The protein tyrosine phosphatase PTP-PEST mediates hypoxia-induced endothelial autophagy and angiogenesis via AMPK activation

Shivam Chandel, Amrutha Manikandan, Nikunj Mehta, Abel Arul Nathan, Rakesh Kumar Tiwari, Samar Bhallabha Mohapatra, Mahesh Chandran, Abdul Jaleel, Narayanan Manoj and Madhulika Dixit
DOI: 10.1242/jcs.250274

Editor: John Heath

Review timeline
Original submission: 18 June 2020
Editorial decision: 10 August 2020
First revision received: 21 October 2020
Accepted: 23 November 2020

Original submission

First decision letter

MS ID#: JOCES/2020/250274

MS TITLE: Protein tyrosine phosphatase-PEST (PTP-PEST) mediates hypoxia-induced endothelial autophagy and angiogenesis through AMPK activation.

AUTHORS: Shivam Chandel, Amrutha Manikandan, Nikunj Mehta, Abel Arul Nathan, Rakesh Kumar Tiwari, Samar Bhallabha Mohapatra, Mahesh Chandran, Abdul Jaleel, Narayanan Manoj, and Madhulika Dixit
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The study "Protein tyrosine phosphatase-PEST (PTP-PEST) mediates hypoxia-induced endothelial autophagy and angiogenesis through AMPK activation" is a very interesting and important study by implicating PTP-PEST as an important regulator of hypoxia-induced and regulated AMPK activity and thereby affecting critical processes like autophagy and angiogenesis. The results of the study are technically sound and of outstanding importance to researchers in the field and to the broad readership of JCS.

Comments for the author

The manuscript reads well and it is easy to follow. I have only minor comments to be addressed before publication.
1. page 5 lane 113: The authors claim that they performed HIF-1a immunoblotting, however this data was is not shown in the manuscript. I would recommend to add these blots.
2. The cytoplasmic localisation of PTP-Pest was analysed, as demonstrated in the supplementary data. Can the authors comment on the subcellular localisation of PHD-2 which might an impact on the demonstrated results. J Cell Sci. 2012 Nov 1;125(Pt 21):5168-76 should be discussed in that context.
3. page 6 lane 143: the citation regarding the program "panther" is missing.
4. Regarding the tube formation assay: Can the authors give detailed informations about the used media/FCS etc.
5. page 15. lane 353: This is an important information, may be chip assays might be valuable 6. The most of the figure legends have to be improved 6.1 figure 1: 1D: can the authors add densitometric data of IB:PTP-PEST. The authors claimed to see almost no differences. I can´t follow the estimation by eye.
6.2 1C the authors should note the cell lines used for HIF-1a and beta-actin immunoblotting 6.3 1A Can the authors add an extended cutout of the HIF-1a gel. It would be of interest to estimate possible changes of the phosphorylated HIF-1a signals.
6.4 figure 3G: The authors should comments on the differences between PTP-PEST signals. Signal intensity is varying
6.5 figure 7: I would recommend to add AMP and LKB1 and CAMKKbeta to the sketch
6.6 page 30. N=? the number of experiments should be annotated.
6.7 page 31. A detailed describtions of the figure is missing in the figure legend.

Reviewer 2

Advance summary and potential significance to field

Under hypoxia PTP-PEST-AMPK interaction is lost leading to angiogenesis .

Comments for the author

In this manuscript the authors inverstigated the role of PTP-PEST in the endothelial response to hypoxia. They found that hypoxia (1% oxygen) increases protein levels and catalytic activity of PTP-PEST in primary endothelial cells. PTP-PEST interacted with AMP-activated protein kinase alpha subunits (AMPK α1 and α2) under normoxia but not in hypoxia. Knock-down of PTP-PEST abrogated hypoxia mediated tyrosine dephosphorylation and activation of AMPK (Thr172 phosphorylation) and blocked hypoxia-induced autophagy and also attenuated endothelial cell migration and capillary tube formation . They conclude that PTP-PEST is a regulator of hypoxia-induced AMPK activation and endothelial autophagy to promote angiogenesis.development and embryonic lethality.

Although this is a potentially interesting manuscript several points remain to be elucidated.

Fig. 1C: This figure is unclear.. why is HIF1a showing up there? Also the description in the results section is not matching the figure. Please clarify.
Fig. 1D: How was the IP performed? Was there a specific antibody against phosphorylated PTP-PEST used? This is not clear from the figure labeling,
Does metformin also recover endothelial migration and angogenesis?
How ist the role of HIF1a in the hypoxic angiogenis response regulated by PTP-PEST? Please provide data,
The authors conclude that the proposed pathways play a role in development and embryonic lethality without providing data. Please provide data or omit these parts.

First revision

Author response to reviewers' comments

Reviewer 1

Advance Summary and Potential Significance to Field: The study "Protein tyrosine phosphatase-PEST (PTP-PEST) mediates hypoxia- induced endothelial autophagy and angiogenesis through AMPK activation" is a very interesting and important study by implicating PTP-PEST as an important regulator of hypoxia-induced and regulated AMPK activity and thereby affecting critical processes like autophagy and angiogenesis. The results of the study are technically sound and of outstanding importance to researchers in the field and to the broad readership of JCS.

Reviewer 1 Comments for the Author:

The manuscript reads well and it is easy to follow. I have only minor comments to be addressed before publication.

1. page 5 lane 113: The authors claim that they performed HIF-1a immunoblotting, however this data was is not shown in the manuscript. I would recommend to add these blots.

Response: As suggested by the reviewer, we have now included this data in Fig. 1C of the revised manuscript.

2. The cytoplasmic localisation of PTP-Pest was analysed, as demonstrated in the supplementary data. Can the authors comment on the subcellular localisation of PHD-2 which might an impact on the demonstrated results. J Cell Sci. 2012 Nov 1;125(Pt 21):5168-76 should be discussed in that context.

Response: We thank the reviewer for this pertinent suggestion and bringing our attention to the above-mentioned article. PHD-2 can hydroxylate proteins at proline residues present in 'PEST' motifs to mediate their proteasomal degradation [1], [2]. Since PTP-PEST carries four PEST motifs in tandem, it is possible that PTP-PEST may be a target of PHD2 mediated degradation. Nuclear localization of PHD2 is necessary for induction of its hydroxylase activity as reported in the mentioned paper [3] and it is found to be higher in the nuclear fraction during hypoxia [4]. Based on these papers and our observation of cytoplasmic retention of PTP-PEST during hypoxia as seen in Fig. S1A, it is possible that under hypoxic conditions, nuclear PHD-2 fails to hydroxylate cytoplasmic PTP-PEST thereby enhancing the protein stability of this phosphatase. Hence, one of the reasons for increased protein stability of cytosolic PTP-PEST during hypoxia could be the enhanced activity of PHD-2 in the nuclear compartment. We have now included these points in the discussion section of the revised manuscript (lines: 427-435).

3. page 6 lane 143: the citation regarding the program "panther" is missing.

Response: We thank the reviewer for pointing out this oversight, we have now included this citation in the revised manuscript.

4. Regarding the tube formation assay: Can the authors give detailed informations about the used media/FCS etc.

Response: As suggested, we have included these details on media and serum used in the 'Results' (lines: 318-323), 'Materials and Methods' (lines: 602-608) and the 'Figure Legends' (lines: 972-979) sections of the revised manuscript.

5. page 15. lane 353: This is an important information, may be chip assays might be valuable

Response: India is presently seeing a rapid increase in the number of COVID-19 cases and currently our Institute is under lock-down till end of December 2020 and all the research labs and administrative services are closed till end of this year. We will be in a position to comment on the feasibility of performing these experiments only in the month of January 2021, provided, the institute opens and we are able to place orders for the required reagents such as ChIP compatible HIF-1α antibody. At this stage of lock-down, with administration and freight facilities closed, we are unable to place orders till the pandemic situation improves. We also wish to bring to the reviewers notice that at this stage we do not know if the increase in PTP-PEST is due to an increase in transcription or due to an increase in protein stability. Considering these challenging times, we will appreciate if the reviewer can exempt us from the need of doing this experiment since the primary focus of this paper is to elucidate the novel role of PTP-PEST in hypoxia-induced AMPK activation, autophagy and angiogenesis.

6. The most of the figure legends have to be improved

Response: We thank the reviewer for pointing out this shortcoming and as suggested, we have provided detailed figure legends for all the figures in the revised manuscript.

6.1 figure 1: 1D: can the authors add densitometric data of IB:PTP-PEST. The authors claimed to see almost no differences. I can´t follow the estimation by eye.

Response: As suggested, we have now included the densitometric data (for figure 1D) as bar graph in Fig S1B of the revised manuscript.

6.2 1C the authors should note the cell lines used for HIF-1a and beta-actin immunoblotting

Response: As required, these details are now included in Fig. 1C of the revised manuscript as well as in the 'Results' (lines: 115-124) and 'Figure legends' (lines: 871-873) sections of the revised manuscript.

6.3 1A Can the authors add an extended cutout of the HIF-1a gel. It would be of interest to estimate possible changes of the phosphorylated HIF-1a signals.

Response: As suggested, an extended cut out of the HIF-1α blot is now included in Fig. 1A of the revised manuscript.

6.4 figure 3G: The authors should comments on the differences between PTP- PEST signals. Signal intensity is varying

Response: We wish to clarify to the reviewer that in this figure (Fig. 3G) we are demonstrating the effect of blockade of hypoxia-induced PTP-PEST increase, on activation of AMPK and its target ACC. Hence, in lane two for the PTP-PEST blot, we see an increase in PTP-PEST in response to hypoxia in cells infected with control lentivirus. This hypoxia-induced increase in PTP-PEST is however blocked in cells treated with PTP-PEST shRNA lentivirus (lane 4). So, in the Fig. 3G, lane 1 and lane 2 are for cells treated with control virus while, lane 3 and 4 are for cells treated with PTP-PEST ShRNA, with lane-4 demonstrating abrogation of hypoxia-induced increase in PTP-PEST expression. Since the knockdown for PTP-PEST worked for samples represented in lane 3 and 4, the protein levels of PTP-

PEST in these two lanes is lower than that seen in lane 1 and 2. The equal loading of gels was infact confirmed through Western blotting for ß-actin in this figure.

6.5 figure 7: I would recommend to add AMP and LKB1 and CAMKKbeta to the sketch

Response: We thank the reviewer for this suggestion. We have now included AMP, LKB1 and CAMKKß to the sketch in Fig. 7 of the revised manuscript.

6.6 page 30. N=? the number of experiments should be annotated.

Response: We have now provided this detail in all the figure legends of the revised manuscript.

6.7 page 31. A detailed description of the figure is missing in the figure legend.

Response: We thank the reviewer for pointing this out. A detailed description of the figure is now provided in the figure legend of Fig. 7 in the revised manuscript (lines: 988-1002).

Reviewer 2 Comments for the Author:

In this manuscript the authors investigated the role of PTP-PEST in the endothelial response to hypoxia. They found that hypoxia (1% oxygen) increases protein levels and catalytic activity of PTP-PEST in primary endothelial cells. PTP-PEST interacted with AMP-activated protein kinase alpha subunits (AMPK a1 and a2) under normoxia but not in hypoxia. Knock-down of PTP-PEST abrogated hypoxia mediated tyrosine dephosphorylation and activation of AMPK (Thr172 phosphorylation) and blocked hypoxia-induced autophagy and also attenuated endothelial cell migration and capillary tube formation. They conclude that PTP- PEST is a regulator of hypoxia-induced AMPK activation and endothelial autophagy to promote angiogenesis. development and embryonic lethality. Although this is a potentially interesting manuscript several points remain to be elucidated.

1. Fig. 1C: This figure is unclear. why is HIF1a showing up there? Also the description in the results section is not matching the figure. Please clarify.

Response: We wish to clarify to the reviewer that the purpose of Fig. 1C as mentioned in lines: 115-122 of the revised manuscript, was to determine if hypoxia increases the expression of PTP-PEST in other cell lines as well, such as in HEK293, HASMC, HeLa and Huh7. Fig. 1C thus confirms that hypoxia-induced increase in PTP-PEST is a universal phenomenon. Western blotting for HIF-1α was done to confirm induction of hypoxia and hence an increase in its protein levels is used as an internal control to confirm hypoxia. For all the blots shown in Fig. 1A (performed on HUVECs) and 1C (performed on cell lines), equal loading was confirmed by blotting for ß-actin and confirmation of hypoxia was done through HIF- 1α. Although in the original manuscript, the data for ß-actin and HIF-1α was not shown for all the cell lines in Fig. 1C, we have now included the ß-actin and HIF-1α data for all the cell lines in revised Fig. 1C.

2. Fig. 1D: How was the IP performed? Was there a specific antibody against phosphorylated PTP-PEST used? This is not clear from the figure labeling,

Response: Immunoprecipitation (IP) was performed using PTP-PEST AG10 monoclonal antibody (CST, USA). This PTP-PEST AG10 antibody is specific for PTP-PEST and it does not cross react with other protein tyrosine phosphatases. In this experiment, no specific antibody against phosphorylated PTP-PEST was used. In fact, it is presently unknown if PTP-PEST is phosphorylated in response to hypoxia. The purpose of this experiment was to specifically pull down endogenous PTP-PEST from primary endothelial cells and then perform phosphatase assay. Specificity of PTP-PEST pull down was confirmed by employing corresponding IgG isotype antibody as negative control as mentioned in the 'Methods' section of the manuscript (lines: 518-527). Immunoprecipitation of PTP-PEST was followed by phosphatase assay to determine effect of hypoxia on its catalytic activity. Detailed protocol for this experiment is provided in the 'Materials and Methods' section of the manuscript (lines: 518-534) as suggested by the reviewer, it is briefly explained even in the figure legend (lines: 873-877) of the revised manuscript.

3. Does metformin also recover endothelial migration and angiogenesis?

Response: AMPK plays an indispensable role in hypoxia-induced angiogenesis [5]– [8]. In the current study we demonstrate that PTP-PEST is necessary for hypoxia mediated activation of AMPK, in order to promote autophagy and consequent angiogenesis (Fig. 3G, Fig. 4 and Fig. 6 respectively). Given that metformin, a known activator of AMPK, recovered autophagy (Fig. 4 C-E) in PTP-PEST knocked down HUVECs and the fact that autophagy inducer rapamycin recovered angiogenesis in absence of PTP-PEST (Fig. 6), we believe metformin will also recover endothelial migration and angiogenesis under these settings. In fact others have already demonstrated that metformin promotes migration of HUVECs [9] as well as angiogenesis in experimental models of hypoxia such as stroke [10], [11]. These aspects of metformin are now included in the discussion section of the revised manuscript (lines: 399-402).

Under normal circumstances, we would have willingly executed these experiments in PTP-PEST knocked down primary endothelial cells. However, our Institute and research labs are currently closed till end of this year due to the COVID-19 pandemic and presently there is no clarity on when will it reopen for experimental research. Hence, we will not be able to perform these experiments. Considering this unprecedented pandemic situation and the uncertainty associated with it in these difficult times, we will appreciate if the reviewer in the interest of time could exempt us from performing these experiments and allow us to submit the revised manuscript with all the other doable corrections.

4. How is the role of HIF1a in the hypoxic angiogenesis response regulated by PTP- PEST? Please provide data.

Response: We would like to clarify to the reviewer that the results of the present study demonstrate that hypoxia enhances the expression and activity of PTP-PEST to promote endothelial autophagy and consequent angiogenesis. The reviewer may note that this study is not about regulation of HIF-1α in hypoxic angiogenesis by PTP-PEST.

5. The authors conclude that the proposed pathways play a role in development and embryonic lethality without providing data. Please provide data or omit these parts.

Response: The reviewer may kindly note that both in the abstract and the discussion section of the manuscript we have concluded that the proposed pathway is required for hypoxia-induced AMPK activation, autophagy and angiogenesis (lines: 53-55, and 463-465). Nowhere in the discussion have we concluded or indicated that the PTP-PEST mediated AMPK activation is necessary for embryonic development. Reviewer may also note that it is well known that PTP-PEST is essential for embryonic development, since PTP-PEST knockout mice are embryonically lethal due defective cardiovascular development and impaired endothelial network in the yolk sac [12], [13]. These listed phenotypes of the PTP-PEST knock-out mice and the fact that hypoxia promotes angiogenesis, served as motivation for the current study to determine the role of PTP-PEST in hypoxia-induced endothelial responses as mentioned in the 'Introduction' section of the manuscript (lines: 100-105).

References:

[1] M. Rechsteiner and S. W. Rogers, "PEST sequences and regulation by proteolysis," *Trends Biochem. Sci.*, vol. 21, no. 7, pp. 267–271, 1996, doi: 10.1016/S0968-0004(96)10031-1.

[2] M. Strowitzki, E. Cummins, and C. Taylor, "Protein Hydroxylation by Hypoxia- Inducible Factor (HIF) Hydroxylases: Unique or Ubiquitous?," *Cells*, vol. 8, no. 5, p. 384, 2019, doi: 10.3390/cells8050384.

[3] F. K. Pientka *et al.*, "Oxygen sensing by the prolyl-4-hydroxylase PHD2 within the nuclear compartment and the influence of compartmentalisation on HIF-1 signalling," *J. Cell Sci.*, vol. 125, no. 21, pp. 5168–5176, 2012, doi: 10.1242/jcs.109041.

[4] U. Berchner-Pfannschmidt *et al.*, "Nuclear oxygen sensing: Induction of endogenous prolyl-

hydroxylase 2 activity by hypoxia and nitric oxide," *J. Biol. Chem.*, vol. 283, no. 46, pp. 31745–31753, 2008, doi: 10.1074/jbc.M804390200.

[5] I. Filippi *et al.*, "Different Adaptive Responses to Hypoxia in Normal and Multiple Myeloma Endothelial Cells," *Cell. Physiol. Biochem.*, vol. 46, no. 1, pp. 203–212, 2018, doi: 10.1159/000488423.

[6] S. M. Jeon, "Regulation and function of AMPK in physiology and diseases," *Exp. Mol. Med.*, vol. 48, no. 7, p. e245, 2016, doi: 10.1038/emm.2016.81.

[7] Y. C. Long, J. R. Zierath, Y. C. Long, and J. R. Zierath, "AMP-activated protein kinase signaling in metabolic regulation Find the latest version : Review series AMP-activated protein kinase signaling in metabolic regulation," vol. 116, no. 7, pp. 1776–1783, 2006, doi: 10.1172/JCI29044.1776.

[8] G. R. Y. De Meyer, M. O. J. Grootaert, C. F. Michiels, A. Kurdi, D. M. Schrijvers, and W. Martinet, "Autophagy in vascular disease," *Circ. Res.*, vol. 116, no. 3, pp. 468–479, 2015, doi: 10.1161/CIRCRESAHA.116.303804.

[9] S. Bakhashab *et al.*, "Proangiogenic effect of metformin in endothelial cells is via upregulation of VEGFR1/2 and their signaling under hyperglycemia- hypoxia," *Int. J. Mol. Sci.*, vol. 19, no. 1, pp. 1–18, 2018, doi: 10.3390/ijms19010293.

[10] Q. Jin *et al.*, "Improvement of functional recovery by chronic metformin treatment is associated with enhanced alternative activation of microglia/macrophages and increased angiogenesis and neurogenesis following experimental stroke," *Brain. Behav. Immun.*, vol. 40, pp. 131–142, 2014, doi: 10.1016/j.bbi.2014.03.003.

[11] V. R. Venna, "Chronic metformin treatment improves post-stroke angiogenesis and recovery after experimental stroke," *Eur J Neurosci*, vol. 39, no. 12, pp. 2129–2138, 2014, doi: 10.1111/ejn.12556.

[12] J. Sirois *et al.*, "Essential function of PTP-PEST during mouse embryonic vascularization, mesenchyme formation, neurogenesis and early liver development," *Mech. Dev.*, vol. 123, no. 12, pp. 869–880, 2006, doi: 10.1016/j.mod.2006.08.011.

[13] C. M. Souza, D. Davidson, I. Rhee, J. P. Gratton, E. C. Davis, and A. Veillette, "The phosphatase PTP-PEST/PTPN12 regulates endothelial cell migration and adhesion, but not permeability, and controls vascular development and embryonic viability," *J. Biol. Chem.*, vol. 287, no. 51, pp. 43180–43190, 2012, doi: 10.1074/jbc.M112.387456.

Second decision letter

MS ID#: JOCES/2020/250274

MS TITLE: Protein tyrosine phosphatase-PEST mediates hypoxia-induced endothelial autophagy and angiogenesis via AMPK activation

AUTHORS: Shivam Chandel, Amrutha Manikandan, Nikunj Mehta, Abel Arul Nathan, Rakesh Kumar Tiwari, Samar Bhallabha Mohapatra, Mahesh Chandran, Abdul Jaleel, Narayanan Manoj, and Madhulika Dixit
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors adequately improved the given manuscript in regard to the comments of both reviewers.

Comments for the author

The authors adequately improved the given manuscript in regard to the comments of both reviewers. Now, I would like to recommend the manuscript for publication.

Reviewer 2

Advance summary and potential significance to field

The authors answered and improved the manuscript in most cases thus contributing to a potential advance in the field.

Comments for the author

The authors adequately answered to most of the issues addressed. I can see the Point that due to Corona additional experiments will be difficult. However, I do not agree that ambryonic development have not been addressed since this is a Major Point in the introduction.
Either the authors Point to that in the discussion or Focus more on adult angiogenesis.