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Hypoxia induced ELF3 promotes tumor angiogenesis through IGF1/IGF1R

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1st Editorial Decision **4th May 2021**

Dear Dr. Kwon,

Thank you for submitting your manuscript to EMBO Reports. We have now received two referee reports, which are included below.

Referees express interest in the proposed role of ELF3 in hypoxia induced tumor angiogenesis. However, they also raise concerns that preclude publication in this journal. They point out concerns regarding the methodologies used and conclusiveness of the dataset. As such, they do not recommend publication in EMBO Reports. Given such input from these recognized experts who are also experienced referees, we cannot publish your manuscript.

Thank you in any case for the opportunity to consider this manuscript. I am sorry that I cannot communicate more positive news, but nevertheless hope that you will find our referees' comments helpful.

Kind regards,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The work by Seo et al. describes upregulation of the transcription factor ELF3 in ovarian cancer cells during hypoxia which promotes tumor angiogenesis by enhancing IGF1/IGF1R-signaling in endothelial cells and increased expression of VEGFA. The identification of ELF3 as a hypoxia-regulated transcription factor that promotes tumor angiogenesis is interesting. However, the study is incomplete, lacking essential quantification of data, primarily resting on in vitro experiments. A role for ELF3 in ovarian cancer progression has been described previously but the emphasis has been on the correlation between high ELF3 expression and better overall survival. The rationale for the current study is thereby questioned. Comments are given below.

1. Please define n in the figure legends. Now, it is unclear whether each assay has been performed 3 independent times; it may also have been 3 wells in the same experiment, for example. Moreover, blotting must be done 3 independent times and then quantified. Please also provide quantifications of the angiogenic array in Figure 5A. The upregulation of the angiogenic factors should be normalized to controls present on the array.
2. The role of ELF3 in tumor progression remains contradictory and the transcription factor is described as a tumor suppressor in certain cancer and an oncogene in others. The authors need to address this contradiction and cite the literature. For example, Yeung et al. reported on the positive correlation between Elf3 expression and survival in ovarian cancer. See Oncotarget. 2017 Mar 7; 8(10): 16951-16963. The Human Protein Atlas (proteinatlas.com) states that Elf3 is not prognostic in ovarian cancer. In contrast, in hepatocellular carcinoma, expression of Elf3 is associated with poor prognosis. See Zheng et al. Cell Death and Disease 2018. See also Zhang et al. Aging (Albany NY), 2021 Jan 25;13(2):3112-3145. These published data undermine the rationale for the current study design.
3. By using a bioinformatic approach the authors identified 31 transcription factors that contained at least five HRE and which were overexpressed during hypoxia, as compared to normoxia. Next, authors compared expression levels in ovarian cancer and chose ELF3 based on the highest expression. A more relevant approach would be to choose the transcription factor with the highest overexpression during hypoxia. Please provide a heatmap showing the 31 transcription factors and indicate the upregulation of ELF3 during hypoxia. In addition, authors need to validate that ELF3 is induced by HIF1-alpha.
4. The study rests on in vitro models in spite of that the Elf3 fl/fl mouse is available from Jackson Labs. It would increase the general interest in the study if in vivo analyses would were done, for example on any effects of Elf3 on metastatic spread?
5. It is a problem that the siCtrl has such marked effects on its own. See levels of VEGFR2, FAK, pAKT, peNOS/eNOS in the siCtrl condition compared to untreated controls in Fig 6. There seems to be no difference between the siCtrl and siElf3 conditions in the levels of these proteins, still the authors describe the effects as specific to the loss of Elf3.

Minor comments
6. Figure 4 was not included in the uploaded files and could not be reviewed.

7. Please provide immunostainings of the aortic rings to convince that cells growing out are endothelial and not vSMCs.

8. The importance of calcium in modulating the interaction between ELF3 and CAM should be explored in more detail. In Figure 2D, in addition to CaCl2 and WF7 treatment, please include experiments using a calcium chelator.

9. In Figure 3A and S3D, is this indirect co-culture or treatment with CM? The schematic Figure S3C indicates indirect co-culture.

10. In Figure 3F, the bottom image is out of focus and the sprouts are difficult to see. Please replace with another image.

11. Please correct "vesicular permeability". Should be "vascular permeability".

Referee #2:

The authors investigated the contribution of ELF3, a transcription factor, in epithelial ovarian cancer. Authors used a broad range of techniques to identify that: a) ELF3 is upregulated by hypoxia, under the control of HIF-1alpha; b) ELF3 controls the expression of pro-angiogenic molecules, including VEGFA, IGF1; c) ELF3 transcriptional activity is partially regulated by calmodulin.
The authors performed an extensive work to elucidate the details of the interactions between hypoxia, ELF3, IGF1 and the overall contribution to angiogenesis. Despite these efforts, the main point missing in this manuscript is the relevance of the findings in the context of tumor angiogenesis. Moreover, the logic of experiments in the manuscript is difficult to follow. For instance, Figure 2 and Figure 4 seemed to fit better after current figure 5. Also, quantitative information is missing in many experiments, only providing qualitative data, as representative images. In addition, some important controls are missing in key experiments.
Overall, the reviewer appreciates the novelty of the findings, however a major revision of the current form is necessary prior to publication.

Major comments:
1- Strikingly, the manuscript version that the reviewer was able to download lacked Figure 4 (it is currently unclear if this was a mistake from the authors or from the journal).

2- SI Appendix , Fig. S1A and S1B: Pulldown experiments should be performed using an anti-IgG to control for unspecific binding.

3- Fig.2C: Additional controls should be performed. For instance, a negative control for luciferase activity when expressing Cluc-CAM or Nluc-ELF3FL alone, and Cluc + Nluc linked to unrelated proteins and/or alone.
The luciferase assay is not properly described in the main text. Authors are expressing a split luciferase that can only be activated when targeted proteins interact. Material and methods and supplementary files provide good evidence. However, the main text and main figure is insufficient to understand the experiment. These details should be made clearer in the text.

4- In several experiments, the authors used CaCl2 or W7 to activate or inhibit Calmodulin, respectively. In these assays, it is not possible, however, to conclude that effects of CaCl2 on ELF3 activity are strictly dependent on calmodulin. For instance, authors wrote "The luciferase activity changed according to the degree of Ca2+ binding to CaM, increasing significantly with CaCl2 treatment compared with EBS alone and decreasing considerably with W7 treatment (Fig. 2G). Therefore, CaM interacts directly with ELF3 to a greater degree when bound to Ca2+. This ELF3 and Ca2+-CaM complex enhances the nuclear translocation of ELF3 and its function as a transcription factor." To confirm this claim, authors should deplete calmodulin using siRNA and repeat the same type of experiment, or at least have perform CaCl2 stimulation together with the CaM inhibitor W7. Moreover, in SI Appendix Fig. S2E, the control for CaM-agarose without CaCl2 stimulation is missing, thus it is not possible to conclude that CaCl2 increases calmodulin-ELF3 interaction.
It is also very much unclear if the link between calmodulin-ELF3 is relevant for the biological effects downstream of hypoxia.

5- An assay to measure the effects of ELK3 activity in angiogenesis in vivo is missing. The methods that the authors employed were not sufficiently detailed. For instance, aortic ring assays were not very conclusive, as no vessel were clearly observed in the representative images. Aortas have several other type of cells that can react to chemokines and migrate into matrigel, as fibroblasts or SMCs. Authors should confirm the identity of migrating cells using an endothelial cell marker. Also, the authors should perform an analysis of vessel density in matrigel plugs (Fig.3G) to confirm differences in vascularization, in addition to the hemoglobin concentration (Fig.3H). A tumor model would be the most interesting model to strengthen the relevance of ELF3 in tumor angiogenesis.

6- The authors suggest that ELF3 controls IGF1 expression, through binding motif number 4 (Fig. 5E-G). However, deletion of that specific site does not abrogate luciferase activity when compared to control IGF1 promoter. Thus, the authors cannot conclude that IGF1 regulates IGF1 expression through that specific site.

Minor comments:

1- Reference to Fig.1A in this sentence is incorrect.
To experimentally evaluate whether ELF3 is a direct target of HIF1α, we executed chromatin immunoprecipitation (ChIP) and luciferase reporter assays using the predicted HRE region of the ELF3 promoter in Fig. 1A .

2- In (SI Appendix , Fig. S1C), ELF3 levels seems to be upregulated prior to HIF1a stabilization when cells are treated with CoCl2. This suggests that CoCl2 can directly activate ELF3 expression independent of HIF1a. Can authors clarify this discrepancy.

Our manuscript is slightly different from last time.

We have been working to validate a novel role of ELF3 as an imperious inducer of angiogenesis in epithelial ovarian cancer. We have also tried to develop PPI inhibitors capable of inhibiting the ELF3-CaM interaction and to verify their antiagiogenic efficacy *in vitro* and *in vivo*. In order to solidify and clarify the logic presented in the manuscript, we decided to present the angiogenic activity of ELF3 according to the correlation between ELF3 and IGF1 in this manuscript first. We will then publish data related to the ELF3-CaM interaction after completing finding a highly potent PPI inhibitor for the ELF3-CaM interaction and using it as a tool to demonstrate the importance of the ELF3-CaM interaction on angiogenesis. Therefore, since this manuscript does not include the interactive part of the ELF3-CaM, we have omitted responses to the reviewer's comments.

Referee #1:

The work by Seo et al. describes upregulation of the transcription factor ELF3 in ovarian cancer cells during hypoxia which promotes tumor angiogenesis by enhancing IGF1/IGF1R-signaling in endothelial cells and increased expression of VEGFA. The identification of ELF3 as a hypoxia-regulated transcription factor that promotes tumor angiogenesis is interesting. However, the study is incomplete, lacking essential quantification of data, primarily resting on in vitro experiments. A role for ELF3 in ovarian cancer progression has been described previously but the emphasis has been on the correlation between high ELF3 expression and better overall survival. The rationale for the current study is thereby questioned. Comments are given below.

1. Please define n in the figure legends. Now, it is unclear whether each assay has been performed 3 independent times; it may also have been 3 wells in the same experiment, for example. Moreover, blotting must be done 3 independent times and then quantified. Please also provide quantifications of the angiogenic array in Figure 5A. The upregulation of the angiogenic factors should be normalized to controls present on the array.

Answer: Each analysis was performed at least three times independently. In the case of qPCR analysis, the average value was used by repeating one experiment in three wells, and this way was independently repeated three or more times and normalized. The number of repeated experiments was indicated in red in the legend of each figure as n = 5 independent repetitions, for example. We also performed three independent blots and then

Protein lysate-1

	A	B	C	D	E	F	G	H
1					1.3	1.1	1.0	0.7
2					1.3	1.3	1.2	0.8
3	0.9	1.2	1.6	1.1	0.9	1.1	1.0	1.1
4	0.8	1.1	1.7	1.1	0.9	1.0	1.0	1.2
5	0.9	1.1	1.1	0.9	0.9	1.0	1.0	1.0
6	0.9	1.0	1.0	0.8	0.8	1.0	1.0	0.9
7								
8								

Protein lysate-2

	A	B	C	D	E	F	G	H
1					1.4	1.1	0.9	0.9
2					1.5	1.0	1.0	0.9
3	1.2	1.0	1.0	1.1	1.5	1.3	1.1	1.3
4	1.1	1.0	1.0	1.1	1.4	1.4	1.3	1.2
5	1.1	1.0	0.9	1.6	1.6	1.4	1.1	1.1
6	1.1	1.0	0.8	1.5	1.7	1.3	1.4	1.0
7	1.2	1.3	1.0					
8	1.2	1.1	1.1					

	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	ANG	EGF	ENA-78	bFGF
2	POS	POS	NEG	NEG	ANG	EGF	ENA-78	bFGF
3	GRO	IFN gamma	IGF-1	IL-6	IL-8	Leptin	MCP-1	PDGF-BB
4	GRO	IFN gamma	IGF-1	IL-6	IL-8	Leptin	MCP-1	PDGF-BB
5	PLGF	RANTES	TGF beta1	TIMP-1	TIMP-2	THPO	VEGF	VEGF-D
6	PLGF	RANTES	TGF beta1	TIMP-1	TIMP-2	THPO	VEGF	VEGF-D
7	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS
8	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS

	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	ANGPT1	ANGPT2	PLG	Endostatin
2	POS	POS	NEG	NEG	ANGPT1	ANGPT2	PLG	Endostatin
3	G-CSF	GM-CSF	I-309	IL-10	IL-1 alpha	IL-1 beta	IL-2	IL-4
4	G-CSF	GM-CSF	I-309	IL-10	IL-1 alpha	IL-1 beta	IL-2	IL-4
5	I-TAC	MCP-3	MCP-4	MMP-1	MMP-9	PECAM-1	TIE-2	TNF alpha
6	I-TAC	MCP-3	MCP-4	MMP-1	MMP-9	PECAM-1	TIE-2	TNF alpha
7	uPAR	VEGF R2	VEGF R3	BLANK	BLANK	BLANK	NEG	POS
8	uPAR	VEGF R2	VEGF R3	BLANK	BLANK	BLANK	NEG	POS

quantified them. Quantification of blots was presented near the blotting in each figure.

Figure 5A in this comment was changed to Figure 2C in this manuscript.

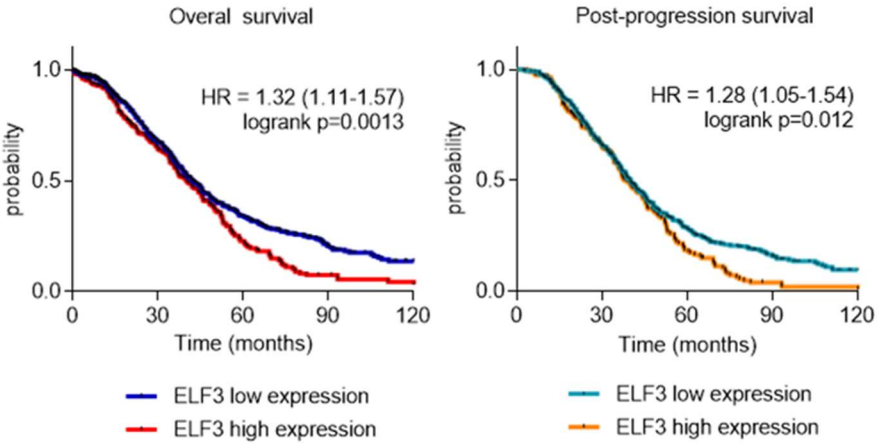
Angiogenic antibody arrays are normalized according to this manufacturer's protocol. Quantification of angiogenesis arrays was expressed as fold change relative to controls. These results are shown in Figure 2 source data.

2. The role of ELF3 in tumor progression remains contradictory and the transcription factor is described as a tumor suppressor in certain cancer and an oncogene in others. The authors need to address this contradiction and cite the literature. For example, Yeung et al. reported on the positive correlation between Elf3 expression and survival in ovarian cancer. See Oncotarget. 2017 Mar 7; 8(10): 16951-16963. The Human Protein Atlas (proteinatlas.com) states that Elf3 is not prognostic in ovarian cancer. In contrast, in hepatocellular carcinoma, expression of Elf3 is associated with poor prognosis. See Zheng et al. Cell Death and Disease 2018. See also Zhang et al. Aging (Albany NY), 2021 Jan 25;13(2):3112-3145. These published data undermine the rationale for the current study design

Answer: We read the papers suggested by the reviewer and checked "The Human Protein Atlas (proteinatlas.com)."

ELF3 is a transcription factor that acts bidirectionally as an oncogene or a tumor suppressor gene depending on the type of cancer. In the Human Protein Atlas, ELF3 was found to be highly expressed not only in ovarian cancer, but also in breast, prostate, and lung cancer, which is known to act as an oncogene. (Figure 1B and Figure EV3 of this manuscript). Additionally, ELF3 was more amplified in ovarian cancer than in normal tissues in the GSE36668 data set (Figure 1C in this manuscript).

Although not mentioned in the manuscript, we identified a poor prognosis with overall survival and post-progression survival in patients with high ELF3 expression (GSE201510). We used various public databases to confirm that ELF3 could act as an oncogene in ovarian cancer.



In addition, two papers explaining the role of ELF3 suggested by the reviewer were published from opposing viewpoints in ovarian cancer. That can arouse various views or interests from researchers.

However, the recently published papers reported that ELF3 overexpression induces pathogenesis and cisplatin resistance of ovarian cancer (Xu et al, 2021; Zhang et al., 2021; Liu et al, 2021; Kuang & Li, 2021). Therefore, we think that these papers can support our findings.

The papers presented above are briefly described in the introduction to this manuscript.

Reference

Xu H, Wang H, Li G, Jin X, Chen B (2021) The Immune-Related Gene ELF3 is a Novel Biomarker for the Prognosis of Ovarian Cancer. *Int J Gen Med* 14: 5537-5548

Zhang Y, Wang X, Chen X (2021) Identification of core genes for early diagnosis and the EMT modulation of ovarian serous cancer by bioinformatics perspective. *Aging (Albany NY)* 13: 3112-3145

Liu Y, Wang S, Zhou R, Li W, Zhang G (2021) Overexpression of E74-like transformation-specific transcription factor 3 promotes cellular proliferation and predicts poor prognosis in ovarian cancer. *Oncol Lett* 22: 710

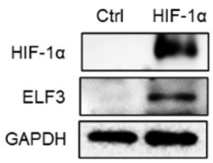
Kuang L, Li L (2021) E74-like factor 3 suppresses microRNA-485-5p transcription to trigger growth and metastasis of ovarian cancer cells with the involvement of CLDN4/Wnt/beta-catenin axis. *Saudi J Biol Sci* 28: 4137-4146

3. By using a bioinformatic approach the authors identified 31 transcription factors that contained at least five HRE and which were overexpressed during hypoxia, as compared to normoxia. Next, authors compared expression levels in ovarian cancer and chose ELF3 based on the highest expression. A more relevant approach would be to choose the transcription factor with the highest overexpression during hypoxia. Please provide a heatmap showing the 31 transcription factors and indicate the upregulation of ELF3 during hypoxia. In addition, authors need to validate that ELF3 is induced by HIF1-alpha.

Answer: We took a different approach to find transcription factors that could induce angiogenesis in hypoxia-exposed ovarian cancer. This section of this manuscript discussed in detail ***'ELF3 expression was upregulated in ovarian cancer under hypoxic conditions'*** (Figure 1). Briefly, we selected genes overexpressed in ovarian cancer cell lines exposed to hypoxia compared to normoxia. Among them, we filtered out 51 transcription factors and found that ELF3 was highly expressed when comparing the expression of transcription factors in a public database of ovarian cancer patients.

To explain the overexpression of ELF3 under hypoxic conditions, it was presented based on multi omics-related papers and experimental data that HIF1α can regulate ELF3 expression.

Although not presented in the manuscript, we confirmed that ELF3 expression was increased when HIF1α was artificially overexpressed.



4. The study rests on in vitro models in spite of that the Elf3 fl/fl mouse is available from Jackson Labs. It would increase the general interest in the study if in vivo analyses would were done, for example on any effects of Elf3 on metastatic spread?

Answer: Our study focused on the role of ELF3 on angiogenesis.

We confirmed angiogenic activity following ELF3 expression using *in vitro* and *ex vivo* models that reflect angiogenesis. This time, we further confirmed the angiogenic activity following ELF3 expression *in vivo* xenograft models using SKOV3-shELF3 or -shCtrl cells (please see Figure 6 in this version of the mansucript). We believe that these results can sufficiently support our logic.

5. It is a problem that the siCtrl has such marked effects on its own. See levels of VEGFR2, FAK, pAKT, peNOS/eNOS in the siCtrl condition compared to untreated controls in Fig 6. There seems to be no difference between the siCtrl and siElf3 conditions in the levels of these proteins, still the authors describe the effects as specific to the loss of Elf3.

Answer: Since each group (Ctrl and ELF3) or (shCtrl and shELF3) was loaded in different SDS-PAGE gel and detected at different times, we think blots pointed by the reviewer can be just an experimental error, not the effect of shCtrl. To supplement the comments, we again performed western blotting on the same SDS-PAGE gel and quantified phosphorylated protein levels compared to total protein levels. In this version of the manuscript, all western blot results were quantified and presented as bar graphs showing each data point.

6. Figure 4 was not included in the uploaded files and could not be reviewed.

Answer: We don't know what went wrong, but at that time we were still able to see and download Figure 4 from the EMBO reports homepage. This time, to prevent such a mistake, we checked the converted pdf file after uploading the manuscript to make sure that there was no missing figures.

7. Please provide immunostainings of the aortic rings to convince that cells growing out are endothelial and not vSMCs.

Answer: To supplement the comments, the aortic rings were fluorescently stained using the CD31 antibody as an endothelial marker and the α-SMA antibody as a vSMC marker (Fig. 4*E* and Fig. 5*D*). The degree of vascular sproutings was quantified.

9. In Figure 3A and S3D, is this indirect co-culture or treatment with CM? The schematic Figure S3C indicates indirect co-culture.
Answer: Figure 3*A* and Figure S3*C* and S3*D* in this comment were changed to Figure 4*D* and Figure 4*B* and Figure EV.2*B* sequentially in this manuscript.

The experiments of Figures 4*D* and Figure EV.2*B* were co-cultured according to the method described in Figure 4*B*.

10. In Figure 3F, the bottom image is out of focus and the sprouts are difficult to see. Please replace with another image.

Answer: Figure 3*F* in this comment was changed to Figure 5*D* in this manuscript.

We re-performed the aortic ring assay and quantified the degree of vascular sprouting (Fig. 5D).

11. Please correct “vesicular permeability”. Should be “vascular permeability”.

Answer: Thanks, it is corrected.

Referee #2:

The authors investigated the contribution of ELF3, a transcription factor, in epithelial ovarian cancer. Authors used a broad range of techniques to identify that: a) ELF3 is upregulated by hypoxia, under the control of HIF-1alpha; b) ELF3 controls the expression of pro-angiogenic molecules, including VEGFA, IGF1; c) ELF3 transcriptional activity is partially regulated by calmodulin.
The authors performed an extensive work to elucidate the details of the interactions between hypoxia, ELF3, IGF1 and the overall contribution to angiogenesis. Despite these efforts, the main point missing in this manuscript is the relevance of the findings in the context of tumor angiogenesis. Moreover, the logic of experiments in the manuscript is difficult to follow. For instance, Figure 2 and Figure 4 seemed to fit better after current figure 5. Also, quantitative information is missing in many experiments, only providing qualitative data, as representative images. In addition, some important controls are missing in key experiments.
Overall, the reviewer appreciates the novelty of the findings, however a major revision of the current form is necessary prior to publication.

1- Strikingly, the manuscript version that the reviewer was able to download lacked Figure 4 (it is currently unclear if this was a mistake from the authors or from the journal).

Answer: We don't know what went wrong, but at that time we were still able to see and download Figure 4 from the EMBO reports homepage. This time, to prevent such a mistake, we checked the converted pdf file after uploading the manuscript to make sure that there was no missing figures.

2- SI Appendix , Fig. S1A and 5F: Pulldown experiments should be performed using an anti-IgG to control for unspecific binding.

Answer: Figure S1*A* and 5*F* in this comment was changed to Figure 3*B* and Fig.EV 4A in this manuscript. To supplement the comments, we re-performed ChIP analysis using anti-IgG as a negative control (Fig 3*B* and Fig. EV 4*A*).

5- An assay to measure the effects of ELF3 activity in angiogenesis in vivo is missing. The methods that the authors employed were not sufficiently detailed. For instance, aortic ring assays were not very conclusive, as no vessel were clearly observed in the representative images. Aortas have several other type of cells that can react to chemokines and migrate into matrigel, as fibroblasts or SMCs. Authors should confirm the identity of migrating cells using an endothelial cell marker. Also, the authors should perform an analysis of vessel density in matrigel plugs (Fig.3G) to confirm differences in vascularization, in addition to the hemoglobin concentration (Fig.3H). A tumor model would be the most interesting model to strengthen the relevance of ELF3 in tumor angiogenesis.

Answer: To supplement the comments, the aortic rings were fluorescently stained using the CD31 antibody as an endothelial marker and the α-SMA antibody as a vSMC marker (Fig. 4E and Fig. 5D). The degree of vascular sproutings was quantified.

We created xenograft models using SKOV3-shELF3 or -shCtrl cells and measured microvessel density through IHC staining with CD31 (Figure 6H and I).

6- The authors suggest that ELF3 controls IGF1 expression, through binding motif number 4 (Fig. 5E-G). However, deletion of that specific site does not abrogate luciferase activity when compared to control IGF1 promoter. Thus, the authors cannot conclude that IGF1 regulates IGF1 expression through that specific site.

Answer: Figure 5Fand 5G in this comment was changed to Figure 3B and 3D in this manuscript.

It was our mistake. ELF3 binding motif in IGF1 was site 4 (Fig. 3B) that was confirmed by significantly deceased luciferase activity when site 4 was deleted (Fig. 3D).

The position of ** should be in IGFΔsite4 instead of in IGFΔsite3 of Fig. 3D. The marker of ** was unintentionally mispositioned in the process of modifying the picture. It is correct to express significance (**p<0.01) for IGF1△sit4.

The statistical analysis results of raw data used for bar graph of Fig. 3D were obtained using the Kruskal-Wallis test and Dunn's multiple comparisons test of GraphPad Prism as listed below.

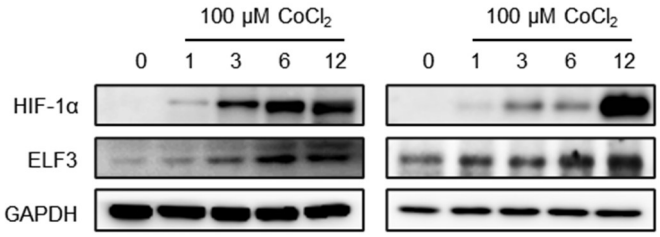
Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
IGF1 vs. IGF1Δsite6	10.00	No	ns
IGF1 vs. IGF1Δsite5	12.80	No	ns
IGF1 vs. IGF1Δsite4	22.20	Yes	**
IGF1 vs. IGF1Δsite3	-3.200	No	ns
IGF1 vs. IGF1Δsite2	8.400	No	ns
IGF1 vs. IGF1Δsite1	5.800	No	ns

Minor comments:
1- Reference to Fig.1A in this sentence is incorrect.
To experimentally evaluate whether ELF3 is a direct target of HIF1α, we executed chromatin immunoprecipitation (ChIP) and luciferase reporter assays using the predicted HRE region of the ELF3 promoter in Fig. 1A .
Answer: Figure 1*A* in this comment was changed to Figure EV. 4*A* in this manuscript.

We agree with the reviewer's point, so this sentence has been modified as follows and presented in the discussion section of the manuscript. *'To experimentally confirm the results of this study, we predicted the hypoxic response element (HRE) region of the ELF3 promoter to refer EPD analysis (p-value<0.0001), and confirmed that HIF-1α directly regulated the expression of ELF3 through ChIP, luciferase reporter gene analysis, and western blotting (Fig. EV4).'*

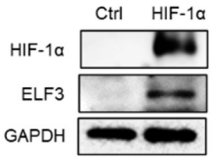
2- In (SI Appendix , Fig. S1C), ELF3 levels seems to be upregulated prior to HIF1a stabilization when cells are treated with CoCl2. This suggests that CoCl2 can directly activate ELF3 expression independent of HIF1a. Can authors clarify this discrepancy.
Answer: Figure S1*C* in this comment was changed to Figure EV. 4*C* in this manuscript.

We detected several times that stabilized HIF1α by treating $CoCl_2$ or exposure to hypoxia (Fig. EV4*C*) induced the increase of ELF3 expression as listed below. Only the western blot presented in Fig. EV4*C* can lead to misinterpretation.



It is well known that HIF1α is stabilized in hypoxic conditions or by treatment of $CoCl_2$. HIF1α stabilized by $CoCl_2$ increased ELF3 promoter activity (Fig. EV4*A*)

Although not presented in the manuscript, we confirmed that ELF3 expression was increased when HIF1α was artificially overexpressed. In conclusion, it can be said that stabilized HIF1α increases ELF3 expression.



Dear Prof. Kwon,

Thank you for submitting your revised manuscript. It has now been seen by both of the original referees.

As you can see, the referees find that the study is significantly improved during revision and recommends publication. However, referees have remaining minor concerns that need to be addressed prior to publication. In particular, please perform the experiment suggested by referee #1 in his/her point 1 (overexpressing VEGFA in shELF3 SKOV3 cells) to clarify the mode of IGF1 action in tumor angiogenesis. Please provide a point-by-point response to all referee comments.

Moreover, I need you to address the editorial points below before I can accept the manuscript.

• As per our guidelines, please add a 'Data Availability Section', where you state that no data were deposited in a public database.
• We note that there are to authors with the initials SH in the Author Contributions section. Please use the abbreviations SHw and Sha to be able to differentiate these authors.
• We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Also, please rename the 'Conflict of Interests' section as 'Disclosure statement and competing interests'.
• Please fill out and include an author checklist as listed in out online guidelines (https://www.embopress.org/page/journal/14693178/authorguide)
• We note that panels of Figure EV4 are not called out in the text.
• EV tables should be uploaded individually using the file type 'Expanded View' and they need to be removed from the manuscript file.
• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.
• The synopsis image needs to be 550px wide and 300-600px high. When your synopsis image is resized accordingly, the labels are too small to read (please see attached). Please provide a synopsis image with larger labels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The authors have performed an ambitious revision. The presentation of their study is now much easier to follow and it convinces on an important role for ELF3 in hypoxia-dependent regulation of IGF1 expression which in turn regulates expression of VEGF and consequent angiogenesis in ovarian cancer. There is however a need for a few remaining adjustments:

1. For Fig 2C, information is missing in the legend. What are the upper and lower panels - different exposures? What do the red and grey boxes indicate?
2. In Fig. 2E, how was normalization done? Please give the number of independent repeats in the figure legend for the data shown here.
3. In Fig. 3B, The IgG ChIP was very high for site 3 in a repeated manner. The authors need to give a brief comment on this in the text.
4. For results shown in Fig 6D, why not plot the number of mice without tumor? This would show the difference between control

and siELF3 in the 5x105 group very clearly.
5. When quantifying the results shown in Fig. 6E-I, the authors must have pooled tumors from the two groups (5x106 and 5x105). This should be stated.

Referee #2:

The authors have substantially reshaped the previous version of the manuscript. The authors have now focused on the role of hypoxia-induced ELF3 in the regulation of IGF1 secretion in ovarian cancer, and its contribution to tumor development and tumor angiogenesis.

The authors consolidated previous data, removed a previous unclear link to calmodulin activity, and performed several new experiments.

The manuscript is now substantially improved and the reviewer is supportive to its publication at EMBO reports.

Nevertheless, there are aspects that could be further clarified, which some involve experimental work. Being a revised manuscript, the reviewer does not consider those experiments mandatory. Yet, the proposed experiments should be considered by the editorial board, and the decision to ask for new experimental data depends on the journal's reviewing policy.

1- The major question that remains unclear is the autocrine or paracrine contribution of IGF1 in the promotion of tumor angiogenesis, and tumor growth.
The authors showed that "In addition, despite the induction of ELF3 overexpression, IGF1 knockdown reduced the expression of VEGF and MMP2 again (Fig. EV1C)." Based on this evidence, the reviewer concludes that IGF1 functions in an autocrine loop to stimulate ovarian cancer cells to secrete VEGF and MMP2, among other factors.
In parallel, the authors also showed that "In the case of angiogenesis-related
signaling pathways in HUVECs, IGF1R and VEGFR2 signaling pathways were significantly activated when incubated with CMs of SKOV3-shCtrl exposed to hypoxia, but not when ELF3 was silenced in SKOV3. In particular, the phosphorylation of IGF1R showed a significant difference according to changes in ELF3-mediated IGF1 expression (Fig. 5A and E)." Based on this evidence, the reviewer concludes that IGF1 and VEGF functions in a paracrine loop to stimulate angiogenic activity.
In addition, xenograft experiments (Fig. 6) showed that ELF3-defficient SKOV3 cells failed to engraft in mice. Yet, those engrafting growth (as measured by the pictures provided) to quite large sizes.
This raises the question that ELF3-defficient SKOV3 cells may have impaired ability to engraft due to autocrine effects of ELF3 LoF (for instance, impaired IGF1-IGFR1 signaling in tumors cells) rather than based on paracrine angiogenic effects. Otherwise, the reviewer would expect a similar number of engrafted tumors, yet, with a dramatically reduced size.

A clarifying experiment would be to overexpress VEGFA in shELF3 SKOV3 cells, to rescue paracrine effects on angiogenesis, without affecting the autocrine effects.

2- Xenograft experiments.
a) There is a mismatch between the text and examples of tumors in Fig. 6C and numbers in Fig. 6A, 5/7 in SKOV3 shCtrl 5*10^5 Fig. 6A vs 6/7 in Fig. 6C.
b) The reviewer has several doubts concerning the representation of the data in Fig. 6B and Fig. 6D. The images of tumors do not fit well with tumor volume curves. Based on the inclusion of non-existent tumors (failed engraftment) in Fig. 6D (values = 0), the reviewer believes that tumor volume curves also contain values of non-existent tumors. This is incorrect. Tumors that do not engraft should not be considered for statistics in Fig. 6B and Fig. 6D, as it represents a completely different parameter. Engraftment potential is demonstrated in Fig. 6A. Growth curves and weight should take only into account existent tumors.

3- The authors should include representative images, as sup. data, for vessels in Matrigel plugs quantified in (Fig. 5F left).

4- There is a misspelling of GAPDH (GPADH) in Fig. EV1.

Dear Editor Deniz Senyilmaz Tiebe,

We thank reviewers for providing helpful comments. We have addressed all the issues pointed out by reviewers and editor below. We hope that the revised manuscript has been improved enough to be published in *EMBO reports*. Any changes made in accordance with reviewer's comments are highlighted in red in the revised manuscript.

To address the editorial points.

As per our guidelines, please add a 'Data Availability Section', where you state that no data were deposited in a public database.

Answer: We added a 'data availability section' and stated as follows.

'This study includes no data deposited in external repositories.'

We note that there are to authors with the initials SH in the Author Contributions section. Please use the abbreviations SHw and Sha to be able to differentiate these authors.

Answer: It has been rewritten to distinguish the author's names.

We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Also, please rename the 'Conflict of Interests' section as 'Disclosure statement and competing interests'.

Answer: The section has been named 'Disclosure Statements and Competing Interests' and states as follows.

'The authors have no conflicts of interest to declare.'

Please fill out and include an author checklist as listed in out online guidelines (https://www.embopress.org/page/journal/14693178/authorguide).

Answer: Yes, we wrote it according to the online guidelines.

We note that panels of Figure EV4 are not called out in the text.

Answer: Figure EV4 was mentioned in the section of discussion.

EV tables should be uploaded individually using the file type 'Expanded View' and they need to be removed from the manuscript file.

Answer: EV tables were removed from the manuscript file and uploaded individually using the 'extended view' file format.

Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.

Answer: We have uploaded a 'Synopsis' PDF file that includes a synopsis blurb and 3 bullet points as described below.

Synopsis blurb :

ELF3 acts as a direct transcription factor for IGF1, enhancing the expression of IGF1. An increase in IGF1 secreted from ovarian cancer cells to endothelial cells promotes vascular sprouting, proliferation, and migration of endothelial cells, leading to angiogenesis. When the microenvironment becomes hypoxic due to the rapid growth of ovarian cancer, this cascade of responses is extremely stimulated due to an increase in ELF3 expression, which further promotes tumor angiogenesis.

Bullet points:

- ELF3 is a key molecule that induces angiogenesis by transcriptionally increasing IGF1 expression.
- The promotion of the angiogenic process due to ELF3-mediated IGF1 increase is more pronounced with increased expression of ELF3 under hypoxia.
- Inhibition of ELF3 expression can be significantly beneficial in the treatment of tumor angiogenesis in epithelial ovarian cancers.

The synopsis image needs to be 550px wide and 300-600px high. When your synopsis image is resized accordingly, the labels are too small to read (please see attached). Please provide a synopsis image with larger labels.

Answer: We have adjusted the size of the synopsis image and labels. Please review whether it

is appropriate. Synopsis image has been uploaded as a separate file of 'Synopsis' PDF file.

Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Answer: As requested, any changes can be tracked in the revised manuscript, and the revised file has been uploaded with track changes activated.

Responses point-by-point to all referee comments

Referee #1:

The authors have performed an ambitious revision. The presentation of their study is now much easier to follow and it convinces on an important role for ELF3 in hypoxia-dependent regulation of IGF1 expression which in turn regulates expression of VEGF and consequent angiogenesis in ovarian cancer. There is however a need for a few remaining adjustments:

1. For Fig 2C, information is missing in the legend. What are the upper and lower panels - different exposures? What do the red and grey boxes indicate?

Answer: Antibodies that target a total of 43 types of angiogenic factors are implanted in Both the upper and lower membranes. A list of 43 angiogenic factors was presented in map format in the EV2 source data file. To distinguish them, we designated them as Membrane 1 or Membrane 2, respectively, in Figure 2C.

Since there was no difference in meaning between the existing red box and gray box, the black box was unified to avoid confusion. The black boxes indicate more than 1.5-fold change in protein expression compared to the control.

We mentioned these descriptions in the Figure 2C legend.

2. In Fig. 2E, how was normalization done? Please give the number of independent repeats in the figure legend for the data shown here.

Answer: The experiment was independently repeated five times. Relative expression of each mRNA was normalized to β-actin and then calculated as fold change compared to the Ctrl or siCtrl groups, respectively. These descriptions were mentioned in the Figure 2E legend.

3. In Fig. 3B, The IgG ChIP was very high for site 3 in a repeated manner. The authors need to give a brief comment on this in the text.

Answer: To supplement the reviewer's comment, we added the following explanation to the section of the results, '*ELF3 transcriptionally regulated IGF1 expression, thereby promoting the secretion of proangiogenic factors*'.

'As a result of ChIP analysis using primers containing each predicted site (Fig. 3A), when immunoprecipitation was performed with a negative control IgG, it was inferred that high expression of site3 was induced by non-specific binding of IgG to site 3 (Fig. 3B). However, upon immunoprecipitation with ELF3, the expression of site 4 was specifically and significantly increased. Therefore, we found that ELF3 strongly binds to site 4 (-1065 bp away from TSS) of the IGF1 promoter.'

4. For results shown in Fig 6D, why not plot the number of mice without tumor? This would show the difference between control and siELF3 in the $5x10^5$ group very clearly.

Answer: Please see the answer for the comment #2-b of reviewer #2. Statistical analysis was modified to exclude non-growing tumors and the legend of Figure 6D was corrected as follows.

'As in (A) and (C), only the grown tumors were weighed and analyzed statistically.'

5. When quantifying the results shown in Fig. 6E-I, the authors must have pooled tumors from the two groups ($5x10^6$ and $5x10^5$). This should be stated.

Answer: To supplement the comments, we noted:

'IHC analysis of SKOV3-shCtrl or -shELF3 tumor sections was performed with tumors pooled from both 5×10^6 and 5×10^5 groups and IHC results were statistically analyzed.'

Referee #2:

The authors have substantially reshaped the previous version of the manuscript. The authors have now focused on the role of hypoxia-induced ELF3 in the regulation of IGF1 secretion in ovarian cancer, and its contribution to tumor development and tumor angiogenesis.

The authors consolidated previous data, removed a previous unclear link to calmodulin activity, and performed several new experiments.

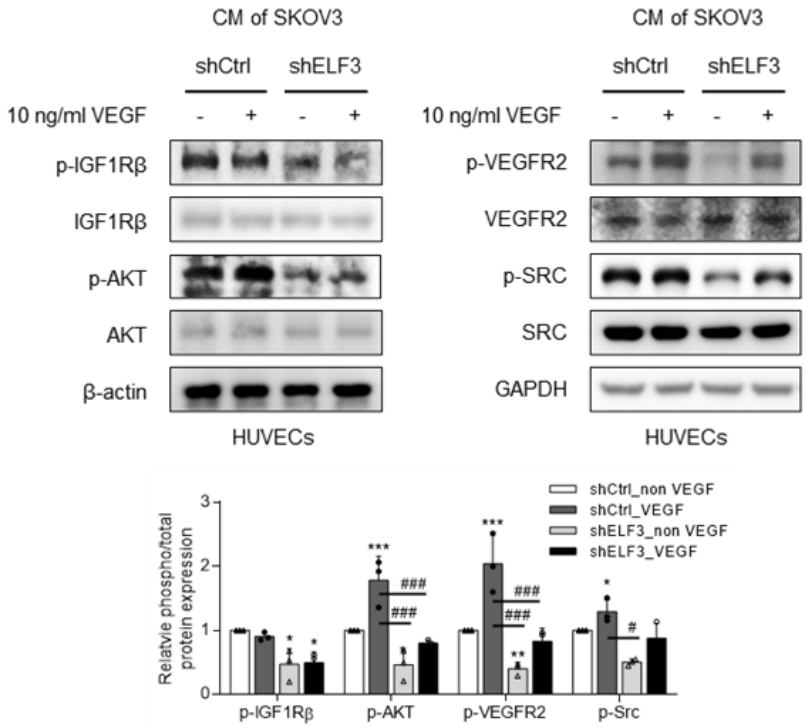
The manuscript is now substantially improved and the reviewer is supportive to its publication at EMBO reports.

Nevertheless, there are aspects that could be further clarified, which some involve experimental work. Being a revised manuscript, the reviewer does not consider those experiments mandatory. Yet, the proposed experiments should be considered by the editorial board, and the decision to ask for new experimental data depends on the journal's reviewing policy.

1. The major question that remains unclear is the autocrine or paracrine contribution of IGF1 in the promotion of tumor angiogenesis, and tumor growth.
The authors showed that "In addition, despite the induction of ELF3 overexpression, IGF1 knockdown reduced the expression of VEGF and MMP2 again (Fig. EV1C)." Based on this evidence, the reviewer concludes that IGF1 functions in an autocrine loop to stimulate ovarian cancer cells to secrete VEGF and MMP2, among other factors.
In parallel, the authors also showed that "In the case of angiogenesis-related signaling pathways in HUVECs, IGF1R and VEGFR2 signaling pathways were significantly activated when incubated with CMs of SKOV3-shCtrl exposed to hypoxia, but not when ELF3 was silenced in SKOV3. In particular, the phosphorylation of IGF1R showed a significant difference according to changes in ELF3-mediated IGF1 expression (Fig. 5A and E)." Based on this evidence, the reviewer concludes that IGF1 and VEGF functions in a paracrine loop to stimulate angiogenic activity.
In addition, xenograft experiments (Fig. 6) showed that ELF3-defficient SKOV3 cells failed to engraft in mice. Yet, those engrafting growth (as measured by the pictures provided) to quite large sizes.
This raises the question that ELF3-defficient SKOV3 cells may have impaired ability to engraft due to autocrine effects of ELF3 LoF (for instance, impaired IGF1-IGFR1 signaling in tumors cells) rather than based on paracrine angiogenic effects. Otherwise, the reviewer would expect a similar number of engrafted tumors, yet, with a dramatically reduced size.

A clarifying experiment would be to overexpress VEGFA in shELF3 SKOV3 cells, to rescue paracrine effects on angiogenesis, without affecting the autocrine effects.
Answer: Applying the reviewer's comments, the experiment was conducted in the presence or

absence of 10 ng/ml recombinant VEGF in HUVECs using conditioned medium (CM) of SKOV3-shCtrl or SKOV3-shELF3 cells. The results obtained are shown below and are included as Figure 4G in the revised manuscript.



We added the following explanation to the section of the results, '*The ELF3/IGF1/IGF1R signaling axis accelerated tumor angiogenesis*'.

'The angiogenic effect was investigated to check whether HUVECs rescued paracrine effects when treated with CM of SKOV3-shCtrl or -shELF3 cells in the presence of recombinant VEGF. Upon treatment with recombinant VEGF, phosphorylation of VEGFR2 was increased compared to each control without recombinant VEGF, but phosphorylation of IGF1R was not changed. Phosphorylation of VEGFR2 was significantly attenuated in CM of SKOV3-shELF3 than in CM of SKOV3-shCtrl upon treatment with recombinant VEGF (Fig. 4G). Thus, IGF1 induced by ELF3 promotes angiogenesis in two ways: via autocrine signaling in the EOCs and paracrine signaling in ECs.'

The discussion section also provided an additional explanation, as shown in bold below.
'Along with changes in ELF3 expression levels, angiogenic activities and signaling, particularly IGF1R signaling, were significantly affected (Fig. 5B-F). That is additional

evidence indicating that ELF3 directly regulates IGF1. Considering that IGF1 indirectly regulates VEGF expression (Fig. EV1A-C), the reduced VEGF is considered to be an effect following IGF1 reduction. **This phenomenon may explain the decreased VEGFR2 downstream signaling in the CM of SKOV3-shELF3 compared to the control despite the artificial addition of VEGF (Fig. 4G).** These results confirmed that tumor angiogenesis was greatly affected by the expression level of ELF3, which activates IGF1.'

2. Xenograft experiments.

a) There is a mismatch between the text and examples of tumors in Fig. 6C and numbers in Fig. 6A, 5/7 in SKOV3 shCtrl 5*10^5 Fig. 6A vs 6/7 in Fig. 6C.

Answer: We made a mistake in Figure 6A and corrected it.

b) The reviewer has several doubts concerning the representation of the data in Fig. 6B and Fig. 6D. The images of tumors do not fit well with tumor volume curves. Based on the inclusion of non-existent tumors (failed engraftment) in Fig. 6D (values = 0), the reviewer believes that tumor volume curves also contain values of non-existent tumors. This is incorrect. Tumors that do not engraft should not be considered for statistics in Fig. 6B and Fig. 6D, as it represents a completely different parameter. Engraftment potential is demonstrated in Fig. 6A. Growth curves and weight should take only into account existent tumors.

Answer: We re-performed statistical analysis after excluding non-growing tumors in referring to reviewer comment. Additionally, the following phrase was added to the legend of Figure 6D. 'As in (A) and (C), only the grown tumors were weighed and analyzed statistically.'

We also corrected the Figure 6D-related contents in the section of '*ELF3 silencing attenuated angiogenesis and tumorigenesis in EOC-xenografted tumors*' of results in the main text.

3. The authors should include representative images, as sup. data, for vessels in Matrigel plugs quantified in (Fig. 5F left).

Answer: We performed the Matrigel plug assay (Figure 5F left) and quantified the degree of vascular formation by measuring the hemoglobin concentration (Fig. 5F right) following the method described in the section of *'Matrigel plug assay and hemoglobin measurement'*. (We don't know if we correctly understand the reviewer's intent.)

4. There is a misspelling of GAPDH (GPADH) in Fig. EV1.

Answer: Thanks, it is corrected.

2nd Revision - Editorial Decision **19th May 2022**

Dear Youngjoo,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

--
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Manuscript Number: EMBOR-2021-52977V3

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Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods_Table EV4

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods_Table EV3

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	Not applicable

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	Not applicable
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	Not applicable
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	Not applicable

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	Not applicable

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Materials and Methods

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	Not applicable
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	Not applicable

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Materials and Methods

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Materials and Methods
Include a statement about **blinding** even if no blinding was done.	Not Applicable	Not applicable
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	Not applicable
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods, Figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	Not applicable
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	Not applicable
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	Not applicable
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods (IACUC20-031)
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	Not applicable

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Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	Not applicable
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	Not applicable
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	Not applicable

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Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	Not applicable
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	Not applicable
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	Not applicable

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	Not applicable
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	Not applicable
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	Not applicable
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Materials and Methods