and description of methods need to be improved before publication since a lot of information is lacking. I find it worthy of publication after the following points are addressed by the authors. 1) Please check English grammar and synthax carefully 2) 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. 3) 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. 4) 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… 5) Also the Results section and Figures and Legends need improvement

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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.

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