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STAT3 signaling induced by the IL-6 family of cytokines modulates angiogenesis

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MS TITLE: STAT3 signaling induced by IL-6 family cytokines modulates angiogenesis

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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 substantial 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.

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.

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Reviewer 1

Advance summary and potential significance to field

This manuscript investigates STAT3 signaling in a context of OSM- and CNTF-effects in EC. Both ligands activate STAT3 but only OSM stimulates ERK and AKT. STAT3 KD reduces OSM and VEGF induced vascular sprouting. Gene expression changes and mitochondrial function are investigated. These parameters do not provide any clear explanation to the functional responses.

The study provides a significant amount of data but these are not easily amenable to interpretation. In fact, a number of contradictions are apparent.

Comments for the author

Specific critique:

1. The discrepancy between OSM and CNTF may relate to ERK and AKT (not proven) but why do the ligands exhibit such differences in sígnaling readout? This is the first question that comes to mind. Also the time kinetics with respect to STAT3 activation differ.

2. It is repeated claimed that OSM activation of ERK and AKT causes increased proliferation but this is not shown. Proliferation is not the only cellular response of relevance to sprouting. Cell migration is equally important.

3. The sprouting assay does not well represent in vivo angiogenesis.

4. Fig 3A. Is there an effect of STAT3 KD on the response to VEGF + OSM? No significance has been indicated. This is a key issue that cannot be easily resolved with the model proposed. The mean is so much higher that an increased number of experiments have to be performed to minimise SEM and blunt the mean difference if significance cannot be obtained.

5. The gene expression changes in response to STAT3 KD are summarised as indicative of increased ERK and AKT signaling but the data do not support this. Again, the data are interpreted to suggest a proliferative response but this has not been measured.

6. Where is the compensatory STAT1 up regulation in response to STAT3 downregulation shown?

Minor: no clear explanation of what CNTFR, CNTF+R are given.

Reviewer 2

Advance summary and potential significance to field

The study while using classical experimental approaches, present significant findings on the the topic of STAT3 regulation by Cytokines, in the regulation of angiogenesis processes. THe authors compare the effect of anti-angiogenic cytokine, CTNF and pro-angiogenic cytokine, OSM on the angiogenic properties/transcriptome and respiration of HUVECs. The paper is generally well-written and intersting for the field.

Comments for the author

While interesting for the field, the study presents some shortcomings.

Major comments:

- Fig1: THe phospho-form should be normalized with total-form of the protein (ie pSTAT3 for total STAT3), as the treatments could impact on the total protein level and not only on the phosphorylation of proteins.

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- Fig4: to claim that the effect observed is mediated by cytokines such as Interferon Gamma, this should be quantified in their system. For example, by performing an Elisa on the supernatant of the cells for Interferon Gamma (or quantitative PCR).
- Fig6/7: the link between respiration/ metabolism with the first experiments is weak. While basing their hypothesis on the litterature, I would validate the accumulation of STAT3 in mitochondria in the system, by either co-staining of STAT3 with mitochondrial markers (COX4 or mitotracker) or immunoblotting of STAT3 on mitochondrial purified fraction (magnetic pulldown or cell fractionation). THis is key to strengthen this weak link in the paper.
- Fig6d: I would be surprised to observed dissociation of the mitochondrial complexes upon growth factor/ cytokine treatment. The complex integrity should be assessed after STAT3 siRNA.
- Fig6e: MitoSOX is usually indicative of severe oxidative stress, and not very sensitive. A better option would be to use sensors such as Mito-roGFP-Orp1, that measure smaller changers in H2O2 in the mitochondria.

Minor comments:

- Fig 1: the effect observed could be due to changed in proliferation. Could the authors block proliferation in the assays / or measure proliferation upon cytokine treatment (MTT or similar)?
- According to phosphosite (https://www.phosphosite.org/proteinAction.action?id=1050), S727-p of STAT3 can be directly linked to ERK activity, while Y705-p is not. I would present both phosphorylation events at the same time in the figures (not S727 after Y705), as s727 potentially takes place before Y705, if a ERK dependent pathway is considered.
- Fig1c: please include other phospho-targets. ERK and AKT are actually linked to different pathways. Assessing activation of MEK1/2 and PI3K could discriminate between the two, and explain what kinase is directly important for STAT3 activation.
- Fig 2a: the clustering is poor in the PCA plot. Could it be due to use of independent pools of HUVECs/ or bench effect? Please clarify.
- Fig 2c/ d: the pathway analysis looks very rudimentary. Could the data be reanalyzed to get a better understanding (for example with REactome or IPA)? For Figure 2d, a volcano plot would be more suitable.
- Fig3: please include the KD of STAT1 in the sprouting experiments, to demonstrate that the effect observed in STAT3 dependent. An alternative would be to present a rescue with KD + overexpression of STAT3.
- Fig7: could the authors explain more what the expectations are for Figure7.
This part is underdeveloped right now.

Reviewer 3

Advance summary and potential significance to field

This MS by Rapp et al. dissects the signaling of the IL-6 family cytokines OSM and CNTS to address the reasons of their opposite effects on angiogenesis. The work is performed exclusively in vitro using HUVEC cells. The results are interesting and to a certain extent novel. However, the failure to report the observations to a physiological or pathological condition in humans or animal models, considerably reduces the enthusiasm as it is not clear under which conditions the endothelial cells would be exposed to either cytokine, not to speak of the soluble CNTFR and of which cells would secrete them. Therefore, the functional significance of the findings is unclear.

Comments for the author

Specific comments:

1) Fig 1C: CNTF -mediated activation of STAT1 is very low, please change the text accordingly (instead of “OSM had a stronger effect”: “CNTF had a very weak effect”.

2) Supplementary Fig S1: why are the time courses different? Please add the 24 h timepoint to the OSM treatments.

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3) The description of transcriptomic results is misleading, first leading to believe they are similar and then underlying the differences. I would suggest to fuse the two chapters and emphasize at the same time the consistency AND the differences.

4) In the GO-term enrichment, please add the number of significantly enriched genes /total number of genes in the category.
5) The migration studies with STAT3 k/d cells should be performed also with CNTF+R+VEGF, and also with the 2nd siRNA.

6) Enriched GO categories in Fig 4A display also “negative regulation of growth”: the authors should comment on this, and perform a proliferation. Indeed, this must be taken into account as it could be a confounding factor in the wound healing assay, which is not performed in the presence of mitomycin C.

7) The authors claim that upon both OSM and OSM+VEGF treatments, STAT3 K/d did not result in enhanced activation of pAKT or pERK. However, the western blot of Fig. 5a shows a reduction of pAKT upon both OSM and OSM+VEGF treatment, and of pERK upon OSM+VEGF in STAT3 silenced cells. Please show quantification of triplicate experiments and statistical analysis.

8) It is well known that there is a compensatory increase in STAT1 phosphorylation upon STAT3 KO in response to IL-6 (Costa Pereira et al., PNAS 2004), so much so that IL-6 triggers and IFNgamma-like response. However, what is relevant in term of IFN-response is the length of phosphorylation rather its extent. STAT1 and STAT3 Y-P should be measured in time course experiments up to 24 hours, before asserting that STAT3 silencing does not affect STAT1 activation.

9) STAT3 Ser-P does not allow accumulation in the mitochondria, as S-A mutants still localize to the organelle, but is required for mitochondrial functions. Please correct.

10) Fig 6C: the statistical analysis is incomplete. Please provide comparisons between: VEGF and CNTF+VEGS vs EBM and OSM alone.

11) The title of the last chapter in the results section is incomplete.

12) S-P STAT3 should be measured in the mitochondria.

13) The discussion lacks of focus and is repetitive.

- Please define the cells used in both text and figure legends from the first experiment. Define EBM in legend.
- Statistics: differences should be indicated with reference to the significance (<0,05, 0,01, 0,001 etc) not always with just 1 star.
- Western blots need to be quantified (minimum 3 experiments) and statistical differences shown.

First revision

Author response to reviewers' comments

We thank the editor and reviewer for the constructive comments and the opportunity to revise our manuscript. Based on the reviewers’ comments we have performed additional experiments and incorporated additional data into the manuscript and figures. For a point-by-point response to each comment please refer to the following text. We believe that the revisions have significantly improved the manuscript and hope that you now find it suitable for publication in your journal “Journal of Cell Science”.

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Reviewer 1

Major comments:

1. The discrepancy between OSM and CNTF may relate to ERK and AKT (not proven) but why do the ligands exhibit such differences in sígnaling readout? This is the first question that comes to mind. Also the time kinetics with respect to STAT3 activation differ.

This is an essential point, and the authors thank the reviewer for bringing it up. It is well known that OSM and CNTF bind to cytokine-specific multimeric receptor complexes. OSM binds to the heterodimer consisting of gp130 and OSM-R (Mosley et al., 1996) and CNTF to the heterotrimer consisting of gp130, Leukemia inhibiting factor receptor (LIF-R) and CNTF-Rα (Davis et al., 1991). Previously published work shows that cell type specific activation of JAKs further defines activated downstream pathways (Stahl et al., 1994) To confirm those published data, we decided to determine distinct JAK/TYK phosphorylation patterns for both cytokines in endothelial cells as these proteins are upstream of STAT, ERK and AKT (Supplemental Fig. S1A). While no difference in pJAK1 und pJAK2 was measurable, only OSM was able to significantly activate pTYK2 (Supplemental Fig. S1A). We updated the text accordingly (line 161-166) and think that this information presents one more vital reason for the discrepancy between OSM and CNTF+R in their signaling patterns. The time kinetics of STAT3 was also streamlined (Supplemental Fig. S1B).

2. It is repeated claimed that OSM activation of ERK and AKT causes increased proliferation but this is not shown. Proliferation is not the only cellular response of relevance to sprouting. Cell migration is equally important.

To follow up on this major comment we added results of proliferation assays using the CyQUANT kit (methods line 593-601). HUVECs were stimulated with OSM or CNTF+R and results shown as novel Fig. 1C. We hope that we therefore resolve this issue by displaying migration and proliferation data right next to the sprouting assay. Our added results of the proliferation assay indeed show that only OSM can induce a significant proliferative effect (Fig. 1C) and we altered the manuscript accordingly (line 142-146).

3. The sprouting assay does not well represent in vivo angiogenesis.

We thank the reviewer for this important remark. We acknowledge that the pure *in vitro* approach of this paper as a limitation in the study. The idea of our project which resulted in the presented manuscript was to have a very close look on molecular level at the differences in the angiomodulatory effect between OSM and CNTF on vascular endothelial cells. We therefore decided to stay in an *in vitro* setting which provides the precision needed to work up the differences. For *in vitro* settings, the assays we have chosen to investigate the effects on angiogenesis are in line with what would be considered good choices (Nowak-Sliwinska et al., 2018). Furthermore, our in vitro assays now cover endothelial cell proliferation, migration and sprouting – all important cell functions that contribute to angiogenesis. The next step in our research will be to follow up on these results and how the findings of this manuscript translate in *in vivo* settings, but we think that this is the scope of a follow up project.

4. Fig 3A. Is there an effect of STAT3 KD on the response to VEGF + OSM? No significance has been indicated. This is a key issue that cannot be easily resolved with the model proposed. The mean is so much higher that an increased number of experiments have to be performed to minimise SEM and blunt the mean difference if significance cannot be obtained.

We added the statistical test between OSM+VEGF with STAT3 knock-down and OSM+VEGF with control siRNA in our statistical model and indicated the result in the novel Fig. 4A (former Fig. 3A).

5. The gene expression changes in response to STAT3 KD are summarised as indicative of increased ERK and AKT signaling but the data do not support this. Again, the data are interpreted to suggest a proliferative response but this has not been measured.

Based on Reviewer 1 and 2’s suggestion we added cell proliferation assays for stimulation and knock-down experiments in this manuscript. Novel supplemental Fig. S4 shows cell proliferation in

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HUVECs exposed to VEGF or OSM+VEGF following STAT3 knock-down or transfection with control siRNA (Supplemental Fig. S4). Cell proliferation in contrast to cell sprouting and migration is indeed decreased in the STAT3 knock-down group. We conclude from this data that proliferation is not the main driver for the observed phenotype of OSM+VEGF after STAT3 knock-down in the spheroid sprouting and migration model. Enhanced vascular endothelial cell migration in a 2D and 3D setting may thus play an important role which is also supported by the RNA Seq data where "regulation of cell adhesion" and "positive regulation of cell migration" belong to the top regulated GO terms. The manuscript was updated accordingly (line 248-252 + 336-342).

6. Where is the compensatory STAT1 upregulation in response to STAT3 downregulation shown?

The reviewer can find the compensatory STAT1 upregulation indirectly on RNA level represented in the top enriched GO terms "cellular response to type I interferon" and "response to interferon gamma" which include STAT1 in Fig. 5A+5B as well as on protein level in Figure 6A-B. On the request of a Reviewer 3, we added western blot analysis for pSTAT1 activity comparing OSM+VEGF stimulated STAT3 knock-down cells with cells just transfected with control siRNA for up to 24h in a time course experiment (Fig. 6B). We measured that initially after 5 min (Fig. 6A), 30min (Fig. 6B) and 1 h (Fig. 6B) no difference could be detected. However, STAT3 knock-down cells show prolonged activation of pSTAT1 for at least 24 h (Fig. 6B) while the control group loses signal before 6 h post stimulation (Fig. 6B). The manuscript was updated accordingly (line 326-335) hoping that data supporting STAT1 activation is now clearly outlined.

Minor comments:

1. no clear explanation of what CNTFR, CNTF+R are given.

To prevent any confusion, we updated the first paragraph of the results section were the abbreviation CNTF+R, meaning CNTF co-stimulated with its soluble CNTF-Rα, gets introduced (line 127-128).

Reviewer 2

Major comments:

1. Fig1: THe phospho-form should be normalized with total-form of the protein (ie pSTAT3 for total STAT3), as the treatments could impact on the total protein level and not only on the phosphorylation of proteins.

We thank the reviewer for this comment. We agree that normalization is usually done using STAT3. At the beginning of the project, we discovered, however, that in our setting a strong increase in pSTAT3 levels, e.g. through stimulation with strong activators like OSM, go along with a strong decrease in STAT3 measured in western blot. We added an example to this response. We also observed this pattern in previously published work ((Bucher et al., 2020), Fig. 3B). We hypothesize that the STAT3 antibody does not detect phosphorylated STAT3. Therefore, STAT3 cannot be used to properly determine the total protein level. We thus chose GAPDH as the better option for normalization in our setting because we did not observe any changes due to treatment and normalizing on native STAT3 would result in an overestimation of the activation.

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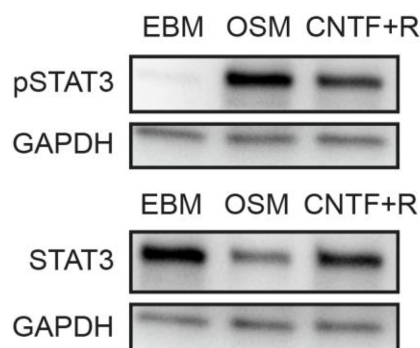
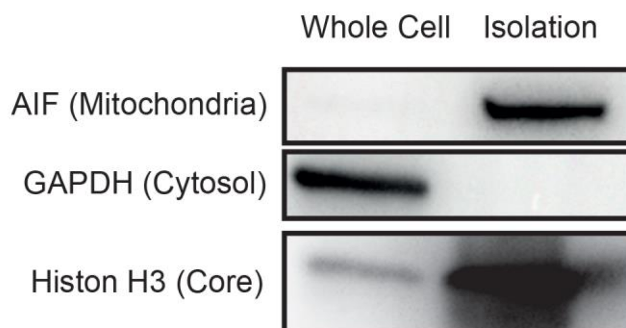


2. Fig4: to claim that the effect observed is mediated by cytokines such as Interferon Gamma, this should be quantified in their system. For example, by performing an Elisa on the supernatant of the cells for Interferon Gamma (or quantitative PCR).

We thank the reviewer for this commend but think this is a misunderstanding. While we measure enriched GO terms related to interferon-gamma in the enrichment analysis (Fig. 5A), we do not believe that this represents increased interferon-gamma levels in response to OSM/VEGF treatment following STAT3 knock-down. We rather believe that it represents increased pSTAT1 activity, a well-known downstream target of interferon-gamma (Bhat et al., 2018), in response to OSM/VEGF treatment following STAT3 knock-down. We updated the part of the discussion about the sequencing data and hope this will minimize any confusion (line 445-457).

3. Fig6/7: the link between respiration/ metabolism with the first experiments is weak. While basing their hypothesis on the litterature, I would validate the accumulation of STAT3 in mitochondria in the system, by either co-staining of STAT3 with mitochondrial markers (COX4 or mitotracker) or immunoblotting of STAT3 on mitochondrial purified fraction (magnetic pulldown or cell fractionation). THis is key to strengthen this weak link in the paper.

To follow up on this important suggestion, we tried both methods suggested by the reviewer. However, unfortunately we did not achieve specific STAT3 staining using standard immunocytochemical methods. Cell fractionation resulted in significant contamination with nuclear proteins in control staining's for nuclear marker so that those results are biased as STAT3 translocates into the nucleus after stimulation as well. An example blot of this is attached to this response. We then turned to track intracellular distribution of STAT3 using overexpression of a previously published Venus-tagged STAT3 protein (Letra-Vilela et al., 2020). Since HUVECs are known to be difficult to transfect, we used Bovine Aorta Endothelial Cells (BAECs) and transfected them with a plasmid coding for a florescence tagged STAT3. Following successful transfection, cells had then been stimulated by OSM and co-stained with mitotracker. The reviewer can find the protocol in the updated method sections (line 632-661). We observed that cells stimulated for 30 min by OSM showed a co- localization of STAT3 within Mitotracker-labelled mitochondria which was not visible in unstimulated cells (Fig. 7A). We updated the manuscript accordingly (line 353-359). Due to this new experiment we decided to omit the old staining of mitochondria in HUVECs after cytokine treatment (OLD Supplemental Fig. S5A) because of redundancy.



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4. Fig6d: I would be surprised to observed dissociation of the mitochondrial complexes upon growth factor/ cytokine treatment. The complex integrity should be assessed after STAT3 siRNA.

We thank the Reviewer for this remark. In the current manuscript, we only performed western blot analyses as basic screening for major changes in total amount of complexes after cytokine treatment in hope of finding an explanation for the increased activity of mitochondria. We agree that a thorough work-up on the yet unsolved mechanism behind the observed increase in metabolic activity, including integrity of complexes and the effect of STAT3 knock-down on HUVECs, is important and will be an interesting analysis. However, we believe that this goes beyond the scope of this already complex story and would be part of one of our follow-up projects which concentrates and dives deeper into the molecular interaction of STAT3 in the mitochondria.

5. Fig6e: MitoSOX is usually indicative of severe oxidative stress, and not very sensitive. A better option would be to use sensors such as Mito-roGFP-Orp1, that measure smaller changes in H2O2 in the mitochondria.

The authors totally agree with the remark made by the reviewer. We updated the text (line 367-368, line 485-488) accordingly to especially point out the drawback of the method we used. However, as we just before, we think that the detailed work up of the molecular function of STAT3 in mitochondria of endothelial cells is the scope of one of our follow-up projects.

Minor comments:

1. Fig 1: the effect observed could be due to changed in proliferation. Could the authors block proliferation in the assays / or measure proliferation upon cytokine treatment (MTT or similar)?

To address this comment, we performed proliferation assays using the CyQUANT proliferation assay (line 593-600). OSM can induce a significant proliferative effect (Fig. 1C), that is partially reduced following STAT3 knock-down (Supplemental Figure S4). We incorporated these results in the revised manuscript (line 142-146 and line 248-252).

2. According to phosphosite (https://www.phosphosite.org/proteinAction.action?id=1050), S727-p of STAT3 can be directly linked to ERK activity, while Y705-p is not. I would present both phosphorylation events at the same time in the figures (not S727 after Y705), as s727 potentially takes place before Y705, if a ERK dependent pathway is considered.

Based on your suggestion, we added the STAT3 S727 data to the signaling analysis in Fig. 2. We also added statistical analysis based on three biological replicates to the updated figure to further enhance the data's quality on the request of reviewer 3. Furthermore, we added a time kinetics for S727 STAT3 (Supplemental Fig. S1B). In contrast to the Y705, S727 shows the peak of activation later, at 15 min post stimulation, which is also the general kinetics of the ERK activation. We updated the manuscript accordingly (line 171-173).

3. Fig1c: please include other phospho-targets. ERK and AKT are actually linked to different pathways. Assessing activation of MEK1/2 and PI3K could discriminate between the two, and explain what kinase is directly important for STAT3 activation.

Due to the request of Reviewer 1 we decided to have a closer look at JAK proteins that are known to play an important role in receptor and STAT phosphorylation (Hu et al., 2021). In HUVECs, we saw no phosphorylation of JAK1 in response to OSM or CNTF+R treatment, pJAK2 levels were also unaltered in response to cytokine stimulation. Interestingly, only OSM was able to significantly activate pTYK2 (Supplemental Fig. S1A) suggesting that pTYK2 phosphorylation plays an important role activating downstream signaling pathways. We updated the text accordingly (line 161-166). Based on our data, we believe that OSM activates multiple distinct signaling pathways in parallel including STAT3, ERK and AKT rather than ERK or AKT leading to STAT3 phosphorylation. However, we did not find conclusive data on OSM- associated intracellular signaling cascades in HUVECs in published papers. Due to the cell specificity of STAT signaling pathways, we would be careful to draw conclusion from papers published in other cell types.

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4. Fig 2a: the clustering is poor in the PCA plot. Could it be due to use of independent pools of HUVECs/ or bench effect? Please clarify.

Indeed, the replicates shown pre group represent true biological replicates with HUVECs from independent batches of pooled HUVECs. This was done on purpose to better represent biological variability. Because the first principal component separates the conditions with a variance of 77% and the second principal component the replicates with a variance of 12%, we still think that the clustering is not bad. Nevertheless, we decided due to this comment and the request of a different reviewer to rephrase the first paragraph about the RNA sequencing to stress that the induced transcriptome of OSM+VEGF and CNTF+R+VEGF has differences but also many similarities (line 178-198).

5. Fig 2c/ d: the pathway analysis looks very rudimentary. Could the data be reanalyzed to get a better understanding (for example with REactome or IPA)? For Figure 2d, a volcano plot would be more suitable.

Based on the Reviewer's suggestion, we added a pathway enrichment analysis using the Reactome database to the analysis (Supplemental Fig. S2) und substituted the scatterplot with a volcano plot in the revised Figure 3D (former Figure 2D). The manuscript was updated accordingly (line 204-209).

6. Fig3: please include the KD of STAT1 in the sprouting experiments, to demonstrate that the effect observed in STAT3 dependent. An alternative would be to present a rescue with KD + overexpression of STAT3.

Based on the Reviewer's suggestion we performed additional siRNA-mediated STAT1 knock-down experiments (Supplemental Figure S3E+F). Western blot analysis showed a sufficient STAT1 knockdown 48 hours after transfection (Supplemental Fig. S3E). In the Spheroid-Sprouting Assay, STAT1 knock-down decreased the baseline sprouting rate (Supplemental Fig. S3F). OSM lost its pro-angiogenic effect following STAT1 knock-down when compared to the EBM group. In contrast to the STAT3 knock-down (Figure 4A), STAT1 knock-down did not results in an excessive increase in spheroid-sprouting in response to VEGF+OSM treatment. In the control siRNA group, VEGF+OSM did not show the previously observed additional pro-sprouting effect compared to VEGF. This may be attributed to high overall sprouting rates during Revision experiments which can lead to a ceiling effect in treatment group with high sprouting rates. Since the STAT1 knock-down groups exhibit overall lower sprouting rates, we believe that those treatment groups were unaffected. Taken together, these data gave us confidence in the validity of or STAT3 knock-down data and we updated the manuscript accordingly (line 263-272)

7. Fig7: could the authors explain more what the expectations are for Figure7. This part is underdeveloped right now.

We thank the Reviewer for the comments particularly on the metabolism associated part of our paper. We extended the paragraph of this figure (revised Fig. 8C-D) and hope this offers a more complete description of our gathered data (line 373-382). We are aware that this paper lacks a mechanistic explanation on molecular level on how OSM enhances mitochondrial as well as glycolytic activity. Due to the solid OSM- associated angiomodulatory phenotype this is subject to ongoing studies. However, we do believe that a further mechanistic work-up is currently beyond the scope of this paper.

Reviewer 3

Major comments:

1. Fig 1C: CNTF -mediated activation of STAT1 is very low, please change the text accordingly (instead of "OSM had a stronger effect": "CNTF had a very weak effect".

The authors thank the Reviewer for this comment. Based on your "Minor comments" No. 3, we quantified Western Blot analysis. Results can now be found in the revised Figure 2A showing that

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also on a semi-quantitative level CNTF-dependent STAT1 activation is weak. The text has been adopted accordingly (line 153-156).

2. Supplementary Fig S1: why are the time courses different? Please add the 24 h timepoint to the OSM treatments.

Indeed, the different time course might confuse the reader. We decided to streamline the time courses by adapting the CNTF+R kinetics to the OSM time course. The old CNTF+R kinetics included for each time point CNTF+R treated samples and untreated samples (only stimulated with Endothelial Basel Medium (EBM)). We think that there is more value in changing the EBM groups to VEGF treated samples (as in in the OSM time kinetics). The reviewer can find the new and streamlined kinetics in the revised Supplemental Fig. S1B.

3. The description of transcriptomic results is misleading, first leading to believe they are similar and then underlying the differences. I would suggest to fuse the two chapters and emphasize at the same time the consistency AND the differences.

The authors agree and adjusted the manuscript according to the Reviewer's proposal (line 178-198).

4. In the GO-term enrichment, please add the number of significantly enriched genes /total number of genes in the category.

We added the requested data as Supplementary Table S3-S10 and updated the legend for each figure which includes a GO-term enrichment. Updated figure legends include information where to find the requested enriched genes/total number of genes in the category.

5. The migration studies with STAT3 k/d cells should be performed also with CNTF+R+VEGF, and also with the 2nd siRNA.

The authors added one replicate for CNTF+R with or without STAT3 knock-down HUVECs in the sprouting assay using the second STAT3 siRNA (Supplemental Fig. S3D). Because the same tendencies could be detected we feel that one experiment is enough to give (together with the OSM data about the second siRNA) confidence in the data. The manuscript has been updated with this new data (line 260-262). Unfortunately, we were not able to perform further migration studies due to technical difficulties in our Core Facility.

6. Enriched GO categories in Fig 4A display also "negative regulation of growth": the authors should comment on this, and perform a proliferation. Indeed, this must be taken into account as it could be a confounding factor in the wound healing assay, which is not performed in the presence of mitomycin C.

We thank the Reviewer for this suggestion and followed up by using a CyQUANT proliferation assay for cytokine stimulation (Fig. 1C) as well as knock-down experiments (Supplemental Fig. S4). OSM enhances vascular endothelial cell proliferation even in the presence of VEGF (Fig. 1C). STAT3 Knock-down significantly decreased endothelial cell proliferation (Supplemental Fig. S4). We therefore assume that proliferation as a confounder is not the reason for the massively increased migration after STAT3 knock-down (Fig. 4B). The manuscript text has been updated (line 248-352).

7. The authors claim that upon both OSM and OSM+VEGF treatments, STAT3 K/d did not result in enhanced activation of pAKT or pERK. However, the western blot of Fig. 5a shows a reduction of pAKT upon both OSM and OSM+VEGF treatment, and of pERK upon OSM+VEGF in STAT3 silenced cells. Please show quantification of triplicate experiments and statistical analysis.

We absolutely agree with the reviewer's comment and repeated the experiment to generate three independent experiments (Fig. 6A). Semi-quantitative analysis of the western blots showed no statistical difference in pAKT or pERK levels following STAT3 knock-down (Fig. 6A).

8. It is well known that there is a compensatory increase in STAT1 phosphorylation upon STAT3 KO in response to IL-6 (Costa Pereira et al., PNAS 2004), so much so that IL-6 triggers and

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IFNgamma-like response. However, what is relevant in term of IFN-response is the length of phosphorylation rather its extent. STAT1 and STAT3 Y-P should be measured in time course experiments up to 24 hours, before asserting that STAT3 silencing does not affect STAT1 activation.

The authors are thankful for the advice to perform time course experiments to detect potential STAT1 activation. In the revised manuscript, we added a proposed time course experiment of HUVECs stimulated with OSM+VEGF for 30 min, 1 h, 6 h and 24 h (Fig. 6B) following STAT3 knock-down or control siRNA treatment. While no difference was detectable until 1 h, after 6 h prolonged pSTAT1 activity was obvious (Fig. 6B). We updated the manuscript (line 326-334) and the abstract accordingly (line 52-54).

9. STAT3 Ser-P does not allow accumulation in the mitochondria, as S-A mutants still localize to the organelle, but is required for mitochondrial functions. Please correct.

The authors updated the text accordingly the reviewer's suggestion (line 348-350).

10. Fig 6C: the statistical analysis is incomplete. Please provide comparisons between: VEGF and CNTF+VEGS vs EBM and OSM alone.

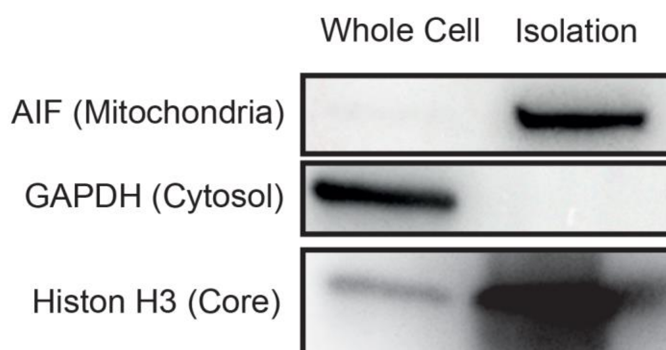
We added statistical analysis between EBM and VEGF (revised Fig. 7C). The other comparison proposed by the reviewer as CNTF+R+VEGF vs OSM alone does not seem to be correct for us because for samples co-stimulated with VEGF, the VEGF groups always represents the control group to which samples should be compared to.

11. The title of the last chapter in the results section is incomplete.

We thank the Reviewer for this remark. The title of the last chapter has been updated (line 372).

12. S-P STAT3 should be measured in the mitochondria.

Based on Reviewers 2 and 3 suggestions we performed additional experiments trying to visualize and quantify STAT3 in mitochondria. Cell fractionation resulted in significant contamination with nuclear proteins in control staining's for nuclear marker so that those results are biased as STAT3 translocates into the nucleus after stimulation as well. An example of our result for cell fractionation is embedded in this response. We thus moved to overexpression of a fluorescent Tagged STAT3 construct (Letra-Vilela et al., 2020) in Bovine Aorta Endothelial Cells. Co-staining with mitotracker was conducted after cytokine treatment. The reviewer can find the protocol in the updated method sections (line 632-660). We observed that cells stimulated for 30 min by OSM showed a co-localization of STAT3 with the stained mitochondria which was not visible in unstimulated cells (Fig. 7A). We updated the manuscript accordingly (line 353-358). Due to this new experiment we decided to omit the old staining of mitochondria in HUVECs after cytokine treatment (OLD Supplemental Fig. S5A) because of redundancy.



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13. The discussion lacks of focus and is repetitive.

We thank you for this criticism and decided to update the discussion the following way. Especially the first two paragraphs about STAT3's role in the cell and the STAT3 knock-down are much needed in our opinion, but we removed repetitive content in the second half of the discussion und fused parts of it together (please keep in mind that deleted content cannot be marked be yellow highlighting in the tracked changes version). The last segment about mitochondria activity is in our opinion also important for the reader. We hope we could improve the discussion by editing it the described way.

Minor comments:

1. Please define the cells used in both text and figure legends from the first experiment. Define EBM in legend.

The legends have been updated to minimize any confusion.

2. Statistics: differences should be indicated with reference to the significance (<0,05, 0,01, 0,001 etc) not always with just 1 star.

We updated all figures the following way: P values < 0.05 were marked with one asterisk. P values of < 0.01 were visualized by two asterisks and smaller value then 0.001 by three asterisks.

3. Western blots need to be quantified (minimum 3 experiments) and statistical differences shown.

Thank you for this important methodological comment. To keep the figures simple, we decided to show representative image of our western blot results throughout the manuscript but added statistical analysis of original western blots that included 3 biological replicates representative for three independent experiments (Fig. 2, Fig. 6, Supplemental Fig. S1). The methods section has been updated accordingly (line 552- 558). Uncut western blots showing biological replicates were submitted separately as by the journal's submission standard.

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Stahl, N., Boulton, T.G., Farruggella, T., Ip, N.Y., Davis, S., Witthuhn, B.A., et al. (1994). Association and Activation of Jak-Tyk Kinases by CNTF-LIF-OSM-IL-6 β Receptor Components. *Science* 263(5143), 92-95. doi: doi:10.1126/science.8272873.

Second decision letter

MS ID#: JOCES/2022/260182

MS TITLE: STAT3 signaling induced by IL-6 family cytokines modulates angiogenesis

AUTHORS: Julian Rapp, Malte Jung, Rhena F. U. Klar, Julian Wolf, Jakob Arnold, Oliver Gorka, Olaf Gross, Clemens Lange, Hansjuergen Agostini, Guenther Schlunck, and Felicitas Bucher
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

Revision satisfactory.

Comments for the author

no further comments.

Reviewer 2

Advance summary and potential significance to field

The authors describe the role STAT3 pathway in angiogenesis, especially describing the therapeutic potential of cytokine, oncostatin M. Every experiments was validated by asssessing the effect on an anti-angiogenic cytokine, ciliary neurotropic factor (CNTF). This paper is of broad interest for the readership of the journal, as the paper describes a novel mechanism of endothelial cell signaling regulation by STAT3 and a balance between two pro and anti angiogenic cytokines. This paper is significant as it could lead to novel therapeutic approaches in angiogenic diseases associated to immune signaling dysregulation.

Comments for the author

The authors have responded to all points in a very detailed manner.
The manuscript is now suitable for publication.

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