

ARNT-Dependent HIF-2 α Signaling Protects Cardiac Microvascular Barrier Integrity and Heart Function Post-Myocardial Infarction

Corresponding Author: Dr Rongxue WU

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Ullah K et al. have submitted the manuscript titled "A Novel ARNT-Dependent HIF-2 α Signaling as a Protective Mechanism for Cardiac Microvascular Barrier Integrity and Heart Function Post-Myocardial Infarction." In their study, the authors provide evidence of the protective effect of the HIF-2 α /ARNT pathway in a murine in vivo model of permanent ligation. Their findings demonstrate the critical role of the endothelial HIF-2 α /ARNT axis as a transcriptional repressor in myocardial infarction, offering new insights into potential therapeutic strategies against heart failure. While these findings are of significant interest and importance, several concerns affect the interpretation of the data and diminish the impact of the current findings. The authors have reported the results of the murine model of permanent ligation. It is suggested to study the role of HIF-2 α in a more relevant model as ischemia-reperfusion injury. The authors must include the limitations of using a permanent ligation model.

Major comments:

-One novel aspect of this study is the assessment of endothelial barrier integrity after MI. The authors used Evans Blue staining to measure cardiac vascular permeability, which requires careful handling to avoid artificial increases in permeability. MRI may provide more robust evidence of vascular permeability.

-The authors have used the permanent ligation model to study HIF-2 α . Please justify the choice of this model over the ischemia-reperfusion model due to clinical relevance and explain the potential differences in HIF-2 α when the myocardium is reperused.

-Clarify using 100% oxygen during the surgical procedure and specify if other gases were provided (include percentages).

The echocardiography images provided do not represent a 28-day post-MI heart. It is recommended that acquisition be performed under consistent echocardiography settings. The EF and FS don't correlate with the images provided.

-The duration of ischemia and hypoxia are crucial for understanding the implications of damage in the in vivo and in vitro studies. The in vitro protocol for hypoxia is not clear and should be included.

-Specify the time points at which inflammation and permeability were assessed in relation to the HIF-2 α /ARNT pathway.

-Discuss the implications and role of HIF-2 α in both cardiomyocytes and endothelial cells.

Minor Points:

Keywords should include "HIF-2 α , Heart failure by HIF-2 α , Heart failure."

The authors should consistently use HIF-2 α , Hif2 α , or HIF2a throughout the text. If there are differences between these terms, they should be clearly defined.

Decide whether to use *ecHif2 α* ^{-/-}, *hif2a*^{-/-}, or *Hif2 α flox/flox*+CRE, as it is currently difficult to follow.

Provide definitions for all abbreviations used in the manuscript.

Ensure that "in vivo" is italicized throughout the text.

It is unclear at what time points the inflammation and permeability of HIF-2 α /ARNT were assessed. I have included this information for clarity.

Reviewer #2

(Remarks to the Author)

In this manuscript entitled "A novel ARNT-dependent HIF-2 α signaling as a protective mechanism for cardiac microvascular barrier integrity and heart function post-myocardial infarction", the authors adopted myocardial infarction model and investigated the roles of HIF-2 α signaling in vascular endothelial cells. The in vivo study showed that vascular integrity was disrupted in endothelial cell specific HIF-2 α deficient mice. As a result, left ventricular systolic function was impaired, cardiac fibrosis was more prominent in HIF-2 α deficient mice. As a molecular process, the authors identified that genes related to endothelial barrier function such as *cdh-5*, *tip-1* and *angpt-2* were downregulated in HIF-2 α deficient mice. The author also focused on the expression of inflammatory cytokines including IL-6. By performing in vitro analyses, the authors addressed that ARNT suppressed IL-6 expressions in HIF-2 α deficient cells. Overall, the manuscript is well written, and in vivo study showed the homeostatic function of HIF-2 α in vascular integrity. However, the reviewer has several concerns on this manuscript as follows.

Major comments

1. The main results of the in vivo myocardial infarction model are the roles of HIF-2 α in vascular homeostasis. The reviewer considers that the molecular process by which vascular integrity is disrupted in HIF-2 α deficient mice has to be analyzed in more detail, since. Is reduction of *cdh-5* transcript related to the phenotype of MI model? How does IL-6 participate in the vascular disruption in HIF-2 α deficient mice?
2. It is unclear why the authors focused on IL-6 instead of *cdh-5* in the in vitro analysis. If the authors were to elucidate the molecular processes by which HIF-2 α deletion disrupt vascular integrity, they should focus on the genes related to endothelial barrier function.
3. In figure 7-9, the authors analyzed the roles of ARNT in IL-6 gene expression. How does HIF-2 α affect the roles of ARNT? Is HIF-2 α required for the function of ARNT? If so, how does HIF-2 α affect ARNT activity? The consensus idea is that the heterodimer of HIF-2 α /ARNT binds to hypoxia responsible element. If ARNT alone, but not HIF-2 α bind to the DNA sequence and suppresses IL-6 expression, how about the consensus sequence at ARNT binding sites?
4. ARNT not only binds to HIF-2 α , but also HIF-1 α , Clock or NPAS2. How does these transcription factors affect the function of ARNT? Not only the western blot, ChIP-PCR data of HIF-1 α or other transcription factors are required to elucidate the roles of ARNT in IL-6 gene expression.

Minor comments

1. In figure 1D, which genes or primers pair did the authors used to detect the deletion efficiency? They need to evaluate the primers detecting deleted exon of HIF-2 α flox mice compared to the non-deleted exon of HIF-2 α gene.
2. In Figure 5, the author used ARNT mutant vector. The author need to describe more detailed information of the ARNT mutant.
3. In supplementary figure 1c, HIF-1 α ^{-/-} should be corrected to HIF-2 α ^{-/-}.

Reviewer #3

(Remarks to the Author)

In the manuscript by Ullah and Ai et al. titled "A Novel ARNT-Dependent HIF-2 α Signaling as a Protective Mechanism for Cardiac Microvascular Barrier Integrity and Heart Function Post-Myocardial Infarction," the authors investigated the role of HIF-2 α in endothelial cells in maintaining blood vessel integrity following myocardial infarction. The researchers used mice with a specific deletion of HIF-2 α in *Cdh5*-expressing endothelial cells. They found that these mice showed normal function at baseline. After myocardial infarction, mice lacking HIF-2 α exhibited higher mortality, increased leakage from heart blood vessels, inflammation, decreased heart function, and worsened fibrotic remodeling. The study also demonstrated that increasing the levels of ARNT, an essential partner of HIF-2 α , noticeably reduced these effects. Interestingly, the researchers found that ARNT, not HIF-2 α , directly attaches to the IL6 gene promoter to attenuate its expression. This manuscript provides valuable insights into the repressive role of the endothelial HIF-2 α /ARNT axis in gene expression during ischemia, offering potential new avenues for treating heart failure.

Major Comments:

In Figure 2, the authors describe increases in heart weight, fibrosis, and immune cell infiltration. However, a more thorough assessment and quantitation of these claims should be addressed.

Minor comments:

Error in the spelling of “normoxia” in Figure 1 panel C. Please check other figures as well.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

In this revised manuscript, the authors responded to the comments raised by the reviewer. The reviewer has no further concern on this manuscript.

Reviewer #3

(Remarks to the Author)

The authors have addressed my major and minor comments sufficiently.

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Response to Reviewer Comments

Dear Dr Wu,

Your manuscript entitled "A Novel ARNT-Dependent HIF-2 α Signaling as a Protective Mechanism for Cardiac Microvascular Barrier Integrity and Heart Function Post-Myocardial Infarction" has now been seen by 3 referees, whose comments are appended below. You will see from their comments copied below that while they find your work of potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the manuscript for publication but would be interested in considering a revised version that addresses these important concerns.

We hope you will find the referees' comments useful as you decide how to proceed. Should further experimental data or analysis allow you to address these criticisms, we would be happy to look at a substantially revised manuscript.

In particular, please note that the following revisions would be necessary for us to contact our referees again: please carefully address the concerns from reviewer #1 regarding the permanent ligation model used. Please also pay particular attention to reviewer #2's concerns regarding the mechanistic data supporting vascular integrity disruption by HIF2 α . The reviewer offered constructive feedback on how to strengthen these data.

Response:

Dear Dr. Ummarino and Board Committee Members,

Thank you for your thorough review and constructive feedback on our manuscript titled "A Novel ARNT-Dependent HIF-2 α Signaling as a Protective Mechanism for Cardiac Microvascular Barrier Integrity and Heart Function Post-Myocardial Infarction," submitted to Communications Biology. We truly appreciate the positive comments and valuable suggestions provided by you and the reviewers.

We have carefully addressed all the major concerns raised in the initial review, particularly regarding the choice of the permanent ligation model and the mechanistic data surrounding HIF-2 α 's role in vascular integrity, as highlighted by reviewers #1 and #2. We have included additional justification for our model choice and expanded our analysis to further clarify the molecular processes, with a particular focus on CDH5 and IL-6. Additionally, we have incorporated further data as recommended to strengthen our findings. All changes made to the manuscript have been tracked in yellow, and a point-by-point response to each reviewer's

Reviewer 1

Comments -The authors have used the permanent ligation model to study HIF-2 α . Please justify the choice of this model over the ischemia-reperfusion model due to clinical relevance and explain the potential differences in HIF-2 α when the myocardium is reperfused.

Response:

Thank you for your valuable suggestion regarding the ischemia-reperfusion (I/R) model. We agree that the I/R model closely mimics reperfusion injury in patients and offers essential insights into acute ischemic injury. However, for this study, we chose the permanent ligation (PL) model to focus specifically on the long-term effects of sustained ischemia and chronic heart failure, which better aligns with our research objectives. The PL model allowed us to evaluate the protective role of the HIF-2 α /ARNT pathway over prolonged ischemia, emphasizing its relevance in chronic myocardial infarction. By contrast, the I/R model would primarily highlight acute changes in HIF-2 α /ARNT signaling.

In the revised manuscript (Page 7), we have clarified our rationale for using the PL model and acknowledged its limitation in not capturing the reperfusion phase. To address this, we conducted *in vitro* hypoxia-reoxygenation (HR) experiments using human microvascular endothelial cells (HMVECs) to simulate ischemia-reperfusion injury (see Figure 1, L and M). These experiments allowed us to assess the role of HIF-2 α in maintaining endothelial barrier function during conditions that resemble reperfusion injury.

While incorporating both PL and I/R models would provide a more comprehensive analysis, this approach is beyond the scope of the current study. However, we are actively exploring the use of the I/R model in ongoing research and plan to present these findings in future publications. Thank you again for the suggestion.

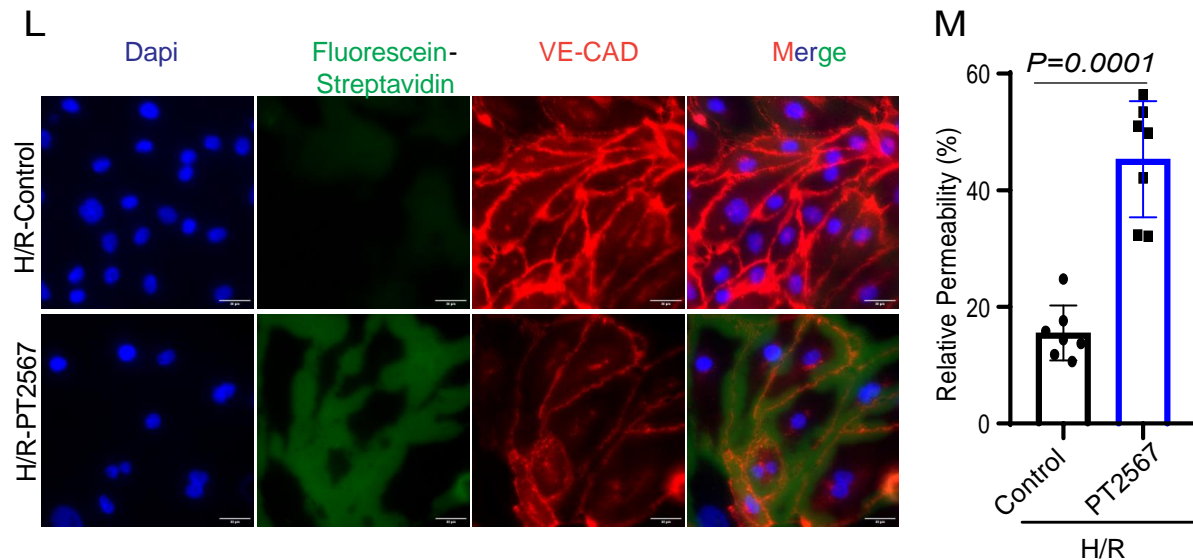


Figure 1L, M: *In vitro* permeability assay in a hypoxia-reperfusion (HR) model. Primary Human Microvascular Endothelial Cells (HMVECs) were treated with the HIF-2 α inhibitor PT2567 and subjected to hypoxia (overnight) followed by reoxygenation for 6 hours. The nuclei were stained with DAPI (blue), VE-Cadherin was stained with a specific antibody (red), and fluorescein-streptavidin (green) was used to indicate cell permeability. Increased green fluorescence represents enhanced leakage across the endothelial monolayer. Scale bar: 30 μ m. The bar graph on the right quantifies permeability, showing a significant increase in the PT2567-treated group compared to the control ($P =$

0.0001). *Fluorescence Intensity Analysis: ImageJ was used to measure mean fluorescence intensity. The relative intensity of the green channel (fluorescein-streptavidin) was compared between the PT2567-treated group and the control group to assess changes in permeability.*

Comments: One novel aspect of this study is the assessment of endothelial barrier integrity after MI. The authors used Evans Blue staining to measure cardiac vascular permeability, which requires careful handling to avoid artificial increases in permeability. MRI may provide more robust evidence of vascular permeability

Response:

Thank you for your insightful comment regarding the potential use of MRI to assess vascular permeability. In our study, we employed a multi-faceted approach to evaluate endothelial barrier integrity in response to HIF-2 α expression under hypoxic conditions. Specifically, we used three complementary methods: Evans Blue staining for in vivo assessment, ex vivo permeability assays with primary cardiac microvascular endothelial cells (CMVECs) from both eHIF-2 α knockout and control mice measured via the ECIS system to evaluate trans-endothelial resistance (TER), and a new in vitro permeability assay. The ECIS analysis demonstrated a significant reduction in TER under hypoxic conditions, indicating compromised barrier function, which we further confirmed by observing reductions in VE-cadherin expression and junction integrity.

We agree that MRI would offer robust evidence of vascular permeability. Unfortunately, our animal MRI machine is undergoing repairs, which has postponed any further MRI-based data collection. Given the robust findings from our current methodologies, we hope this will provide sufficient evidence without delaying publication. Thank you for your understanding and support in this matter.

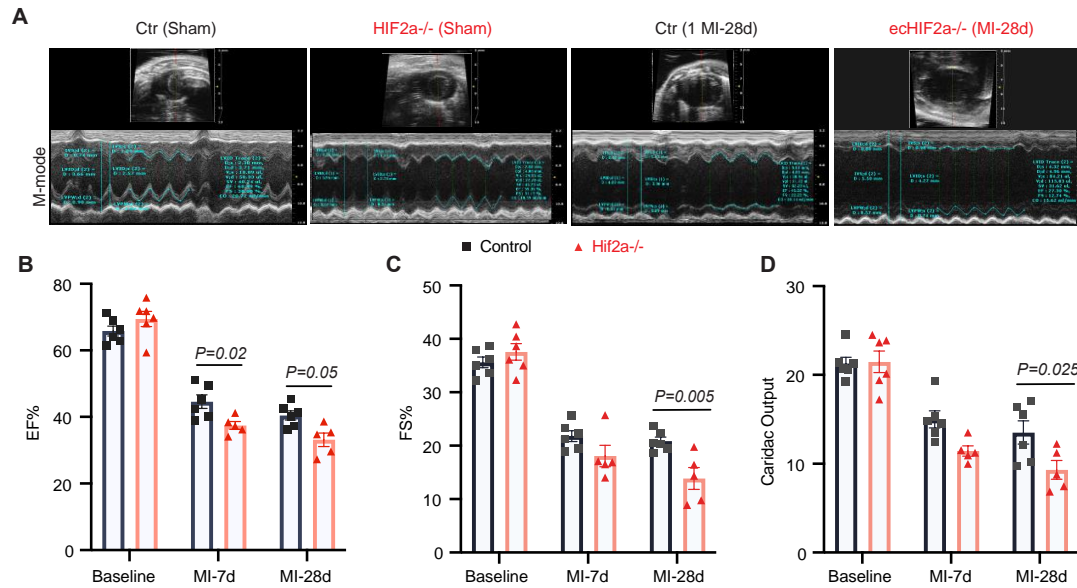
Comment -Clarify using 100% oxygen during the surgical procedure and specify if other gases were provided (include percentages)

Response:

Thank you for your valuable feedback. During the surgical procedure, we administered 100% oxygen, in line with our standard protocol, to ensure proper oxygenation. Anesthesia was maintained with 1.5% isoflurane, delivered in a mixture with 100% oxygen throughout the procedure. This clarification has been added to the Methods section on Page 10 of the revised manuscript.

Comment -The echocardiography images provided do not represent a 28-day post-MI heart. It is recommended that acquisition be performed under consistent echocardiography settings. The EF and FS don't correlate with the images provided.

Response: Thank you very much for highlighting this issue. Some images were indeed captured using a different version of the echocardiography machine, which may have resulted in varying settings. We have carefully reviewed all echocardiography images and selected representative ones that accurately correspond to the EF and FS values reported. These updated images are now included in the revised figure seen below.



Comment: The duration of ischemia and hypoxia are crucial for understanding the implications of damage in the in vivo and in vitro studies. The in vitro protocol for hypoxia is not clear and should be included.

Response:

We appreciate the reviewer's insightful comment regarding the in vitro hypoxia protocol. To address this, we have included a detailed description on page 12 of the revised manuscript. Briefly, cells were seeded in 6-well plates, and after 24 hours, they were exposed to 1% oxygen balanced with 5% CO₂ and 95% N₂ for 16 hours in an InVivo 2400 hypoxia workstation (Ruskin Technologies). Cells were then harvested immediately after the 16-hour hypoxia exposure. We hope this clarification improves understanding of our in vitro hypoxia model and adequately addresses the reviewer's comment. Thank you for the valuable feedback.

Comments- Specify the time points at which inflammation and permeability were assessed in relation to the HIF-2α/ARNT pathway.

Response: We thank the reviewer for your thoughtful comments. The time points for assessing inflammation and permeability in relation to the HIF-2α/ARNT pathway have been clarified in the revised manuscript. Specifically, in vivo and ex vivo permeability measurements were conducted 24 hours post-MI. Inflammation markers were evaluated both at an early stage, 12 hours post-MI, and at 28 days post-MI to capture both acute and chronic responses. We hope this clarification adequately addresses your concern. Thank you for your valuable feedback.

Reviewer 2

Comments- Overall, the manuscript is well written, and in vivo study showed the homeostatic function of HIF-2 α in vascular integrity. However, the reviewer has several concerns on this manuscript as follows. The main results of the in vivo myocardial infarction model are the roles of HIF-2 α in vascular homeostasis. The reviewer considers that the molecular process by which vascular integrity is disrupted in HIF-2 α deficient mice has to be analyzed in more detail, since. Is reduction of cdh-5 transcript related to the phenotype of MI model? How does IL-6 participate in the vascular disruption in HIF-2 α deficient mice.

Response: Thank you for your insightful feedback and comments on our manuscript. We have carefully considered your concerns and made revisions accordingly. Below are detailed responses to each point:

Reviewer comments 1: The molecular process by which vascular integrity is disrupted in HIF-2 α -deficient mice needs to be analyzed in more detail. Is the reduction of cdh-5 transcript related to the phenotype of the MI model?

Response:

Thank you for your insightful comment. The reduction of cdh-5 (VE-cadherin) transcript is indeed associated with the phenotype observed in the myocardial infarction (MI) model. Loss of HIF-2 α in mouse endothelial cells exacerbates the reduction of cdh-5 expression, as shown in our gene sequencing data from HIF-2 α -deficient mouse hearts. Furthermore, in vitro data from human and mouse endothelial cells confirmed that HIF-2 α regulates VE-cadherin expression at both the transcriptional and protein levels, as demonstrated by western blotting and immunofluorescence analysis. These findings underscore the critical role of HIF-2 α in maintaining endothelial barrier integrity through the regulation of VE-cadherin expression.

Reviewer comments 2: How does IL-6 participate in the vascular disruption in HIF-2 α -deficient mice?

Response:

We have also observed that HIF-2 α deletion leads to an upregulation of inflammatory cytokines in serum, particularly IL-6, but not IL-1 β or TNF- α . IL-6 can directly or indirectly act on vascular endothelial cells. [1] IL-6 is known to disrupt endothelial barrier function through several mechanisms, including its effects on VE-cadherin [2]. It is reported that specifically, IL-6 promotes the phosphorylation of VE-cadherin and associated proteins like β -catenin, which leads to the internalization or degradation of VE-cadherin, thereby weakening cell-cell adhesion and increasing vascular permeability. Additionally, IL-6 activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, which further contributes to endothelial dysfunction by altering endothelial cell behavior [3]. Over-activation of this pathway increases vascular permeability and compromises the endothelial barrier. In our study, deletion of IL-6 was able to restore VE-cadherin expression that had been impaired by HIF-2 α deletion (Figure 6J seen below). We plan to explore more signaling pathways related to this process in our ongoing research.

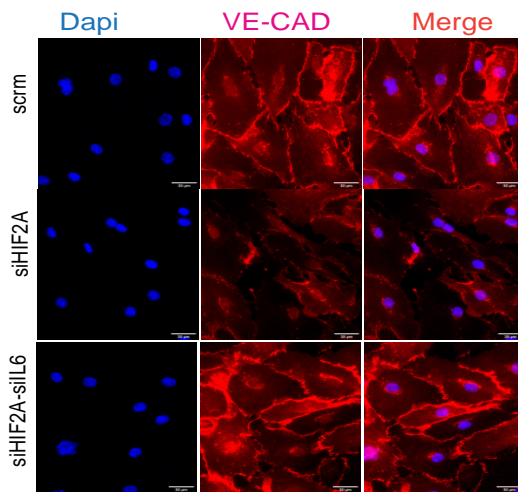


Figure 6J. Role of IL-6 Knockdown in Restoring VE-Cadherin (VE-CAD) Expression. Immunofluorescence staining of VE-Cadherin (VE-CAD) expression in human cardiac microvascular endothelial cells (HCMECs) following HIF-2 α knockdown by siRNA, with and without IL-6 knockdown (siRNA). DAPI (blue) indicates nuclei, and VE-CAD (red) marks cell-

Reviewer comments 3- It is unclear why the authors focused on IL-6 instead of cdh-5 in the in vitro analysis. If the authors were to elucidate the molecular processes by which HIF-2a deletion disrupt vascular integrity, they should focus on the genes related to endothelial barrier function.

Response:

Thank you for your valuable feedback. We appreciate the suggestion to focus on genes directly related to endothelial barrier function. We agree that cdh-5 (VE-cadherin) plays a crucial role in maintaining endothelial integrity. In our *in vitro* experiments, we indeed examined the role of HIF-2 α in regulating cdh-5 expression under various conditions (Fig. 1f-h, Fig. 3g, Fig. 5b, Fig. 6a-c). Our findings show that HIF-2 α deficiency leads to reduced cdh-5 expression, which correlates with increased vascular permeability and VE-cadherin disruption.

Additionally, we examined IL-6's role in this process, as IL-6 is known to influence VE-cadherin function by promoting phosphorylation and internalization, weakening endothelial junctions. Our study shows that IL-6 expression is elevated in HIF-2 α -deficient cells, and deletion of IL-6 in HMVECs partially restores the HIF-2 α -deficiency-induced VE-cadherin disruption under hypoxic conditions. We propose that HIF-2 α regulates endothelial barrier function indirectly, partially via IL-6, though other factors may also contribute to the regulation of cdh-5. The dual regulation provides a more comprehensive understanding of how HIF-2 α maintains vascular integrity, addressing your concern regarding our focus on IL-6 alongside cdh-5 in the in vitro analysis.

Reviewer comments 4- In figure 7-9, the authors analyzed the roles of ARNT in IL-6 gene expression. How does HIF-2a affect the roles of ARNT? Is HIF-2a required for the function of ARNT? If so, how does HIF-2a affect ARNT activity? The consensus idea is that the heterodimer of HIF-2a/ARNT binds to hypoxia responsible element. If ARNT alone, but not HIF-2a bind to the DNA sequence and suppresses IL-6 expression, how about the consensus sequence at ARNT binding sites.

“How does HIF-2a affect ARNT activity?” “Is HIF-2a required for the function of ARNT? If so, how does HIF-2a affect ARNT activity?”

Response:

HIF-2 α (Hypoxia-Inducible Factor-2 alpha) modulates ARNT (Aryl Hydrocarbon Receptor Nuclear Translocator) activity by enhancing its DNA-binding ability and regulatory function, especially under hypoxic conditions. Our study demonstrates that HIF-2 α might be essential for ARNT's ability to effectively repress IL-6 expression; deletion of either HIF-2 α or ARNT leads to increased IL-6 expression.

HIF-2 α affects ARNT activity by enabling ARNT to bind efficiently to the hypoxia response element (HRE). We included new data in Figure 6 (see below) to illustrate how the deletion of one HIF partner can influence the protein expression of the other. data from HUVECs revealed that deletion of either HIF2 α or ARNT did not alter HIF1 α levels, nor did ARNT levels change under hypoxia with HIF1 α or HIF2 α deletion (Fig. 6A-G, seen below). Notably, either HIF1 α or ARNT deletion led to elevated HIF2 α protein levels. Typically, ARNT requires a partner, such as HIF-2 α (or HIF-1 α), to form a functional heterodimer, which then binds the HRE and regulates transcription. Without HIF-2 α , ARNT alone cannot sufficiently bind the HRE or recruit the necessary co-factors for transcriptional repression, which leads to increased IL-6 expression. Although ARNT can form alternative complexes, such as homodimers or those with other transcriptional partners, these do not appear to compensate for the absence of HIF-2 α in IL-6 regulation. This indicates that the specific HIF-2 α /ARNT interaction is essential for effective IL-6 control under hypoxic conditions. Further investigation in our lab will delve deeper into these mechanisms.

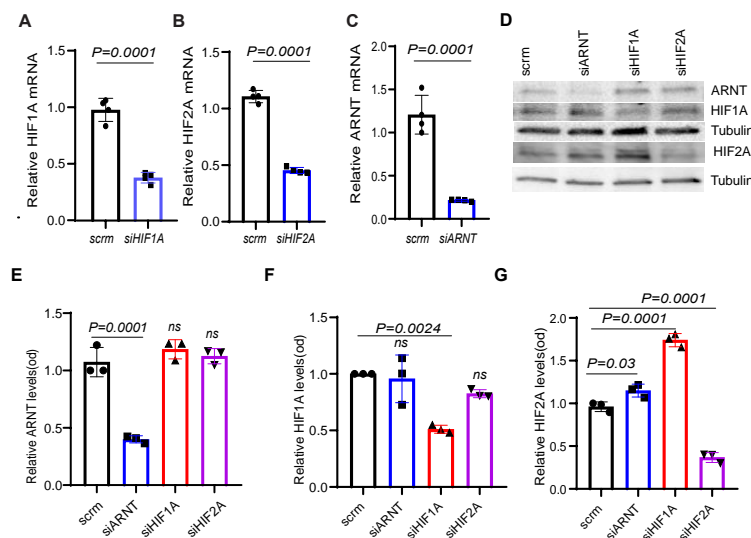


Figure 6 Regulation of HIF Pathway Components and IL-6 in Endothelial Cells: **A-C:** qPCR analysis reveals the significant reduction in mRNA levels of HIF1A, HIF2A, and ARNT in HUVECs following transfection with the specified siRNAs, indicating the effectiveness of the gene silencing approach. **D:** Representative Western blot demonstrating the impact of siRNA-mediated knockdown on ARNT, HIF1A, and HIF2A protein levels in HUVECs exposed to 1% oxygen for 16 hours. **E-G:** Quantitative analysis of protein levels for ARNT, HIF1A, and HIF2A using ImageJ, based on three to four independent experiments.

Comments: The consensus idea is that the heterodimer of HIF-2 α /ARNT binds to hypoxia responsible element. If ARNT alone, but not HIF-2 α bind to the DNA sequence and suppresses IL-6 expression, how about the consensus sequence at ARNT binding sites.

Response: Yes, the consensus understanding is that the HIF-2 α /ARNT heterodimer typically binds to hypoxia-responsive elements (HREs) to activate target gene expression. However, in the regulation of IL-6, only ARNT binds directly to the DNA sequence, not HIF-2 α . HIF-2 α may regulate IL-6 indirectly by modulating ARNT's activity. It's most likely that ARNT regulates IL-6 through the typical HRE binding sites, though novel binding sites may also be involved, which would require further investigation. In our current study, the mechanisms by which ARNT

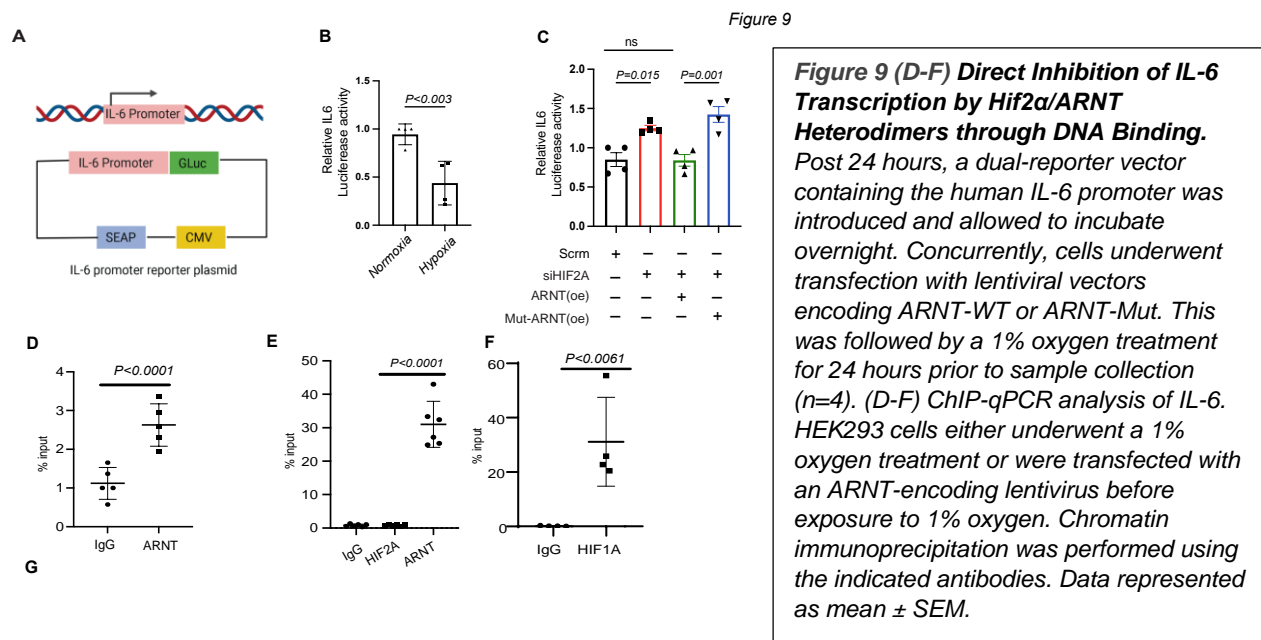
regulates IL-6 were beyond our scope, but we aim to explore these in future research. Thank you very much for the review's great input.

Comments 5- ARNT not only binds to HIF-2 α , but also HIF-1 α , Clock or NPAS2. How does these transcription factors affect the function of ARNT? Not only the western blot, ChIP-PCR data of HIF-1 α or other transcription factors are required to elucidate the roles of ARNT in IL-6 gene expression.

Response: We appreciate the reviewer's suggestion to further investigate the interactions between ARNT and other transcription factors, such as HIF-1 α . Indeed, transcription factors like Clock and NPAS2 can modulate ARNT function by influencing the stability and activity of ARNT-containing heterodimers, as reported in previous studies [4, 5]. Additionally, these heterodimers can impact ARNT expression levels.

In our revised manuscript (see Fig 6), our data indicate that ARNT protein levels remain unchanged when either HIF1 α or HIF2 α is deleted. However, deletion of either HIF1 α or ARNT appears to lead to increased HIF2 α protein levels, possibly due to compensatory mechanisms. Furthermore, under hypoxic conditions, the loss of HIF2 α reveals that the HIF1 α /ARNT complex alone is not sufficient to repress IL-6 expression. This observation aligns with previous findings, where HIF-1 α was shown to bind directly to the IL-6 promoter in B cells, promoting IL-6 production [6]

In the updated manuscript, Figure 9, D-F, our chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) analysis further supports this, demonstrating that both ARNT and HIF1 α , but not HIF2 α , directly bind to the IL-6 promoter. This suggests that HIF2 α may regulate IL-6 via ARNT at the IL-6 DNA binding site, implying a potential repressive role for the ARNT/HIF2 complex in modulating IL-6 expression under hypoxic conditions.



In the manuscript by Ullah and Ai et al. titled “A Novel ARNT-Dependent HIF-2 α Signaling as a Protective Mechanism for Cardiac Microvascular Barrier Integrity and Heart Function Post-Myocardial Infarction,” the authors investigated the role of HIF-2 α in endothelial cells in maintaining blood vessel integrity following myocardial infarction. The researchers used mice with a specific deletion of HIF-2 α in Cdh5-expressing endothelial cells. They found that these mice showed normal function at baseline. After myocardial infarction, mice lacking HIF-2 α exhibited higher mortality, increased leakage from heart blood vessels, inflammation, decreased heart function, and worsened fibrotic remodeling. The study also demonstrated that increasing the levels of ARNT, an essential partner of HIF-2 α , noticeably reduced these effects. Interestingly, the researchers found that ARNT, not HIF-2 α , directly attaches to the IL6 gene promoter to attenuate its expression. This manuscript provides valuable insights into the repressive role of the endothelial HIF-2 α /ARNT axis in gene expression during ischemia, offering potential new avenues for treating heart failure.

Major Comments:

In Figure 2, the authors describe increases in heart weight, fibrosis, and immune cell infiltration. However, a more thorough assessment and quantitation of these claims should be addressed.

Response:

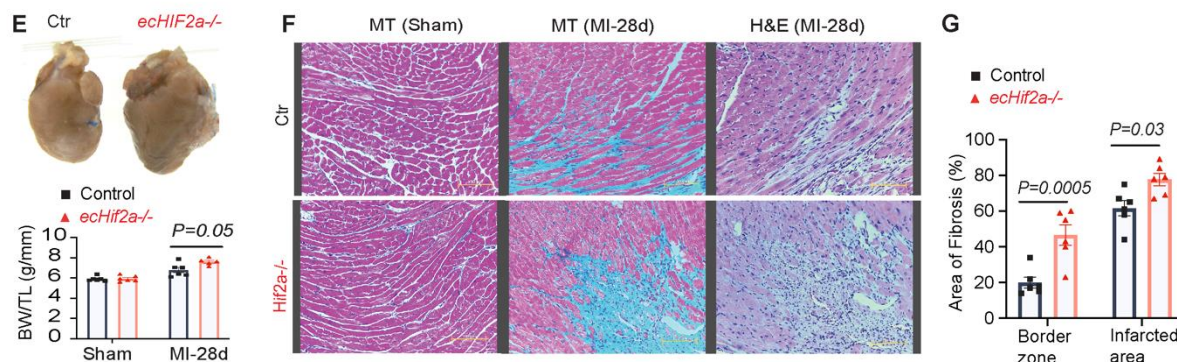


Figure 2: (E) Representative images of hearts at 28 days post-MI. Summary of heart weight normalized to tibia length is shown under the (F) Histological analysis of heart sections stained with hematoxylin-eosin (H&E) and Masson's trichrome (MT). (G) Quantitative analysis of fibrosis, using ImageJ software.

Thank you for the thoughtful input. In the revised manuscript, we have expanded our analysis in Figure 2 (E, F) to provide a more comprehensive assessment. We now show heart weights normalized to corresponding tibia lengths, which highlights a significant increase in heart weight in *ecHIF2 α -/-* mice compared to controls post-MI. This increase likely reflects compensatory hypertrophic changes during the remodeling process following MI.

Additionally, we quantified fibrosis in both the infarcted area and the border zone. Our data indicate that endothelial cell-specific HIF2 α deletion leads to a significant increase in fibrosis, consistent with the observed upregulation of genes involved in fibrosis, as identified in our RNA

sequencing analysis of ecHIF2 α –/– mouse hearts. This additional quantification and analysis underscore the role of HIF2 α in modulating cardiac remodeling and fibrosis post-MI.

Regarding immune cell infiltration. In our study, we observed clear immune cell infiltration in H&E-stained sections, indicative of an inflammatory response. This finding is supported by significantly elevated serum IL-6 levels in ecHIF2 α –/– mice post-MI, along with RNA sequencing data showing upregulated IL-6 and other inflammatory genes in ecHIF2 α –/– hearts post-MI. These results align with our in vitro data indicating that HIF2 α negatively regulates IL-6 expression, suggesting that HIF2 α deletion amplifies the pro-inflammatory environment, contributing to immune cell recruitment post-MI.

Minor

comments:

Error in the spelling of “normoxia” in Figure 1 panel C. Please check other figures as well.

Response: It has been corrected. Thank you.

References

1. Kang, S. and T. Kishimoto, *Interplay between interleukin-6 signaling and the vascular endothelium in cytokine storms*. Exp Mol Med, 2021. **53**(7): p. 1116-1123.
2. Valle, M.L., et al., *Inhibition of interleukin-6 trans-signaling prevents inflammation and endothelial barrier disruption in retinal endothelial cells*. Exp Eye Res, 2019. **178**: p. 27-36.
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6. Kou, K., et al., *Hypoxia-inducible factor 1 α /IL-6 axis in activated hepatic stellate cells aggravates liver fibrosis*. Biochem Biophys Res Commun, 2023. **653**: p. 21-30.