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# Tumor-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway

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**Review timeline:**

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| Submission date: | 29 February 2012 |
| Editorial Decision: | 28 March 2012 |
| Revision received: | 30 May 2012 |
| Editorial Decision: | 13 June 2012 |
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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 28 March 2012

Thank you for submitting your research interesting paper on the role of tumor-secreted miRNAs to promote angiogenic activity in endothelial cells for consideration to The EMBO Journal editorial office.

The attached comments indicate obvious scientific merits of your study but also point to some current weaknesses that should be addressed during major revisions. As you will see, the demands expressed from the referees vary significantly. I thus urge you to focus on the most critical experimentation that, as far as I can see center around better support for the functional and crucial contribution of miR-9 in this process (as mentioned by all refs), thorough documentation of the elicited angiogenic effects (ref#2 point 1, ref#1 point 5) and necessary corroboration of the MV-transfer/uptake (to address ref#1 point 6, first part of point1; ref#2 point2 and ref#3 point 1). Addressing particularly these remarks should improve the paper to the level that should convince these scientists from the general significance of your findings. Please do not hesitate to get in touch in case of further questions (preferably via E-mail) and inform us on the progress to offer more time for necessary experimentation. I hope that specifying our demands facilitates rather efficient proceedings and look forward to receive your appropriately revised dataset.

I do have to formerly remind you that The EMBO Journal considers only one round of major revisions with the ultimate decision solely dependig on content and strength of the final manuscript.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1:

The present manuscript investigates the paracrine activity of tumor cells on endothelial cells. The authors showed that tumor cells can secrete miR-9 which is taken up by endothelial cells and regulates cell migration. The authors additionally identified Soc5 as novel target of miR-9, which controls the JAK-STAT pathway. In vivo inhibition of miR-9 or pharmacological inhibition of JAK reduced tumor growth. The manuscript comprises several interesting novel information, however, at the same time it has several major weaknesses and the experiments are in part not sufficiently controlled. The statistical analysis of the data as provided is incorrect and incomplete. Therefore, various additional experiments are required to support the authors conclusion and improve the manuscript to the level of the EMBO Journal.

Major critique

1. The experiment showing that miR-9 inhibition in tumor cells prevents the microRNA inducing activity of the supernatant does not rule out the possibility that miR-9 inhibition changes the properties of the tumor cells to release paracrine signals or affect the biological composition and properties of the microvesicles, which are well known for their angiogenesis regulating function. Therefore, additional experiments should be provided to confirm that the transfer of miR-9 is indeed the mechanism underlying the observed effects. At least, the copy numbers of miRs should be measured in the microvesicles and the recipient endothelial cells. The concentration shown in the supernatants of human tumor cells (figure 2D) is rather low and it is unclear whether this concentration can be sufficient to elicit a meaningful biological effect. E.g. even for transfection protocols much higher concentrations of microRNAs are usually needed.
2. Additionally, it is unclear whether miR-9 inhibition or siDrosha treatment might influence tumor cell survival thereby affecting the number of cells releasing miRs. Therefore, control experiments documenting that the cell density is similar need to be provided.
3. Tumor cells express various miRs beside miR-9 (e.g. miR-17-92 cluster etc) that might also regulate endothelial cell functions. What makes miR-9 so specific? Are all oncomirs increased in the tumor cell-derived microvesicles? Is miR-9 selectively enriched compared to other tumor-derived miRs? These data should be provided to support that miR-9 is indeed a specific mediator between tumor cells and endothelial cells.
4. The interpretation of the experiment showing that the heparin sepharose column retains the miR inducing activity is unclear. If miR-9 is indeed embedded in microvesicles, are the entire microvesicles bound to the column? If so, what is the heparin-binding protein that is expressed at the surface of the microvesicles? Or is miR-9 bound to a heparin-binding protein at the surface of the microvesicles? It is also unclear how the samples were handled. Are microvesicle permealized before injecting to the column?
5. The authors claim that anti-miR-9 inhibits tumor growth by reducing angiogenesis. However, the data provided to support this conclusion is limited and a more thorough analysis should be performed. It is known that counting capillaries does not necessarily correlate with blood flow. Therefore, the number of perfused vessels should be evaluated and higher magnification images should be shown with double stainings for endothelial and smooth muscle markers.
6. With the anti-miR experiment the authors cannot distinguish between a cell intrinsic effect in the tumor cells and a paracrine effect on endothelial cells. At least the effects on tumor cells (e.g. proliferation, cell death) should be analyzed.
7. The figure legends are superficial and need to be improved: the number of the experiments should be given for all experiments. Moreover, it is unclear which group was compared by the statistical analysis. This is particularly worrying if one does not know whether the anti-miR-9 group is significant against the control. Moreover, it is unclear whether the migration data provided in figure 3 are biological replicates and whether significant differences are seen between groups. Since multiple groups are compared, the t-test is not appropriate and the statistical analysis has to be corrected for multiple testing (e.g. ANOVA)

Minor points

1. It is surprising to see the large heterogeneity of miRs expressed in endothelial cells after co-culture with different tumor cells (shown in figure 1b). Are these changes correlating with the expression profile of the tumor cells? This would suggest that tumor cells export their microRNA signature. Are all tumor cell lines exhibit pro-angiogenic effects? If not, is there any correlation of

the biological function with miR-9 levels?
2. Members of the miR-17-92 cluster were also shown to control the JAK-STAT pathway in endothelial cells. Any involvement of this cluster in the endothelial migration assays? At least, this should be discussed.
3. On page 6/7/8 the authors describe a long list of experiments, which characterize the miR inducing factor(s) released by tumor cells. However, several experiments are just described and no data are provided. I would recommend shortening this paragraph and providing the data for the crucial experiments (such as showing the effect of cytokines on miR-9 expression) as one supplementary figure. All statements which are not supported by data can be discussed but should be removed from the result section.

Referee #2:

The authors describe a pro-angiogenic mechanism in which tumor-derived microRNAs are delivered to endothelial cells by microvesicles. Among the different microRNAs, miR-9 could induce migration of endothelial cells and miR-9 antagomirs reduced tumor vascular density and delayed tumor growth. Molecularly, miR-9 reduced SOCS 5 levels and activated JAK-STAT signaling. JAK inhibitors suppressed miR-9 induced cell migration and reduced tumor growth in vivo. Previous studies showed already the angiogenic potential of microvesicles (MV) derived from tumors but did not characterize the microRNAs involved or their signaling pathway. The authors provide novel findings revealing that tumor-secreted miR-9 activates the JAK-STAT signaling pathway in tumor endothelial cells. Nevertheless, the effect of microRNAs (miR-9) on the angiogenic process and on endothelial cell behavior is poorly characterized.
1. Anti-angiogenic therapy is designed to be beneficial in treating cancer. It is clear that not only a reduction in tumor blood vessels, but also normalization of the abnormal tumor vessels is advantageous. More profound insight in the effect of microRNAs on the angiogenic process is thus required. The authors provide in vitro data on endothelial cell migration, proliferation and survival, but the effect of microRNAs (miR-9) on angiogenic sprouting should be provided, using an appropriate ex vivo/in vitro model. More specifically, is there an increase in the number of tip cells, are there more branching points, is the number of filopodia increased, and is cell proliferation increased?
2. Figure 2F shows that MV are taken up by endothelial cells, but this assay is based on the isolation of MV. To reduce possible technical effects of this procedure, the transfer of MV from tumor cells to endothelial cells should be analyzed in a co-culture transwell system with labeled tumor cells in the upper compartment. Transfer of dye or GFP should be detected in the endothelial cells. To confirm that microRNAs are transferred by MV, some rough characterization of these structures should be performed. More specifically, analysis of microRNA levels after degradation of MV with proteinase K or a phospholipid membrane disruptor, followed by RNAse treatment should confirm that a certain type of vesicles is involved.
3. Figure 3C: the quality of the pictures is not optimal and certainly the increase in lamellipodia is hard to detect. Confocal images with higher magnifications should be provided. Are the effects of knock-down or overexpression of miR-9 on endothelial cell migration statistically significant (Fig 3D and E)?
4. Figure 4: In the in vivo tumor model, can an effect of anti-miR-9 on tumor cells be excluded. The staining of the blood vessels in Fig 4E is hardly detectable.
5. Figure 5: The contribution of SOCS5 as downstream target of miR-9 should be confirmed by absence of increased phosphorylation of STAT and JAK in response to SOCS5 transfection in miR-9 treated cells. It is mentioned that SOCS5 was 'significantly' decreased in response to miR-9 (p12), while no quantification of the Western blots is shown.

Referee #3:

General Comments
In their manuscript entitled "Tumor-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway", Zhuang et al., presents data that 1) identifies

miR-9 as a microRNA secreted by multiple cancer cell lines, 2) reveal that miR-9 is released from cells in micro-vessicles, 3) reveal miR-9 to be sufficient for enhanced endothelial recruitment by cancer cells, 4) Reveal that inhibiting miR-9 in vivo by injection of miR-9 inhibitor reduces tumor growth and angiogenesis, 5) Identify the JAK-STAT pathway as being downstream of miR-9 in endothelial cells, 6) implicate SOCS5 as a potential target of miR-9, and 7) reveal that therapy with a novel JAK-STAT small molecule inhibitor inhibits tumor growth. Overall this is an important and very interesting discovery that warrants publication in EMBO. Circulating miRNAs are being increasingly implicated in various biological processes. As such, the current both implicates a circulating miRNA in an important pathologic process of tumor angiogenesis and delineates downstream signaling molecules regulated by this miRNA.
Recommendations and points:
1. The authors have nicely shown that microvessicles from cancer cells transfected with an inhibitor of miR-9 are less effective at recruiting endothelial cells than control MV's. Could the authors show that inhibition of miR-9 in cancer cells (antagomir for example) reduces endothelial migration in a trans-well assay? This would be done without purification of the MV's and would reveal a positive role for endogenous amounts of miR-9 emanating from cancer cells in this process.
2. The effect of drosha KD on miR-9 levels in endothelial cells are modest (supp. Fig. 2C), could the authors comment on potential reasons for this in the text. Additionally, the effect of drosha KD on endothelial migration is not complete (Fig. 3B). This suggests that potentially non-miRNA factors (secreted proteins) may be mediating the remaining effects. Could the authors comment on this in the text.
3. The authors have used an insightful and novel approach to identify the miR-9 target SOCS5. Did the authors perform standard mutagenesis and loss-of-function analysis for miR-9 in luciferase assays?
4. Although the current work is conceptually distinct and novel from previous work on cancerous miRNAs as regulators of endothelial recruitment, the authors should cite recent work on miRNA-regulated endothelial recruitment in cancer progression.

1st Revision - Authors' Response 30 May 2012

Response to Reviewers

Referee #1

*The present manuscript investigates the paracrine activity of tumor cells on endothelial cells. The authors showed that tumor cells can secrete miR-9 which is taken up by endothelial cells and regulates cell migration. The authors additionally identified Soc5 as novel target of miR-9, which controls the JAK-STAT pathway. In vivo inhibition of miR-9 or pharmacological inhibition of JAK reduced tumor growth. The manuscript comprises several interesting novel information, however, at the same time it has several major weaknesses and the experiments are in part not sufficiently controlled. The statistical analysis of the data as provided is incorrect and incomplete. Therefore, various additional experiments are required to support the authors conclusion and improve the manuscript to the level of the EMBO Journal.*

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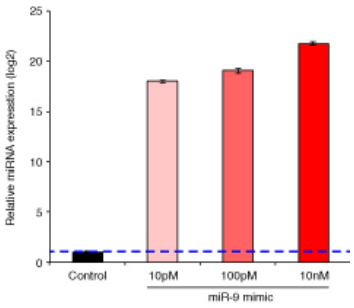
*protocols much higher concentrations of microRNAs are usually needed.*

We appreciate the referee's comments and suggestions. As recommended, we have performed additional experiments to gain further insights into the effects of miR-9 inhibition on tumor cells and microvesicles. First, we found that anti-miR-9 does not affect SK23 tumor cell growth (Supplementary Fig. 3B). Second, we performed experiments with antibody arrays which include 55 angiogenesis-related proteins in SK23-derived microvesicles. MiR-9 inhibition had no detectable effects on such proteins (Supplementary Fig. 3C), indicating that anti-miR-9 does not affect the biological composition of the microvesicles. Finally, we measured miR-9 levels in SK23 cells, microvesicles and recipient HUVECs. In all three circumstances, miR-9 expression was significantly reduced by anti-miR-9 (Supplementary Fig. 2F). Together, these data suggest that direct transfer of miR-9 is the likely cause of the observed changes in endothelial cells.

We used 10nM of miRNA mimics for miR-9 overexpression in our experiments. The miR-9 concentrations in the supernatants of tumor cells ranged from ~10 to ~1000pM. We tested whether these concentrations of miR-9 would be sufficient for miR-9 transfection in HUVECs and found that even 10pM miR-9 mimic results in a marked increase in miR-9 levels (Figure attached below). Although the efficiency of miR-9 transfer is much lower than transfection, we believe that the concentration employed is sufficient to elicit biological effects, as evidenced by our subsequent functional and biochemical analyses.




*2. Additionally, it is unclear whether miR-9 inhibition or siDrosha treatment might influence tumor cell survival thereby affecting the number of cells releasing miRs. Therefore, control experiments documenting that the cell density is similar need to be provided.*

We performed these important control experiments and added the data in Supplementary Fig. 3B. Neither anti-miR-9 nor siDrosha significantly affected tumor cell growth 24h or 72h post transfection. This was true of both SK23 and HM7 cells.

*3. Tumor cells express various miRs beside miR-9 (e.g. miR-17-92 cluster etc) that might also regulate endothelial cell functions. What makes miR-9 so specific? Are all oncomirs increased in the tumor cell-derived microvesicles? Is miR-9 selectively enriched compared to other tumor-derived miRs? These data should be provided to support that miR-9 is indeed a specific mediator between tumor cells and endothelial cells.*

We certainly agree that other miRNAs play a role in regulating tumor angiogenesis. Dews et al. (cited in our manuscript) reported that Myc-induced miR-17-92 cluster in tumor cells increases angiogenesis by a paracrine mechanism. We have been able to detect members of miR-17-92 in SK23 microvesicles, suggesting that indeed many tumor-derived miRNAs are present in MVs. However, unlike miR-9, the levels of miR-17-92 in endothelial cells did not change in response to tumor cells (Supplementary Table1). Therefore, miR-9 and miR-17-92 likely regulate tumor-vessel interaction through different mechanisms.

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*4. The interpretation of the experiment showing that the heparin sepharose column retains the miR inducing activity is unclear. If miR-9 is indeed embedded in microvesicles, are the entire microvesicles bound to the column? If so, what is the heparin-binding protein that is expressed at the surface of the microvesicles? Or is miR-9 bound to a heparin-binding protein at the surface of the microvesicles? It is also unclear how the samples were handled. Are microvesicle permealized before injecting to the column?*

The reviewer asks excellent questions. However, mass spectrometry identified many proteins in miR-9 inducing fractions eluted from heparin sepharose. Therefore, extensive biochemical purification and characterization will be required to elucidate the biological significance of our observations, which is clearly beyond the scope of this submission. Nevertheless, we believe that the heparin-binding property is intriguing and may be potentially physiologically relevant.

As shown in Supplementary Fig. 2E, we found that miR-9 carried by microvesicles is resistant to RNase treatment, suggesting that miR-9 is embedded in MVs. For column purification, tumor conditioned medium was subjected to heparin-sepharose column without any prior permealization.

The heparin column specifically bound miR-9 carriers that target endothelial cells. This finding suggests that specific heparin-binding proteins in complex with miR-9 mediate this process.

*5. The authors claim that anti-miR-9 inhibits tumor growth by reducing angiogenesis. However, the data provided to support this conclusion is limited and a more thorough analysis should be performed. It is known that counting capillaries does not necessarily correlate with blood flow. Therefore, the number of perfused vessels should be evaluated and higher magnification images should be shown with double stainings for endothelial and smooth muscle markers.*

The referee's point is well taken. We performed FITC-lectin perfusion to evaluate functional tumor vessels. As shown in Supplementary Fig. 4A, we found that either anti-VEGF or anti-miR-9 significantly reduced FITC-lectin labeled vessels, indicating that the number of perfused vessels was decreased.

We now show higher-quality images in Fig. 4E, with double staining for CD31 (endothelial marker) and Desmin (smooth muscle marker). Anti-miR-9 did not significantly affect Desmin coverage in tumor vessels.

*6. With the anti-miR experiment the authors cannot distinguish between a cell intrinsic effect in the tumor cells and a paracrine effect on endothelial cells. At least the effects on tumor cells (e.g. proliferation, cell death) should be analyzed.*

As recommended, we performed Ki67 and cleaved caspase3 staining to quantify tumor cell proliferation and apoptosis, respectively. We found that miR-9 inhibition led to decreased cell proliferation but did not affect apoptosis (Supplementary Fig. 4B). Since targeting miR-9 did not affect HM7 growth in cell culture (Supplementary Fig. 3B), we reasoned that decreased proliferation was more likely caused by inhibition of tumor angiogenesis.

*7. The figure legends are superficial and need to be improved: the number of the experiments should be given for all experiments. Moreover, it is unclear which group was compared by the statistical analysis. This is particularly worrying if one does not know whether the anti-miR-9 group is significant against the control. Moreover, it is unclear whether the migration data provided in figure 3 are biological replicates and whether significant differences are seen between groups. Since multiple groups are compared, the t-test is not appropriate and the statistical analysis has to*

*be corrected for multiple testing (e.g. ANOVA)*

We thank the referee for the insightful suggestions. We made efforts to improve the figure legends. We provide now a clearer description of statistical analysis in figure legends and Methods and added more labels in the figures. We also indicated the number of experiments and biological replicates.

Minor points

*1. It is surprising to see the large heterogeneity of miRs expressed in endothelial cells after co-culture with different tumor cells (shown in figure 1b). Are these changes correlating with the expression profile of the tumor cells? This would suggest that tumor cells export their microRNA signature. Are all tumor cell lines exhibit pro-angiogenic effects? If not, is there any correlation of the biological function with miR-9 levels?*

As hierarchical clustering in Fig. 1B suggests, endothelial cells from five different human tissues have surprisingly distinct miRNA expression. Tumor cell stimulation only affected a subset of miRNAs including miR-9 (Supplementary Table1), which may reflect the miRNA expression profile of the tumor cells. All tumor cell lines require angiogenesis to grow in vivo, although it is difficult to precisely quantify their angiogenic capabilities.

*2. Members of the miR-17-92 cluster were also shown to control the JAK-STAT pathway in endothelial cells. Any involvement of this cluster in the endothelial migration assays? At least, this should be discussed.*

We agree with the referee. We added a reference to the work by Doebele et al. on miR-17-92 and JAK1 in the discussion. These authors reported that members of the miR-17-92 cluster exhibit an endothelial cell-intrinsic anti-angiogenic function by targeting JAK1. However, in our study, we did not find that tumor cells result in significant change in miR-17-92 levels in endothelial cells. Consistent with Doebele's finding, our data suggest that JAK-STAT functions as a pro-angiogenic pathway, which is regulated by miRNAs in endothelial cells.

*3. On page 6/7/8 the authors describe a long list of experiments, which characterize the miR inducing factor(s) released by tumor cells. However, several experiments are just described and no data are provided. I would recommend shortening this paragraph and providing the data for the crucial experiments (such as showing the effect of cytokines on miR-9 expression) as one supplementary figure. All statements which are not supported by data can be discussed but should be removed from the result section.*

We added Supplementary Fig. 2C to show the effects of several angiogenic factors on miRNA expression, and revised the text as the referee recommended.

Referee #2

*The authors describe a pro-angiogenic mechanism in which tumor-derived microRNAs are delivered to endothelial cells by microvesicles. Among the different microRNAs, miR-9 could induce migration of endothelial cells and miR-9 antagomirs reduced tumor vascular density and delayed tumor growth. Molecularly, miR-9 reduced SOCS 5 levels and activated JAK-STAT signaling. JAK inhibitors suppressed miR-9 induced cell migration and reduced tumor growth in vivo. Previous*

*studies showed already the angiogenic potential of microvesicles (MV) derived from tumors but did not characterize the microRNAs involved or their signaling pathway. The authors provide novel findings revealing that tumor-secreted miR-9 activates the JAK-STAT signaling pathway in tumor endothelial cells. Nevertheless, the effect of microRNAs (miR-9) on the angiogenic process and on endothelial cell behavior is poorly characterized.*

*1. Anti-angiogenic therapy is designed to be beneficial in treating cancer. It is clear that not only a reduction in tumor blood vessels, but also normalization of the abnormal tumor vessels is advantageous. More profound insight in the effect of microRNAs on the angiogenic process is thus required. The authors provide in vitro data on endothelial cell migration, proliferation and survival, but the effect of microRNAs (miR-9) on angiogenic sprouting should be provided, using an appropriate ex vivo/in vitro model. More specifically, is there an increase in the number of tip cells, are there more branching points, is the number of filopodia increased, and is cell proliferation increased?*

We thank the referee for raising the important issue of vessel normalization. We performed an in vivo lectin perfusion experiment to assess tumor vessel functions. We found that both anti-VEGF and anti-miR-9 significantly reduce FITC-lectin labeled vessels, indicating that the functional tumor vasculature is compromised in accordance with decrease of vessel density. We now present these data in Supplementary Fig. 4A.

Following the referee's suggestion, we analyzed the effects of miR-9 on HUVEC bead sprouting assays. As shown in Fig. 3F, miR-9 increased sprouting growth by ~2 fold, which suggests an increase in the number of tip cells and/or branching activities of endothelial cells.

*2. Figure 2F shows that MV are taken up by endothelial cells, but this assay is based on the isolation of MV. To reduce possible technical effects of this procedure, the transfer of MV from tumor cells to endothelial cells should be analyzed in a co-culture transwell system with labeled tumor cells in the upper compartment. Transfer of dye or GFP should be detected in the endothelial cells. To confirm that microRNAs are transferred by MV, some rough characterization of these structures should be performed. More specifically, analysis of microRNA levels after degradation of MV with proteinase K or a phospholipid membrane disruptor, followed by RNAse treatment should confirm that a certain type of vesicles is involved.*

We thank the referee for the excellent suggestions. Indeed, we have been able to observe transfer of dye from DiI labeled SK23 cells into co-cultured HUVECs in a transwell system. We added this new data in Fig. 2F.

Consistent with previous reports, miRNAs in purified microvesicles were resistant to RNase treatment. Pre-incubation with 0.5% SDS, but not proteinase K, sensitized these miRNAs to RNase digestion. We added these data in Supplementary Fig. 2E.

*3. Figure 3C: the quality of the pictures is not optimal and certainly the increase in lamellipodia is hard to detect. Confocal images with higher magnifications should be provided. Are the effects of knock-down or overexpression of miR-9 on endothelial cell migration statistically significant (Fig 3D and E)?*

We now provide higher magnification images that we believe provide a better illustration of the lamellipodia structure.

We performed Student t-test on the end time point of the migration assays. Compared with control,

the effects of knock-down or overexpression of miR-9 on cell migration are statistically significant ($p<0.05$). We revised the figure 3 to reflect this point.

*4. Figure 4: In the in vivo tumor model, can an effect of anti-miR-9 on tumor cells be excluded. The staining of the blood vessels in Fig 4E is hardly detectable.*

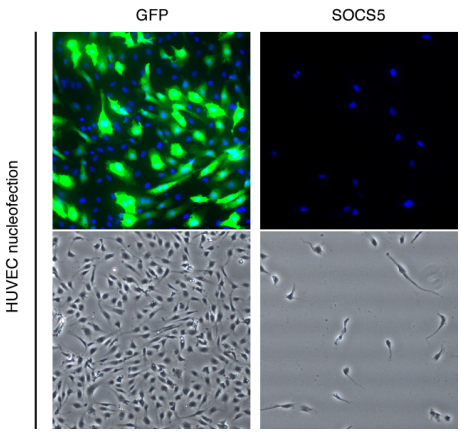
We observed decreased proliferation and unaltered apoptosis of tumor cells in the presence of anti-miR-9 (Supplementary Fig. 4B). Because we found that targeting miR-9 does not affect HM7 growth in cell culture (Supplementary Fig. 3B), we reasoned that decreased proliferation is more likely caused by inhibition of tumor angiogenesis, although effects of anti-miR-9 on tumor cells in vivo cannot be completely ruled out. Please see our response to a similar point raised by reviewer # 1.

In revised Fig. 4E, we provide images with higher magnification, to better illustrate vessel staining.

*5. Figure 5: The contribution of SOCS5 as downstream target of miR-9 should be confirmed by absence of increased phosphorylation of STAT and JAK in response to SOCS5 transfection in miR-9 treated cells. It is mentioned that SOCS5 was 'significantly' decreased in response to miR-9 (p12), while no quantification of the Western blots is shown.*

We agree with the referee that the suggested SOCS5 transfection studies would be valuable in principle. Unfortunately, we encountered some unexpected technical challenges in the execution of such experiments. Although we obtained good transfection efficiency by nucleofection (~70% as estimated by GFP expression), most SOCS5 transfected cells died after 48 hours in multiple experiments. Please see images attached below. We did not detect any SOCS5 expression in the surviving cells, suggesting that SOCS5 is deleterious to HUVECs, which is consistent with its proposed function to suppress JAK-STAT activity. We also tested alternative transfection reagents, with similar results. Nevertheless, our current data in Figure 5 strongly suggests that SOCS5 is directly targeted by miR-9, which correlates with activation of JAK-STAT pathway. Since SOCS proteins have been well studied as negative regulators of JAK-STAT, we believe SOCS5 is at least one mediator of miR-9 induced JAK-STAT activation.

The referee has a valid point regarding the Western blots. We performed densitometry analysis on SOCS5 Western blots using ImageJ and found that miR-9 results in an approximately 50% reduction in SOCS5 levels.



Referee #3

*In their manuscript entitled "Tumor-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway", Zhuang et al., presents data that 1) identifies miR-9 as a microRNA secreted by multiple cancer cell lines, 2) reveal that miR-9 is released from cells in micro-vessicles, 3) reveal miR-9 to be sufficient for enhanced endothelial recruitment by cancer cells, 4) Reveal that inhibiting miR-9 in vivo by injection of miR-9 inhibitor reduces tumor growth and angiogenesis, 5) Identify the JAK-STAT pathway as being downstream of miR-9 in endothelial cells, 6) implicate SOCS5 as a potential target of miR-9, and 7) reveal that therapy with a novel JAK-STAT small molecule inhibitor inhibits tumor growth. Overall this is an important and very interesting discovery that warrants publication in EMBO. Circulating miRNAs are being increasingly implicated in various biological processes. As such, the current both implicates a circulating miRNA in an important pathologic process of tumor angiogenesis and delineates downstream signaling molecules regulated by this miRNA.*

Recommendations and points:

*1. The authors have nicely shown that microvessicles from cancer cells transfected with an inhibitor of miR-9 are less effective at recruiting endothelial cells than control MV's. Could the authors show that inhibition of miR-9 in cancer cells (antagomir for example) reduces endothelial migration in a trans-well assay? This would be done without purification of the MV's and would reveal a positive role for endogenous amounts of miR-9 emanating from cancer cells in this process.*

We appreciate the referee's suggestion and performed trans-well migration assays of HUVEC in co-culture with cancer cells. As shown in Supplementary Fig. 3A, inhibition of miR-9 in SK23 cells reduced endothelial cell migration by ~50%. The efficiency of miR-9 antagomir is shown in Supplementary Fig. 2F.

*2. The effect of drosha KD on miR-9 levels in endothelial cells are modest (supp. Fig. 2C), could the authors comment on potential reasons for this in the text. Additionally, the effect of drosha KD on endothelial migration is not complete (Fig. 3B). This suggests that potentially non-miRNA factors (secreted proteins) may be mediating the remaining effects. Could the authors comment on this in the text.*

Neither siDrosha nor anti-miR-9 could completely inhibited miR-9 expression in tumor cells. Such incomplete inhibition of miR-9 in tumor cells likely explains the residual expression of miR-9 levels in endothelial cells. We added this potential explanation in the revised text.

The same reasons could account for the incomplete inhibition of endothelial cell migration in Fig. 3. In addition, we detected multiple angiogenic factors including VEGF in purified microvesicles, which are not affected by anti-miR-9 (Supplementary Fig. 3C). We agree with the referee that these secreted proteins may mediate the remaining effects. We also discussed these points in the text.

*3. The authors have used an insightful and novel approach to identify the miR-9 target SOCS5. Did the authors perform standard mutagenesis and loss-of-function analysis for miR-9 in luciferase assays?*

We thank the reviewer for the excellent suggestion. We performed mutagenesis on the two predicted miR-9 binding sites at *SOCS5* 3'UTR (details described in Materials and methods). As shown in the revised Fig. 5F, miR-9 overexpression only inhibits the luciferase reporter in frame with wildtype *SOCS5* 3' UTR, but not mutated *SOCS5* 3' UTR, indicating that *SOCS5* is in fact a

direct target of miR-9.

*4. Although the current work is conceptually distinct and novel from previous work on cancerous miRNAs as regulators of endothelial recruitment, the authors should cite recent work on miRNA-regulated endothelial recruitment in cancer progression.*

We now cite the recent report on miR-126-regulated endothelial recruitment in tumor metastasis.

2nd Editorial Decision 13 June 2012

Thank you very much for the revised study that was assessed by one of the original referees that was fully satisfied with the revisions provided.

Please allow me to congratulate to the study and point out that the editorial office will soon be in touch with necessary paperwork related to official acceptance.

Please also notice, that The EMBO Journal encourages the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. This entails presentation of un-cropped/unprocessed scans for the key data of published work. We would be grateful for one PDF-file per figure combining this information. These will be linked online as supplementary "Source Data" files. Please do let me know if you have any questions regarding this initiative AND feel free to check the following URL for a recent example: http://www.nature.com/emboj/journal/v30/n20/suppinfo/emboj2011298as1.html.

I am very much looking forward to efficient proceedings in this matter.

Yours sincerely,

Editor

The EMBO Journal