Peer-Review Record

Hypoxia-induced stabilization of HIF2A promotes cardiomyocyte proliferation by attenuating DNA damage

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Academic Editor: Ali J. Marian

Reviewer 1: Anonymous

Reviewer 2: Anonymous

Round 1

Reviewer 1 Report

The manuscript by Ali et al. describes a role of Hif2a in cardiomyocyte proliferation. First, cardiomyocyte-specific Hif2a loss-of-function mouse model hypoxia-induced experiments show decreased PH3+ cardiomyocytes and greater number of apoptotic cardiomyocytes. Second, over-expression of Hif2a in cardiomyocytes led to increased number of cardiomyocytes in mitosis and newly born cardiomyocytes. Third, after MI, Hif2a overexpression mice showed improved cardiac function and decreased scar size fibrosis. Finally, to delineate the mechanism by which Hif2a can promote cardiomyocyte proliferation, RNA seq analysis showed the most upregulated pathways in the Hif2a-overexpressing hearts were related to angiogenesis, as well as several pathways involved in oxidation-reduction reactions. Follow-up western blot and immunostaining analysis revealed that Hif2a-overexpressing hearts had decreased expression of y-H2AX, reduced activation of the ATM kinase and fewer oxidatively-damaged guanine residues as determined by quantification of 8-oxoG puncta in cardiomyocyte nuclei. It was concluded that ectopic expression of an oxygen-stable Hif2a in cardiomyocytes during normoxia lessens DNA damage and, thereby, suppresses DNA damage-activated pathways that inhibit cell proliferation.

I have the following comments:

1. Data is needed that shows Hif2a KO and over-expression in cardiomyocytes of the mouse models.
2. There is an increase in apoptotic cardiomyocytes due to Hif2a cKO during chronic hypoxic. The authors should also show the number of apoptotic cardiomyocytes decreases when Hif2a is overexpressed in the MI mouse model.
3. The image of a PH3+ cardiomyocyte in Figure 1B shows what appears to be a bi-nucleated cardiomyocyte, with one nuclei positive for PH3! Please use a different representative image where its clear that it's a cardiomyocyte PH3+ nuclei.
4. Please define what is MADM quantification and what's the meaning of TdT+ GFP nuclei shown in Figure 1O.?
5. The figure legends do not reflect the labelling of the figure panels. Or what the different color arrows mean. Please re-write.
6. Figure 2G, the authors do not comment in the main body narrative about the results of Chk1/p-Chk1 or Chk2/p-Chk2.
7. It's very difficult to read the text in Figure 2 panels E and F. Please make larger and clearer.
8. The discussion would benefit from discussing their results in the context of the aging heart, especially considering this is a Cardiovascular aging journal.

Author Response

The manuscript by Ali et al. describes a role of Hif2a in cardiomyocyte proliferation. First, cardiomyocyte-specific Hif2a loss-of-function mouse model hypoxia-induced experiments show decreased PH3+ cardiomyocytes and greater number of apoptotic cardiomyocytes. Second, over-expression of Hif2a in cardiomyocytes led to increased number of cardiomyocytes in mitosis and newly born cardiomyocytes. Third, after MI, Hif2a overexpression mice showed improved cardiac function and decreased scar size fibrosis. Finally, to delineate the mechanism by which Hif2a can promote cardiomyocyte proliferation, RNA seq analysis showed the most upregulated pathways in the Hif2a-overexpressing hearts were related to angiogenesis, as well as several pathways involved in oxidation-reduction reactions. Follow-up western blot and immunostaining analysis revealed that Hif2a-overexpressing hearts had decreased expression of y-H2AX, reduced activation of the ATM kinase and fewer oxidatively-damaged guanine residues as determined by quantification of 8-oxoG puncta in cardiomyocyte nuclei. It was concluded that ectopic expression of an oxygen-stable Hif2a in cardiomyocytes during normoxia lessens DNA damage and, thereby, suppresses DNA damage-activated pathways that inhibit cell proliferation.

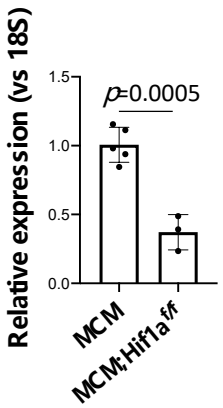
We appreciate the reviewer's meticulous review of our manuscript as well as for the comments below that highlight ways to improve the manuscript.

I have the following comments:

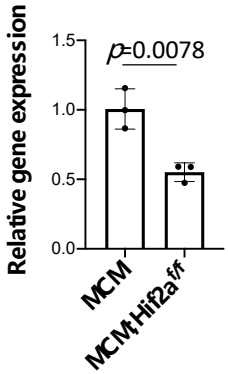
1. Data is needed that shows Hif2a KO and over-expression in cardiomyocytes of the mouse models.

Both of the KO models (Hif1a$^{f/f}$ and Hif2a$^{f/f}$) as well as the Hif2a-OE models are published and previously validated (e.g., Nature Communications volume 9, Article number: 816 (2018) and ***). To show that we achieve knockout and overexpression, respectively, we used qPCR (shown below). These data are now in Fig. 1A and Fig. 1F.

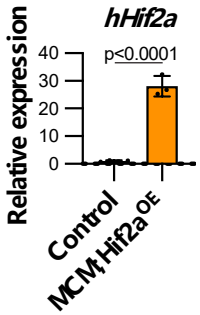
A) qPCR for Hif1a in control and MCM;Hif1a$^{f/f}$ during normoxia



B) qPCR for Hif2a in control and MCM;Hif2a$^{f/f}$ during normoxia



C) qPCR for human Hif2a in control and MCM;Hif2a-OE during normoxia



2. There is an increase in apoptotic cardiomyocytes due to Hif2a cKO during chronic hypoxic. The authors should also show the number of apoptotic cardiomyocytes decreases when Hif2a is overexpressed in the MI mouse model.

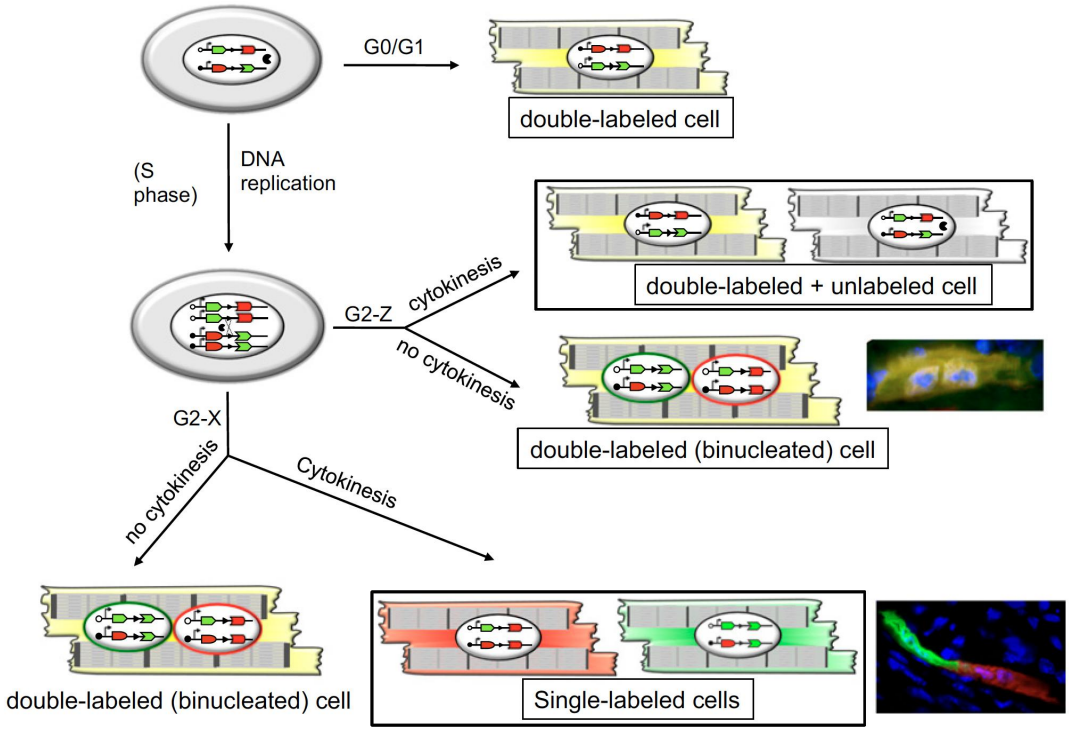
This is an interesting point raised by the reviewer. We noticed the apoptosis phenotype in the hypoxia tissue with the knockouts, but we did not repeat this inquiry with the Hif2a-OE model – either in the uninjured or in the injured setting. Our observation of less apoptosis in the control and MCM;Hif1a$^{f/f}$ compared to MCM;Hif2a$^{f/f}$ suggested that hypoxia can be protective downstream of Hif2a signaling. However, we have not pursued this question after adult MI as it outside the scope of our Brief Report manuscript – whose focus is on proliferation and regeneration – not least due to having reached the word limit of the Brief Report format.

3. The image of a PH3+ cardiomyocyte in Figure 1B shows what appears to be a bi-nucleated cardiomyocyte, with one nuclei positive for PH3! Please use a different representative image where its clear that it's a cardiomyocyte PH3+ nuclei.

We thank the reviewer for pointing this out – and we have accordingly changed the image to a more representative one in the revised manuscript.

4. Please define what is MADM quantification and what's the meaning of TdT+ GFP nuclei shown in Figure 1O.?

Please refer to schematic below this paragraph. Mosaic analysis of double markers (MADM) uses Cre-loxP recombination in trans, and starts with a split GFP and TdT cassette on homologous chromosomes. One chromosome contains the N-terminus of GFP, followed by a LoxP, followed by the C-terminus of TdT. The other chromosome contains the opposite conformation (N-terminus of TdT-LoxP-C-terminus of GFP.) If Cre-LoxP recombination takes place after S phase and there is X-segregation of chromosomes, the nuclei will contain a fully reconstituted copy of TdT or GFP (bottom right arrow). If these two nuclei remain in the same cell (i.e., binucleation), the cell will be double-labeled (bottom left). If the two nuclei are partitioned into two daughter cells (i.e., cytokinesis takes place), then two daughter cells will be labelled with either GFP or TdT. As a result, the only way to achieve single-labeling in MADM – to only have one fluorescent protein expression – is via cell division with completed cytokinesis. The percent of single-labeled cells (revised Fig. 2C-D), therefore, is a surrogate for completed cell division. This technical approach has been utilized in multiple studies in the cardiac regeneration field as a bona vide proof of cytokinesis (e.g., Mohamed et al., 2018, Cell 173, 104 – 116). [Of note, the arrows do not show nuclei but rather cardiomyocytes in cross section. The reporter fluorescent proteins are cytoplasmic in the MADM construct.] (Schematic is from Ali et al. PNAS, 2014 (https://doi.org/10.1073/pnas.1408233111).)



We have edited the manuscript to better clarify this point, which now states, "To determine whether this mitotic activity led to completed cell division, we generated Myh6-MCM;MADM;Hif2aOE mice, which had a two-fold increase in the percent of single-labeled cardiomyocytes compared to control Myh6-MCM;MADM control mice. Since single-labeled cells in the MADM model can only arise through completion of cytokinesis, this finding indicates that twice as many cardiomyocytes were born upon stable Hif2a overexpression (Fig. 2C-D).4"

5. The figure legends do not reflect the labelling of the figure panels. Or what the different color arrows mean. Please re-writes.

We have corrected the figure legends, which were erroneously shifted. The different colors are also described in the legend.

6. Figure 2G, the authors do not comment in the main body narrative about the results of Chk1/p-Chk1 or Chk2/p-Chk2.

We apologize for this oversight, which was due to concern about space. The edited manuscript now states, "Signaling caused by DNA damage activates a series of sensors (Mre11-Rad50-Nbs1 complex), mediators (ATM, ATR/DNA-PKc), and effectors (Chk1, Chk2, etc.) of the damage response. To investigate the DNA damage response in cardiomyocytes following Hif2a overexpression, we examined the activity of both the ATM mediator and effectors Chk1 and Chk2 using immunoblot (Fig. 3C-G). Upon Hif2a-OE, there is a significant decrease in ATM activity (Fig. 3C) without Chk1/2 activation (Fig. 3D-E), using phosphorylation as an indicator of activity. This finding suggests that the overexpression of

Hif2a reduces DNA damage and does not alter DNA damage response or repair (through effector activity).".

7. It's very difficult to read the text in Figure 2 panels E and F. Please make larger and clearer.

We appreciate the reviewer for pointing out this issue. We have formatted the figures in 2E and 2F to make the text more legible (which are now Fig 3A-B).

8. The discussion would benefit from discussing their results in the context of the aging heart, especially considering this is a Cardiovascular aging journal.

We agree with the reviewer, and we have incorporated this into the manuscript. The discussion now states: "Consistent with our findings, a recent study showed that human aging is associated with acquisition of DNA mutations in cardiomyocytes due to oxidative DNA damage and inefficient repair pathway activation.8 Therefore, hypoxia and Hif2a may help overcome some of the deleterious effects of aging in the heart."

Reviewer 2 Report

The study by Ali et al explore the transcriptional mechanisms by which hypoxia stimulates cardiomyocyte proliferation and heart regeneration. The authors deleted either Hif1a or Hif2a and found that Hif2a deletion impairs the effect of hypoxia on cardiomyocyte proliferation. This was accompanied by an increase in apoptosis as well. Interestingly, Hif2a overexpression increased cardiomyocyte numbers and proliferation in the adult heart. The Hif2a overexpression mice were then to subjected to adult MI, which showed improved function and reduced scar compared to control mice. RNAseq analysis shows increase in angiogenesis and antioxidant gene expression following Hif2a overexpression, which was evident in the reduced cardiomyocyte DNA damage in the Hif2a overexpression mice. These results demonstrate an important role for Hif2a in mediating the regenerative effect of hypoxia, and that Hif2a overexpression can be induced to promote adult heart regeneration.

This study provides new mechanistic insights on the role of Hif2a during hypoxia-mediated regeneration. It would be insightful if the authors show levels of cardiomyocyte proliferation and cell size following adult MI in Hif2a overexpression mice.

Author Response

The study by Ali et al explore the transcriptional mechanisms by which hypoxia stimulates cardiomyocyte proliferation and heart regeneration. The authors deleted either Hif1a or Hif2a and found that Hif2a deletion impairs the effect of hypoxia on cardiomyocyte proliferation. This was accompanied by an increase in apoptosis as well. Interestingly, Hif2a overexpression increased cardiomyocyte numbers and proliferation in the adult heart. The Hif2a overexpression mice were then to subjected to adult MI, which showed improved function and reduced scar compared to control mice. RNAseq analysis shows increase in angiogenesis and antioxidant gene expression following Hif2a overexpression,

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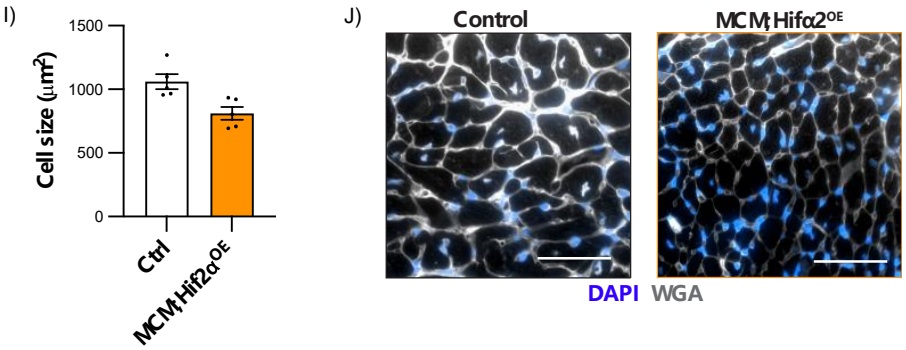
This study provides new mechanistic insights on the role of Hif2a during hypoxia-mediated regeneration. It would be insightful if the authors show levels of cardiomyocyte proliferation and cell size following adult MI in Hif2a overexpression mice.

We appreciate the reviewer's thorough review of our manuscript and for their positive feedback. Regarding the reviewer's questions:

1. Cardiomyocyte cell size after adult MI: We performed WGA staining and quantified the CM cross-sectional area on control and MCM;Hif2a-OE hearts 3 months after adult MI, which show a reduction in cell size. This data is shown below and is in the revised manuscript as Fig. 2I-J.



2. Cardiomyocyte proliferation after adult MI: Due to technical limitations, we were not able to perform this analysis. In general, pH3 staining has established protocols in our lab on frozen tissue. However, following MI, we typically paraffin-embed the hearts for the basic histology analyses we perform after explantation. Our attempts to perform pH3 immunofluorescence staining on these paraffin-embedded tissues were not successful, unfortunately.