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# Targeting Notch signaling by the conserved miR-8/200 microRNA family in development and cancer cells

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**Review timeline:**

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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 10 August 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, referees 1 and 3 express interest in your study: while they do have significant concerns with the work that would need to be addressed in a major revision of the manuscript, they are broadly supportive of publication. Referee 2 is rather less positive, although many of the points he/she raises are similar to those of the other referees. A number of concerns I would like to highlight:

1. All three referees highlight the human cell culture work as requiring significant extension to demonstrate that the ZEB1/miR200c/JAGGED1 links are relevant in the cancer context (e.g. referee 1 second point, and referee 3 point 2). In particular, showing that JAGGED1 over-expression can at least partially rescue the miR-200c over-expression phenotype would be essential.
2. Both referees 1 and 3 raise concerns as to the inconsistent effects of miR-200c and 141 vs. miR200b and 429. This is potentially interesting, and if you are able to provide stronger evidence that the two sets of miRNAs do indeed have different effects, this would be very valuable and I would encourage you to attempt at least some of the analyses suggested by the referees. However, in the case that the results prove inconclusive, I would recommend that these data be excluded from a revised version (or at least that any emphasis on them is minimised).
3. None of the referees are convinced by the data presented in Figure 6 as to the potentially synergistic effects of ZEB1 and Notch signalling in regulating growth and metastasis. Again, these data either need to be strengthened or eliminated.
4. All the referees comment that the discussion is too speculative and that some of the conclusions need to be toned down.

Given the interest expressed by the majority of referees, we would like to give you the opportunity

 

to submit a revised version of your manuscript, but only if you are able to adequately address the concerns raised above (most critical would be point 1), as well as respond to the other criticisms of the referees. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Dominguez et al.

This manuscript describes some interesting findings that are somewhat loosely connected.

The first finding is that Drosophila mir-8 targets Serrate. These results (Figs 1-3) are convincing, even though the loss-of-function of mir-8 only gives a very very subtle Serrate gain-of-function phenotype. These findings support the emerging view that most miRNAs have only subtle loss-of-function phenotypes due to their supportive, rather than instructive, role in orchestrating development.

The second finding is that human mir-200c and mir-141, which are homologous to Drosophila mir-8, target JAGGED1, a Serrate homologue, in cultured human cells (Fig 4). Evidence is then presented that inhibition of JAGGED1 expression largely accounts for the phenotype of mir-200c and mir-141 overexpression in cultured PC3 cells (inhibition of cell proliferation and ZEB1 expression). The authors then claim that overexpression of JAGGED1 should therefore promote cell proliferation and ZEB1 expression in metastatic cancer (Fig 7), but do not show any evidence for this assertion. At the very least, the authors should try to determine whether overexpression of JAGGED1 (with a different 3'UTR) can rescue the effect of mir-200c and mir-141 overexpression in PC3 cells.

The third finding is that the mir-200a, 200b, 429 group appears to be expressed in an opposite fashion and to antagonise mir-200c and mir-141 in a mysterious manner. If the authors wish to improve the relevance of this finding for human cancer, and to justify their model (Fig 7), they should compare the expression patterns of mir-200c and mir-141 versus mir-200a, 200b, 429, JAGGED1 and ZEB1 in cancer patient tissue samples.

The fourth finding is that overexpression of the Drosophila ZEB1 homologue, Zfh1, promotes invasion and metastasis in the fly. Given the extensive work on ZEB1 and EMT in mammals, this finding is not particularly novel. Futhermore, it is completely unclear how this finding relates to the other results from Drosophila regarding mir-8 and Serrate. This finding should be removed from the manuscript and would better serve as the starting point for an investigation into the mechanism by

 

which Zfh1 promotes invasion in Drosophila epithelia.

In summary, the finding that this miRNA-target gene interaction is one of the few that may be conserved across divergent species is an interesting one and is significant enough to warrant publication in EMBO Journal. The additional data that regulation of JAGGED1 may be important for the function of these miRNAs in human cancer remains speculative and the authors should resist the temptation to oversell this data.

Notes.

The font size in many of the figures is too small to read and will be far to small once figures are shrunk for publication.

Both title and abstract are, in my view, not an accurate description of the results contained within the paper. I would suggest a re-write along the following lines:

The Drosophila Serrate gene and its human homologue JAGGED1 are regulated by a conserved family of microRNAs

The Notch receptor is activated by DSL-type ligands including Drosophila Serrate and human JAGGED1. Regulation of Serrate and JAGGED1 expression must be precisely controlled during normal development and can be de-regulated in human cancer. Here we show that expression of the Serrate gene is inhibited by the mir-8 miRNA in Drosophila. Overexpression of mir-8 produces a Serrate loss-of-function phenotype, while mutation of mir-8 causes a very mild Serrate gain-of-function phenotype. We also show that the mir-8 human homologues, mir-200c and mir-141, inhibit expression of JAGGED1 in cultured cells. These miRNAs are known to inhibit cell proliferation and epithelial-to-mesenchymal transition and we provide evidence that inhibition of JAGGED1 expression contributes to these effects. Curiously, other mir-8 family members, mir-200a, 200b and 429, appear to antagonise the ability of mir-200c and 141 to inhibit JAGGED1 expression. Accordingly, mir-200a, 200b and 429 are strongly expressed in metastatic PC-3 prostate cells while mir-200c and 141 are strongly expressed in non-tumourigenic and non-metastatic PNT1A prostate cells. In summary, regulation of Notch ligand expression by the mir-8/200 miRNA family is a conserved mechanism operating in Drosophila development and human cancer.

Referee #2 (Remarks to the Author):

This work has two basic parts. Part 1 is a reasonably complete characterization of Serrate/Jagged1 as a standard, although not previously identified, evolutionarily conserved target of miR-200c regulation. The functional data are consistent with this being a novel, albeit mild, method for regulating Notch signaling. This is solid and valuable due to the fact that the target was identified through in vivo unbiased analyses and not through computational approaches, but it is not alone of sufficient general interest for EMBO journals. Part 2 is a much more flimsy and grasping attempt to argue that this is linked to human metastatic cancer through effects on ZFH1/ZEB1 and a relation to EMT. The evidence for this is a string of relatively weak and weakly related experiments using expression profiling and knockdown/overexpression in various cell culture lines and then a fly 'metastasis' assay. The title as well as the intro raise expectations about an important unanswered question in cancer biology concerning the transition from initial, often dormant micrometastasis into frank metastatic growth following dissemination. But these are not at all fulfilled by this work; they are a serious mismatch with the paper's data. The title and abstract also contain a number of inaccuracies (ZEB1 is not a target of Mir-200; instead it is a repressor Mir 200. Overexpression of ZFH1 causes cell arrest, not dissemnation). This work overall does not merit publication in EMBO J.

Selected comments:

Fig. 1: It is surprising that the data on tumors and metastasis, given the apparent focus of the paper, are only represented by a bar graph.

p. 9 There is an argument that PC-3 cells express high levels of JAG1 because they express low

 

levels of Mir-200c. These experiments are unsatisfying as they are based on quantitation of ECL-developed Western blots, which of course has a much more narrow dynamic range and is much less reliable than the qPCR experiments with which they are compared. Also there is no documentation of how many replicate experiments were performed for each, and no error bars or measures of statistical significance.

p.10. Many factors can block cell growth, and Mir-200c~141 has many additional targets other than JAG1. The authors do not test whether the growth defect can be bypassed for instance by providing a JAG1 expression vector that is insensitive to Mir-200c control. Such an experiment is critical to the case that 'inhibition of tumor growth by miR-200c could be largely attributed to JAG1'.

In several areas there is an implied argument that PNT1A and PC-3 are comparable cell lines allowing valid comparison and investigation of the metastatic character of the latter. For instance, the authors speak on p.10 of 'restoring' expression of Mir200c to PC-3 cells; what they are really doing is overexpressing Mir200c in these cells. The authors provide no citation or evidence to suggest that the two cells lines are not vastly different genomically.

Fig. 5 consists largely of expression profiling in different cell lines which provides little direct evidence. Manipulative experiments to bear out the assumptions are not performed; there are other possible regulatory mechanisms besides those proposed in the text.

Does the Drosophila ZFH1 gene play a role in EMT in the developing fly? Does it regulate E-cadherin?

I do not understand the justification for calling the red cells in Fig. 6 'micrometastasis'. To justify that name the authors would need to show that the cells arise in one tissue and then actively migrate out of it into another. The paper talks about metastatic growth but there is no evidence that the cells are growing following dissemination. Otherwise it is quite misleading. I am also not clear on what we are looking at in Fig. 6GH.

There is no assay for metastatic growth in any human cells or cell lines. The fly 'metastasis assay' cannot be considered a proxy to base claims about the vastly different process of true metastasis, as is implied throughout the paper and by the title.

If ZFH1 is a Notch target, why do the authors need to coexpress a CtBP-binding domain mutant in their assay? The discussion claims that overxpression of ZFH1 also induces 'massive tumor metastases', but the results contradict this.

p.15: what is the evidence or citation that JAG1 is at the top of the tumor metastasis cascade? The authors propose that Notch requirements in bone homeostasis may explain metastatic colonization of bone by prostate cancer, but obviously the body has many other tissues undergoing Notch signaling that are not colonized. In general the discussion contains too much speculation on the basis of quite thin connections.

Referee #3 (Remarks to the Author):

Using an eye-specific gain of function screen in Delta-overexpressing flies, Vallejo and colleagues identified a gene locus that encodes miR-8 as a supressor of the large eye phenotype. The location and type of effect indicated the miR-8 overexpression caused phenotypes characteristic of Ser/Jagged deficiency, which were restored by a Ser transgene and exacerbated by loss of a Ser allele. miR-8 was found to directly target the Ser 3'UTR, confirming the pathway. Hypothesising that the same pathway may apply in human cancers, the authors provide evidence that miR-200c, and less effectively, miR-141, directly target JAG1 in PC-3 prostate cancer cells, resulting in reduced Notch pathway activity and thereby affecting proliferation. JAG1-mediated Notch signaling is found to modestly upregulate ZEB1 and the implications for metasasis are discussed. In addition it is proposed that the 2 human miR-200 gene loci have opposite effects with respect to JAG1. The overall conclusion that miR-200c and 141 target JAG1 is adequately demonstrated but the lack of targeting by miR-200a, b or 429 is not supported by adequate data.

 

The paper has interesting findings but the parts relating to human miR-200 are poorly constructed, making it hard work for the reader to sort out what are valid general conclusions versus cell line-specific single observations. Figure legends in particular tend to lack sufficent experimental detail, or present it unclearly.

1. The finding that the miRs expressed from the miR-200c~141 locus have the opposite effect from miRs expressed from the miR-200b~429 locus is very unexpected because the respective miRs from each locus are very similar in sequence and share seed regions. In particular, miR-200a and 141 are identical from nt 1-16; miR-200c and miR-200b have identical seed regions and differ only at position 11 and 22. Consequently the targeting specificities between the two loci are predicted to be very similar. The conclusion made in this report that they are very different in their targeting of JAG1 needs to be supported by strong data. The targeting of JAG1 by miR-200c is adequately demonstrated, but the lack of targeting by miR-200a, b or 429 is not well supported and relies in part on a lack of effect in PC-3 cells of a pool of LNA anti-sense oligonucleotides against miR-200b, miR-200a and miR-429. This experiment needs a positive control to show adequate effectiveness of the transfection, and needs a comparison with LNA anti-sense oligonucleotides against miR-200c and -141. It is also difficult to know what to make of the second experiment indicating a difference in targeting by the two miR-200 genes (Fig 4E) where inhibition of the miR-200b cluster in PC3 cells leads to decreased JAG1 protein. LNA anti-miRs to miR-200a and miR-429 do not increase JAG1 at all on their own (if anything they increase it), yet they appear to enhance the decrease when included with anti-miR 200b. As far as one can tell, the western blots are single experiments, and there are no statistics given to indicate that the effects of LNA treatments are statistically significant. Further support for the difference in response to miR-200b versus miR-200c could come from comparing the effect of miR-200b to that of miR-200c in the transfection experiments in Fig 5.

2. The statement on p 11 that "miR-200c~141 inhibits cancer cell growth" is very broad for a conclusion drawn from a single type of assay with a single cell line. In fact a number of reports find either no effect of miR-200 on cancer cell proliferation in vitro (eg Dykxhoorn et al PloS ONE 2009, Cochrane et al Mol Cancer Ther 2009) or an increase in proliferation (eg Hyun et al Cell 2009, Yu et al Mol Cancer 2010). Thus the effects of miR-200 on cell growth are very cell dependent. This should be made clear here. Furthermore, the conclusion that the effect of miR-200c~141 inhibits cancer cell growth via its effect on Notch signaling should be supported by demonstration that restoration of JAGGED1 expression overcomes the inhibition of cell growth by miR-200c~141.

3. It is stated on p 10 that PC3 cells express high miR-200b, and on p 11 that "the mesenchymal-like phenotype of PC-3 cells is associated with high levels of the mir-200b~429 cluster". However, we are only shown the level of the mir-200b~429 cluster miRs in relation to the level in PNT1A cells. Is this really a high level, or is it low in both cells, but even lower in PC3 than PNT1A? The data are also presented in a way that allows no comparison between miR-200b and miR-200c in PC3 cells. The absolute levels of the miRs in PC-3 and PNTA1 cells should be shown in comparison to U6 snRNA, and should include a comparison to other mesenchymal and epithelial cells. Without this information it is very hard to consider possible explanations for the surprising contrary effects of miR-200b and miR-200c. A relevant experiment to test the claim that PC-3 cells are mesenchymal despite high functional levels of the mir-200b~429 cluster would be to examine whether they remain mesenchymal after transfection with the mir-200b~429 cluster.

Minor points

Fig 4a legend has insufficient information for easy understanding of the Fig. How was miR-200c/141 or 141 delivered?

P10 "re-expression of mir-200c~141 in PC-3 cells" implies the mir-200c~141 gene is being re-expressed, but in fact it is individual 200c and 141 expression vectors that are used. This should be reworded to make it clear that it is not the intact mir-200c~141 gene that has been transfected.

The highly similar sequences of the miR-200 members makes it possible that antisense oligonucleotides will not distinguish between them. What is the evidence that the LNA anti-miRs used do have specificity for the individual members?

 

2nd Revision - authors' response 12 November 2010

**Referee #1 (Remarks to the Author):**

Dominguez et al.

*1. Evidence is then presented that inhibition of JAGGED1 expression largely accounts for the phenotype of mir-200c and mir-141 overexpression in cultured PC3 cells (inhibition of cell proliferation and ZEB1 expression).*
**R** There is some confusion here, we did not mean to state that JAGGED1 mediates regulation of *ZEB1* by the microRNA mir-200c. Since the decrease in *ZEB1* mRNA levels upon downregulation of endogenous JAG1 by siRNAs or through pharmacological inhibition of NOTCH signaling by GSI is observed in the cells with low and high endogenous expression of the microRNAs *mir-200c* we meant to state that JAG1 via NOTCH signaling positively stimulates *ZEB1* transcription in these cancer cells. This feedforward loop between the two targets of the miR-200c microRNAs would be interconnected with the negative loop between JAG1-miR-200c and also with the double-negative loop between miR-200c-ZEB1 described in other cancer cell contexts (e.g. Bracken et al. 2008; Burk et al. 2008; Wellner et al, 2009). We have rewritten the text to clarify this point.

*2. ...At the very least, the authors should try to determine whether overexpression of JAGGED1 (with a different 3'UTR) can rescue the effect of mir-200c and mir-141 overexpression in PC3 cells.*
**R** We have added the requested experiment in new Figure 5B and Supplementary Figure S4. Transient cotransfection of PC-3 cells with a construct that contains JAG1 ORF without its endogenous 3' UTR (but not the construct of JAG1 containing the 3'UTR sequence) fully rescued cell proliferation inhibition by the microRNAs miR-200c and miR-141. Differences at 24 and 48 hour after transfection were statistically significant (P=0.0057 and P=0.0225), while there were not statistically significant differences between PC3-transduced with the microRNAs and JAG1 construct containing its 3'UTR and the PC-3 expressing only the microRNAs (P=0.7186 at 24 hours and P=0.5530 at 48 hours).

*3. The third finding is that the mir-200a, 200b, 429 group appears to be expressed in an opposite fashion and to antagonize mir-200c and mir-141 in a mysterious manner. If the authors wish to improve the relevance of this finding for human cancer, and to justify their model (Fig 7), they should compare the expression patterns of mir-200c and mir-141 versus mir-200a, 200b, 429, JAGGED1 and ZEB1 in cancer patient tissue samples.*
**R** We did not mean to state that miR-200a, -200b, and -429 antagonize miR-200c and miR-141. We meant that these microRNAs may target a repressor of JAG1 and therefore influenced JAG1 protein levels indirectly.

We have rephrased this section. We also offer a possible mechanism. It came to our notice that PC-3 cells show high PI3K signaling activity and that PI3K stimulates JAG1/NOTCH signaling in PC-3 cells (Wang et al, 2010a,b). Moreover, *FOG2*, a direct target of miR-200b, and a repressor of PI3K signaling is downregulated in prostate cancer (Hyun et al., 2009; citations therein), which would be in agreement with our findings of high miR-200b in the PC-3 cells. Thus, we speculate that miR-200b˜429 could stimulate JAG1 protein via FOG2-PI3K pathway.

Expression data on the microRNAs miR-200, NOTCH ligands, and ZEB1 have been published many times from many different human cancer tissue samples and and cell lines. We have investigated the relationship between miR-200, JAG1 and ZEB1 in cell lines that reflect well adavanced stages of the metastatic disease of prostate and colon cancers, and which mimic the changes of JAG1 and microRNAs expression reported in human samples of the corresponding metastatic diseases.

Particularly useful to this referee' s point are the 60 cell lines of the drug screening panel of human cancer cell lines at the National Cancer Institute (NCI60) represents a catalog of nine different cancers (http://dtp.nci.nih.gov). The group with mesenchymal gene signature shows a consistent relationship between loss of *mir-200c*, loss of E-cadherin and gain of mesenchymal markers ZEB1 and/or its related ZEB2. By contrast, expression of *mir-200b* varied among the group of

 

mesenchymal cells (e.g. see Park, 2009). For example, we noted high expression in the mesenchymal colon cancer cell line SW620 (NCI60 panel, Park et al, 2009) and this cell line has been reported to have JAG1 significantly elevated compared (Suwanjunee Anti-Cancer Drugs 2008). *mir-200b* is overexpressed in pancreatic cancers (Zhang et al, 2009) and ovarian cancer tissue samples, in both types of cancer high JAG1 expression levels and activity have been observed (e.g. Chen et al in Oncotarget (2010).

*4. Furthermore, it is completely unclear how this finding relates to the other results from Drosophila regarding mir-8 and Serrate.*
**R** In response to the Editor and referee's comments we have removed this section from the manuscript and will present elsewhere.

*5. The additional data that regulation of JAGGED1 may be important for the function of these miRNAs in human cancer remains speculative and the authors should resist the temptation to oversell this data.*
**R** We disagree with the referee's comment but have nonetheless carefully revised the text and removed any statement that could be considered an over-interpretation and presented the results in a totally neutral way.

Overall, our findings in *Drosophila* and human cancer cells, along with results from the independent study by the group of Thomas Brabletz strongly suggest that the regulatory circuit linking JAG1 to miR-200c microRNA and to ZEB1 are very likely to be relevant to human cancer.

Notes.

*The font size in many of the figures is too small to read and will be far too small once figures are shrunk for publication.*
**R** Ok, we have now increased the font, labeling and enlarged some images and histograms.

*Both title and abstract are, in my view, not an accurate description of the results contained within the paper. I would suggest a re-write along the following lines…*
**R** We thank the referee for his/her thoughtful comments and suggestions, which have helped us to improve our paper and the text. As suggested and given the excluding of the last part of the study we have changed the title, revised the abstract and Introduction. We have expanded the discussion in the terms suggested by this referee and state the data neutrally to avoid over-interpretations.

**Referee #2 (Remarks to the Author):**

*This work has two basic parts. …Part 2 is a much more flimsy and grasping attempt to argue that this is linked to human metastatic cancer through effects on ZFH1/ZEB1 and a relation to EMT. The evidence for this is a string of relatively weak and weakly related experiments using expression profiling and knockdown/overexpression in various cell culture lines and then a fly 'metastasis' assay.*
**R** The experiments in human cell lines follow up the logic of the experiments in the *Drosophila* part. We have restructured and revised the paper to provide a more cohesive story. We thank the referee for his/her comments and suggestions that revealed some weakness in the writing and/or arguments as well as the suggestion of experiments that helped to improve the manuscript.

*The title as well as the intro raise expectations about an important unanswered question in cancer biology concerning the transition from initial, often dormant micrometastasis into frank metastatic growth following dissemination. But these are not at all fulfilled by this work; they are a serious mismatch with the paper's data.*
**R** I agree with the referee that this is an outstanding question in cancer biology but disagree that our study is unrelated to this issue. There is a growing recognition that metastasis contain cells with properties reminiscent to normal stem cells. Reprogramming cells via the EMT confers normal and tumor cells with invasive capacity and a stem-like nature (Mani et al, 2008; Morels et al, 2008). Loss of microRNAs *mir-200* promotes EMT (29 references) and stemness (Shimono et al, 2009; Wellner et al, 2009) in mammals. Yet, the functional significance of these microRNAs in metastatic growth has remained unclear, given that some metastatic cancer cells re-express the microRNAs.

 

We demonstrate that, in *Drosophila* development and Notch-induced tumorigenesis, the conserved microRNA miR-8 targets Serrate and we functionally link this regulation to growth control. We also obtain functional and expression validation for a conserved miR-200c-JAG1 interaction in human cancer cells. We now demonstrate that miR-200c microRNA and JAG1 acting in a hierarchical order regulate cell proliferation of the human metastasis-prone prostate cancer cells, which concurrently express low levels of endogenous *mir-200c* and *mir-141* genes and high levels of JAG1 (this study).

Previous studies report significant differential expression of *mir-200c* (Vrba et al, 2010), *JAG1* (Santagata et al, 2004), and *ZEB1* (Graham et al, 2008) in normal human prostate tissues and in prostate carcinomas with high Gleason score. Our study links expression and function of JAG1 and the miR-200c microRNA in the regulation of the metastasis-prone prostate cancer cells *in vitro*. PC-3 mimic advanced stage of prostate cancer (please also see Points 2 and 4) and therefore these data along with the extensive analysis in *Drosophila* define links between previously unconnected regulatory factors that we believe relevant to key aspects of metastasis.

*The title and abstract also contain a number of inaccuracies (ZEB1 is not a target of Mir-200; instead it is a repressor Mir 200.*
**R** No, *ZEB1* is a target of miR-200c (>29 references, for example; (Adam et al, 2009; Baffa et al, 2009; Bracken et al, 2008; Burk et al, 2008; Christoffersen et al, 2007; Cochrane et al, 2009; Cui et al, 2008; Gregory et al, 2008; Hurteau et al, 2009; Hurteau et al, 2007; Hurteau et al, 2006; Iorio et al, 2007; Katoh et al, 2008; Korpal et al, 2008; Ladeiro et al, 2008; Li et al, 2009; Mees et al, 2009; Nakada et al, 2008; Nakajima et al, 2006; Olson et al, 2009; Park et al, 2008; Shimono et al, 2009; Taylor et al, 2008; Wang et al, 2007; Xi et al, 2006; Zhang et al, 2008.....) and the referee is correct that ZEB1 is as well a repressor of miR-200 (Bracken et al. 2008; Burk et al, 2008) and these reference are included in the text.

*Overexpression of ZFH1 causes cell arrest, not dissemination).*
**R** For the sake of consistency, this third part will be presented elsewhere.

Selected comments:

*1. Fig. 1: It is surprising that the data on tumors and metastasis, given the apparent focus of the paper, are only represented by a bar graph.*
**R** The bar graph represents a quantitative analysis of tumor inhibition by miR-8 but we could add a supplementary Figure with images of representative tumor eye discs and discs co-overexpressing *mir-8* with the oncogenes if the referee thinks it is necessary.

The phenotypes of disseminated eye tissue (metastases) of Notch and *eyeful* were described in detail previously (Ferres-Marco et al, *Nature* **439**; 430-6, 2006). In brief, the overexpression of the Notch ligand *Delta*, which alone caused a 'enlarged eye' phenotype, coupled to the GS line *eyeful* that enforced expression of two epigenetic repressors named *pipsqueak* and *lola*, produces massive eye tumors (as reflected in the bar histogram) and spreading of tumor cells throughout the body (<30% de the animal exhibit secondary eye growths, Ferres-Marco et al, 2006). The co-overexpression of *mir-8* with these oncogenes (*ey-gal4>UAS-Dl>eyeful/GS(2)SC1*) completely arrested eye tumor growth (as shown in the bar graph) and there were a conspicuous absence of metastatic phenotype in these larvae—no adult flies developed from this combination.

*2. p. 9 ....These experiments are unsatisfying as they are based on quantitation of ECL-developed Western blots, which of course has a much more narrow dynamic range and is much less reliable than the qPCR experiments with which they are compared. Also there is no documentation of how many replicate experiments were performed for each, and no error bars or measures of statistical significance.*
**R** We strongly agree with the referee on the limitations of Western blot analyses but would like to argue that qPCR analyses of mRNA expression would not be taken as a surrogate for protein activity level, given the importance of post-transcriptional regulations including that by microRNAs. However, we are making a qualitative not quantitative argument that JAG1 protein levels and *mir-200c* expression show inverse correlation in PC-3 cells. New Figure 4A shows a representative immunoblot of JAG1 to also compare expression in PC-3 and expression in non-tumorigenic PNT1A (see below). Changes in expression of JAG1 and *miR-200c* in these two cell lines reflect the

reported changes in expression of JAG1 and the microRNAs in normal prostate tissue samples *vs* advanced prostate cancer tissue samples (Santagata et al. 2004; Vrba et al, 2010).

The referee's second point is on the information of how many replicate experiments were done. We have included this information in the figure legends. In particular, data below the blots represent mean values from three independent experiments and in the revised version of the manuscript this is more explicit and a histogram has been added in new Figure 4E. All bar graphs show error bars in the original manuscript but because some error bars are very small they might not be easy to view. In the revised version, in such cases we show values above the bar. Additionally, we have included more statistical analyses throughout the paper.

*3. p.10. The authors do not test whether the growth defect can be bypassed for instance by providing a JAG1 expression vector that is insensitive to Mir-200c control. Such an experiment is critical to the case that 'inhibition of tumor growth by miR-200c could be largely attributed to JAG1'.*
**R** The reviewer is absolutely correct here. This is key piece of data that complement the *in vivo* analyses in *Drosophila* (Figures 1-3). In this revised version we present the rescue experiment that confirm the proposed model. New Figure 5B (see also controls in Supplementary Figure S4) shows the time course of growth of PC-3 cells overexpressing *miR-200c* and *miR-141* alone or in combination with either a construct of *JAG1* expressing vector lacking endogenous *JAG1* 3' UTR (3'UTR—) or a construct containing the *JAG1* 3' UTR (3'UTR +), thus yielding the expression of *JAG1* sensitive to regulation by the microRNAs.

The rescue experiment is described in pages 11 and 12 in the revised manuscript. Thus, we first verified that transient transfection with the *miR-200c* and *miR-141* vectors inhibited cell proliferation of PC-3 cells as we observed with the stably transfected PC-3 cells (Figure 5A). Next, we verified that mature miR-200c and miR-141 were expressed and that they provoked JAG1 downregulation in the transient expression assays (new Supplementary Figure S4). Finally, in the new Figure 5B, we show that expressing *JAG1* ORF without its 3' UTR completely rescued cell proliferation of PC-3 cells expressing the microRNAs (compared growth of the parental PC-3 cells transfected with empty vectors (blue line) with the PC-3 cells expressing the microRNAs and *JAG1* (3'UTR—) (green line). By contrast, PC-3 cells transfected with the construct expressing *JAG1* with its endogenous 3'UTR did not rescue growth inhibition by the microRNAs (orange line).

*4. In several areas there is an implied argument that PNT1A and PC-3 are comparable cell lines allowing valid comparison and investigation of the metastatic character of the latter. For instance, the authors speak on p.10 of 'restoring' expression of Mir200c to PC-3 cells; what they are really doing is overexpressing Mir200c in these cells. The authors provide no citation or evidence to suggest that the two cells lines are not vastly different genomically.*
**R** This is an important remark and two references, Mitchell et al. (2000) and Van Bokhoven et al (2003), relevant to this point have been included in the Material and Method section in the revised manuscript. The highly metastatic PC-3 cells and the immortalized non-tumorigenic prostatic epithelial cell line PNT1A constitutes different types and stages of prostate cancer and are both widely used to study the behavior and molecular mechanisms of human prostate cancer cells. The cell lines are genomically and phenotypically characterized (Van Bokhoven et al 2003) and there is no evidence of generalized microsatellite instability in these cell-lines (Mitchell et al. 2000).

*5. Fig. 5 consists largely of expression profiling in different cell lines which provides little direct evidence. Manipulative experiments to bear out the assumptions are not performed; there are other possible regulatory mechanisms besides those proposed in the text.*
**R** The referee needs to re-inspect old Figure 5 (now new Figures 5 and 6) and old Supplementary Figure S4. New Figures 5 and 6 and supplementary Figures S3-6 present manipulative experiments and differences in cell proliferation, and expression of the gene shown, are statistically significant. The rescue experiments of growth inhibition by the microRNAs by JAG1 without its 3'UTR (new Figure 5B) strengthened our arguments. More statistical analyses have been included in new Figures 5 and 6 as well as Figure 1-4, and have added experiments, which we believe strengthen our original conclusions and emphasizes the usefulness of empirical manipulation in *Drosophila* to identify true conserved mechanisms. If we were making claims based on the human cancer cell data, then we would agree that the arguments are weak. However, we are making a deep analysis in *Drosophila* and in addition present a comparative analysis in a human cancer cell line relevant to the topic in this study. We have validated conserved function and regulation in this cancer cell line and highlight

 

commonalities and differences with other miR-200 family members.

We have also revised the text to present the conclusions in a more neutral way and expanded the discussion to other possible interpretations.

*6. I do not understand the justification for calling the red cells in Fig. 6 'micrometastasis'. To justify that name the authors would need to show that the cells arise in one tissue and then actively migrate out of it into another.*
**R** This third part of the original manuscript will be presented elsewhere. Nonetheless, the eyes of *Drosophila melanogaster* are the only tissue that express the pteridine and ommochrome screening pigments, which are deposited as granules in the photoreceptor cells and the pigment cells of the retina, giving the characteristic red eye color.

The presence of red (eye) cells away from the primary site (head) to distant body parts (thorax, abdomen, etc) is evidence of metastatic spread of tumor cells. Sections in old Figure 6GH showed that eye tumor cells invade and grew within the intestine, muscle, and other internal organs in the fly abdomen. These data will be presented in detail elsewhere.

*7. The paper talks about metastatic growth but there is no evidence that the cells are growing following dissemination. Otherwise it is quite misleading. I am also not clear on what we are looking at in Fig. 6GH. There is no assay for metastatic growth in any human cells or cell lines. The fly 'metastasis assay' cannot be considered a proxy to base claims about the vastly different process of true metastasis, as is implied throughout the paper and by the title.*
**R** We do not want to argue comments on the usefulness or not of *Drosophila vs* other experimental metastasis assays, they are beyond the scope of these discussions here because as suggested by the Editor and referee #1 we will present these data in a separate paper. We have greatly expanded the experiments on fly ZEB1 and we still stand by our conclusions that these studies in *Drosophila* have much to offer to the model of tumor metastasis.

*8. p.15: what is the evidence or citation that JAG1 is at the top of the tumor metastasis cascade?*
**R** NOTCH, WNTs, and HEDGEHOG pathways trigger epithelial-mesenchymal transition through regulation of transcriptional repressors of the SNAIL, ZEB families and TWIST during normal development and in various carcinoma cell lines. JAG1 is a ligand of several NOTCH receptors implicated in cancer metastasis (J Cell Biochem. 2007 Nov 1;102(4):829-39. Cancer metastasis facilitated by developmental pathways: Sonic hedgehog, Notch, and bone morphogenic proteins. Bailey JM, Singh PK, Hollingsworth MA. and Acta Biochim Biophys Sin (Shanghai). 2008 Jul;40(7):643-50. New insights of epithelial-mesenchymal transition in cancer metastasis. Wu Y, Zhou BP).

*10. The authors propose that Notch requirements in bone homeostasis may explain metastatic colonization of bone by prostate cancer, but obviously the body has many other tissues undergoing Notch signaling that are not colonized.*
**R** This of course is to be expected if the vicious circle would depend solely on JAG1 (tumor)-NOTCH (bone) signaling. We have expanded the text to emphasize that the two critical signals, TGF-beta and PDGF, released by bone metastasis are both potent inhibitors of *mir-200c* expression in tumor cells (Gregory et al. 2007; Kong et al., 2009). Moreover, inactivation of RANKL by monoclonal antibodies is being investigated in multiple clinical trials for the prevention and treatment of bone metastases. RANKL is known to induce expression of JAG1

*11. In general the discussion contains too much speculation on the basis of quite thin connections.*
**R** We had presented the data in a complete neutral way and toned down the implications of the data.

**Referee#3 (Remarks to the Author):**

*The overall conclusion that miR-200c and 141 target JAG1 is adequately demonstrated but the lack of targeting by miR-200a, b or 429 is not supported by adequate data.*

*The paper has interesting findings but the parts relating to human miR-200 are poorly constructed, making it hard work for the reader to sort out what are valid general conclusions versus cell line-*

 

*specific single observations. Figure legends in particular tend to lack sufficient experimental detail, or present it unclearly.*
**R** We have substantially improved text of figure legends and revised the text to make it more cohesive and easy to read.

*1. The finding that the miRs expressed from the miR-200c~141 locus have the opposite effect from miRs expressed from the miR-200b~429 locus is very unexpected because the respective miRs from each locus are very similar in sequence and share seed regions. In particular, miR-200a and 141 are identical from nt 1-16; miR-200c and miR-200b have identical seed regions and differ only at position 11 and 22. Consequently the targeting specificities between the two loci are predicted to be very similar.*
**R** Several recent papers report target specificity for microRNAs family members with common seed sequences, and show that regions outside the seed play a key role in regulation of the activity of particular microRNA family members. For instance, specific nucleotides in the precursor (pre-miRNA) of miR- 181a-1 are essential for its specific function (Liu et al. 2008, PLoS-One). The seed sequence is conserved between miR-200c and miR-200b, but their pre-miRNA sequences are divergent.

*2. the lack of targeting by miR-200a, b or 429 is not well supported and relies in part on a lack of effect in PC-3 cells of a pool of LNA anti-sense oligonucleotides against miR-200b, miR-200a and miR-429 This experiment needs a positive control to show adequate effectiveness of the transfection, and needs a comparison with LNA anti-sense oligonucleotides against miR-200c and -141.*
**R** Specificity of LNAs is well established. Nonetheless, we understand the concern of the referee, as we were also very surprised by the concurrent high JAG1 and high miR-200b in PC-3 cells. The results of LNAs on JAG1 protein levels were also unanticipated, but they are absolutely reproducible. As a confirmation of the efficacy of these LNAs please see *ZEB2* positive control (new Supplementary Figure S3D) and discussion below. We also speculate that miR-200b might augment JAG1 indirectly by permitting high PI3K signaling activation in prostate cancer cells. *FOG2*, which represses PI3K and is directly repressed by miR-200b, is downregulated in prostate cancers (Hyun et al, 2009; references therein). Wang et al (2010a, b) report high PI3K signaling and PI·K-mediated stimulation of JAG1/NOTCH signaling by PI3K in PC-3 cells.

*3. It is also difficult to know what to make of the second experiment indicating a difference in targeting by the two miR-200 genes (Fig 4E) where inhibition of the miR-200b cluster in PC3 cells leads to decreased JAG1 protein. LNA anti-miRs to miR-200a and miR-429 do not increase JAG1 at all on their own (if anything they increase it), yet they appear to enhance the decrease when included with anti-miR 200b. As far as one can tell, the western blots are single experiments, and there are no statistics given to indicate that the effects of LNA treatments are statistically significant.*
**R** We apologize for not bringing up the information in the figure legends in our previous manuscript. All experiments are repeated at least three times, but in a few instances an illustrative immunoblot was shown. In the revised manuscript this is more explicit and we also added mean +/- SEM, included histogram of the data obtained from three independent experiments using the LNAs anti-miR-200b, -a, and -429 (new Figure 4E**)** and added more statistical analyses.

We confirmed the efficiency of transfection and the efficacy of the LNAs by assessing the expression of a 'positive' control, *ZEB2* (also called *SIP1*), which contains bindings sites for both miR-200b and miR-200c and has been validated in other cancer cell contexts (e.g. Christoffersen et al, 2007; Hurteau et al, 2008; Park et al, 2008). In the revised version, we show that *ZEB2* expression increased in the presence of the LNAs anti-miR-200b, a, and 429 (new Supplementary Figure S3D).

*4. The statement on p 11 that "miR-200c~141 inhibits cancer cell growth" is very broad for a conclusion drawn from a single type of assay with a single cell line. In fact a number of reports find either no effect of miR-200 on cancer cell proliferation in vitro (eg Dykxhoorn et al PloS ONE 2009, Cochrane et al Mol Cancer Ther 2009) or an increase in proliferation (eg Hyun et al Cell 2009, Yu et al Mol Cancer 2010). Thus the effects of miR-200 on cell growth are very cell dependent. This should be made clear here. Furthermore, the conclusion that the effect of miR-200c~141 inhibits cancer cell growth via its effect on Notch signaling should be supported by demonstration that restoration of JAGGED1 expression overcomes the inhibition of*

 

*cell growth by miR-200c~141.*
**R** We completely agree with the referee that the role of microRNAs mir-200 in cancer cell growth control is not fully clarified, as we pointed out in the Introduction.

Although Cochrane et al, *Mol Cancer Ther* 2009 suggest no effect of mir-200 in cell proliferation, in a recent paper (Cochrane et al, *J Oncol* 2010) show that overexpression of *mir-200c* in the Hey and Hec50 cells decreases cell proliferation. Hyun et al, 2009 show positive stimulation of growth by *mir-200b*, *mir-200a*, and *mir-141* overexpression, but do not show data on *mir-200c* in their Supplementary Figure S1. Others have report recently significant elevated expression of *mir-200b* and *mir-200a* in pancreatic cancer tissue samples compared with normal pancreatic tissue and positive stimulation of growth by miR-200b in this cancer cell type (Li et al, 2010). Yu et al, *Mol Cancer* 2010 report that exogenous overexpression of *mir-200c* in pancreatic cancer cells PANC-1 enhances cell proliferation. These findings are surprising given the key role of NOTCH signaling in pancreatic cancer (e.g review by Mysliwiec and Boucher, *Adv Med Sci*. 2009; Targeting Notch signaling in pancreatic cancer patients--rationale for new therapy; 54(2):136-42).

In our revised manuscript we present our data in a more neutral way. Nevertheless, the results in *Drosophila* and human PC-3 cells point to a conserved growth suppression function of miR-8 and 200c through inhibition of Notch signaling, which is consistent with the reported stemness-inhibitory function of miR-200c in normal and cancer stem cells (Shimono et al, 2009; Wellner et al, 2009). Our paper also might hint to how different miR-200 family members may influence growth in opposing ways.

In addition, in the new revised version, we have stated clearly that experiments referred to PC-3 cells. We have rephrased sentence in page 11 (original manuscript) to “Together these data suggest that, like its *Drosophila* counterpart, human miR-200c~141 inhibits cell growth of PC-3 cells at large extent by restricting ligand-mediated activation of NOTCH signaling” [On page 12 in the revised version of the manuscript].

Regarding the absence of effect of mir-200c overexpression in murine breast cancer cells (Dykxhoorn et al PloS ONE 2009), we speculate that a mechanism similar to that in HCT-116 cells may operate in metastatic murine breast cancer cells that re-gain epithelial status.

*5. It is stated on p 10 that PC3 cells express high miR-200b, and on p 11 that "the mesenchymal-like phenotype of PC-3 cells is associated with high levels of the mir-200b~429 cluster"...*
**R** This is an interesting point that needed further clarification (see page 12, in the revised manuscript). Although most previously reports emphasize the redundant and cooperative roles of miR-200 microRNAs in MET, we noted that expression of *mir-200c~141*, but not that of the microRNAs of the *mir-200b~429* cluster, reflects a more consistent relationship between mesenchymal state, loss of *Ecadherin,* and gain of *Vimentin*, among the NCI60 panel (http://dtp.nci.nih.gov). Thus, while the subgroup of NCI60 cell lines previously classified as mesenchymal state have little or undetectable expression of *mir-200c~141*, the expression of the *mir-200b~429* cluster varied among the group of mesenchymal cell lines (e.g. see Park et al, 2008). These observations do not diminish an important role of miR-200b in MET, but it underscores the non- complete functional redundancy between the miR-200c and mi-200b microRNAs.

Minor point

*Fig 4a legend has insufficient information for easy understanding of the Fig. How was miR-200c/141 or 141 delivered?*
**R** We apologize for this. The revised version of the manuscript expanded the information in figure legends. In all experiments, except in new Figure 5B and Supplementary Figure S4, PC-3 are stably transfected with the mirVec-vectors expressing *mir-200c* and *mir-141* precursor sequences (Supplementary Material and Methods).

*P10 "re-expression of mir-200c~141 in PC-3 cells" implies the mir-200c~141 gene is being re-expressed, but in fact it is individual 200c and 141 expression vectors that are used. This should be reworded to make it clear that it is not the intact mir-200c~141 gene that has been transfected.*
**R** The referee is absolutely right that we have made a misused of the word “re-expression”. We have changed this in the text and figure legends to avoid misunderstandings.

 

*The highly similar sequences of the miR-200 members makes it possible that antisense oligonucleotides will not distinguish between them. What is the evidence that the LNA anti-miRs used do have specificity for the individual members?*
**R** LNAs are highly specifics and widely used. Please see also comments in Points 2 and 3.

2nd Editorial Decision 26 November 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-75232. It has now been seen again by referee 1; the other referees were unfortunately unavailable to look at the revised manuscript. As you will see, referee 1 finds the manuscript to be significantly improved over the previous version, and is fully supportive of publication. However, he/she still finds that the abstract and introduction do not accurately reflect the content of the paper, and therefore feels that further revision of the text would be important before eventual acceptance. While I do recognise that your study does have implications for cancer and metastasis, and understand that you want to discuss this, I also agree with this referee that the results presented do not directly address this, and would therefore ask you to go through and revise the manuscript once more with this referee's comments in mind.

REFEREE REPORT

Referee 1:

Dominguez et al.
My original assessment of this manuscript was as follows:

"In summary, the finding that this miRNA-target gene interaction is one of the few that may be conserved across divergent species is an interesting one and is significant enough to warrant publication in EMBO Journal. The additional data that regulation of JAGGED1 may be important for the function of these miRNAs in human cancer remains speculative and the authors should resist the temptation to oversell this data."

The new manuscript has been improved by the addition of new data to firm up the fact that inhibition of JAGGED1 by mir200c/141 contributes to the anti-proliferative effect of these microRNAs in the cultured PC-3 cell line. Thus, JAGGED1 is a functionally important target in this cell line.

There is no data in this manuscript that deals with human cancer tissue samples or mouse models of cancer metastasis. So, it remains a problem that the authors spend large parts of the text discussing human cancer metastasis and the potential relevance of their results to it. Instead, the authors should resist the temptation to oversell their data in this way and focus on what their data actually show: that the mir8/200c/141 miRNA family regulates expression of the Notch ligands Serrate/JAGGED1.

For example, in the abstract:

"Metastatic growth, the main cause of cancer mortality, remains poorly understood. Recent studies show that reprogramming cells via the epithelial-mesenchymal transition (EMT) confers them with invasive capacity and a stem-like nature. Loss of microRNAs mir-200 promotes both EMT and stemness in mammals, but how these processes are mechanistically couple is unclear."

The authors do not address any of these questions in their paper. They do not show that regulation of JAGGED1 by mir200c/141 contributes to its ability to inhibit EMT and stemness in cancer cells in culture, let alone in metastatic growths in vivo. What this authors show is that regulation of JAGGED1 by mir200c/141 does occur and contributes to its ability to inhibit cell proliferation in PC-3 cultured cells. Since mir200c/141 regulates ZEB1 and Bmi1, it may be that JAGGED1 is not an important target for mir200c/141 activity in vivo - so the authors should not build up the reader's expectation that it might be.

 

Similarly, the first three paragraphs of the introduction deal mainly with metastasis. This should be removed and replaced with an explanation of Notch ligands and the need for careful regulation of their expression in many contexts, possibly including cancer. Any extensive speculation about the possible importance of JAGGED1 being regulated by mir200c/141 in human cancer metastasis should occur only in the discussion section.

Thus, the manuscript is only acceptable following a satisfactory revision of the text.

Additional Correspondence 3 December 2010

Following our discussion on Wednesday, I have now had the chance to read your manuscript carefully once more, and would like to give you some more input on how I think it needs to be revised for publication. I am returning the manuscript to you for a formal revision - to allow you to make the change to figure 1 that we discussed: namely, including a graph showing the effects of miR-8 overexpression on metastasis in the Delta-overexpressing fly.

In terms of the abstract, my primary objection is that the initial part implies that you will be addressing the question of how miR200 regulates stemness, whereas in fact what you are looking at is proliferation. While I agree that your study does bring together previous work on this miRNA in regulating EMT with the identified function here in proliferation, I would ask that you modify the abstract to remove the suggestion that you are addressing questions of stemness in metastatic cancers.

I do share the referee's concerns about the over-emphasis on metastasis in human cancers in the introduction. While I understand your frustration that you were asked by the referees to remove most of the data dealing with metastasis in flies, this means that your study is now less concerned with the implications for metastasis than with how the identified regulatory network impacts on proliferation (albeit in metastatic cancer cell lines, but this does not necessarily imply a specific role in re-initiation of proliferation after dissemination). I therefore think that significant parts of the introduction - particularly the first paragraph - do not belong in the introduction, but should be confined to the discussion where you speculate on how the miR200-Jag-ZEB axis impacts on metastasis. Of course, a brief introduction to metastasis is fine - particularly in the context of how miR200 is already known to regulate EMT - but it is too lengthy at this point. I would instead encourage you to extend your introduction on the role of the Notch pathway in cancer - this is currently very brief (two sentences) but is after all the starting point for your study, and should be introduced in more detail. In this section, you also jump very rapidly from what is known from the existing literature to what you show in this study, and the transition is not immediately obvious.

Please take these comments on board when revising your manuscript: I do agree that your study has potentially important implications for how a metastasis actually becomes established, but this is speculative - at least based on the data that will be presented in the final paper - and it needs to be clear to the readers that this is so.

I hope this message clarifies the changes that need to be made for your study to be accepted here: we are definitely positive about publication, but it is important first that the text accurately reflects the data and scope of the study.

Additional Correspondence 14 December 2010

Thank you for your email on Friday 3rd December. I appreciate your comments to our revised manuscript and am happy to see that you are positive about publication in EMBO Journal.

As I said I very much appreciate your comments and by following them up I believed the manuscript has been improved and also strengthens the conclusions. I removed the two paragraphs

 

in the introduction and make changes in the discussion which like the introduction is now more focused.
In this revised manuscript I have been removed the allusion to stemness in conflicting sentences. The findings might be relevant to stem cell regulation but I agree with your that the paper deals with cell proliferation control. I apologize for this, it was not intended this implied argument.
The data on tumor metastasis suppression by miR-8 that was only presented in Figure 1 legend has been moved to the main text and Figure 1 itself has been improved substantially, and I do not see why I did not plan this figure in this way in the first time.
Referee #3 stated in his/her report that ŒIt is surprising that the data on tumors and metastasis, given the apparent focus of the paper, are only represented by a bar graph¹.
We are now presented the qualitative and quantitative data on miR-8 in tumor and metastasis suppresssion.
Figure 1B-C has been substituted with B and C (eye tumor and metastasis in the Drosophila cancer paradigm Œeyeful¹ used in the genetic screen and the suppression of tumor metastasis by the miR-8 in the GS(1)SC1 line) Figure 1D now shows examples of the representative eye tissue (wild type control) eye tumor and the eye tumor suppression by miR-8 microRNA and the graph.
The data on Delta overgrowth and its suppression by miR-8 are now presented as data not shown.
I have to apologize that there were an error in Figure 1 legend, Œeyeful¹ flies show >15% metastasis, not >25% as it was in the figure legend. The overexpression of miR-8 completely inhibited metastasis (0 metastasis in n=100 animals)

Finally, I therefore think that the part of the tumor metastasis on Notch and Zfh1 would be presented in this study and not elsewhere. Thomas Brabletz is an expert on zeb1 and he thinks the data are crucial and highlight the significance of co-regulation of the two targets of the microRNA. I think Referee#1 also acknowledge the data are valuable but did not see the connection with the other parts because these results were poorly communicated by me and they were highly speculative the significance. I suggest to present plainly and only the relevant data with Zfh1.

I therefore pasted below the abstract and the introduction (it needs so more work), the part on Notch and Zfh1 tumor metastasis would be very brief and only marginally mentioned in the Discussion.

Abstract

Notch signaling is crucial for the correct development and growth of
numerous organs and tissues and when subverted it can cause cancer. Loss of miR-8/200 microRNAs is commonly observed in advanced tumors and correlates with their invasion and acquisition of stem-like properties. Here we show that this microRNA family controls Notch signaling activation in Drosophila and human cells. In an overexpression screen, we identified the Drosophila miR-8 as a potent inhibitor of Notch-induced overgrowth and tumor metastasis. Gain and loss of mir-8 provoked developmental defects reminiscent of impaired Notch signaling and we demonstrated that miR-8 directly inhibits Notch ligand Serrate. Likewise, miR-200c and miR-141 directly inhibited JAGGED1, impeding proliferation of human metastatic prostate cancer cells. It has been suggested that JAGGED1 may also be important for metastases. Although in metastatic cancer cells, JAGGED1 modestly regulated ZEB1, a key miR-200c¹s target in invasion, studies in Drosophila revealed that only concurrent overexpression of Ser and Zfh1/ZEB1 induced formation of metastases. Together these data define a new way to attenuate or boost Notch signaling that may have clinical interest.

The data on Zfh1 and Notch would be presented as follow

Concurrent overexpression of Zfh1/ZEB1 with Notch and tumor metastasis in Drosophila

Our data together with previous studies suggest that the loss of mir-200c microRNA may facilitate the formation of metastases via the pleiotropic action on growth-promoting and EMT-inducing target genes that include JAG1 and ZEB1. Alternatively, and as our data suggest, miR-200 may serve as a node in a network by targeting ZEB1 and an upstream regulator JAG1, thereby facilitating enhancement of the Notch-ZEB1 axis to foster proliferation and invasion. The Notch-Zeb1 axis may be conserved in Drosophila, which encodes a fly homolog of ZEB1, the Zinc finger homeodomain 1 gene (zfh1) (Postigo et al, 1999) that is a direct target of Notch signalling (Krejci et al, 2009) and a predicted target of miR-8 (http://www.flyrnai.org/cgi-bin/DRSC_MinoTar.pl), although this

 

remains to be validated. By analogy to ZEB1, zfh1 regulates myogenic differentiation, cell migration and somatic stem cell self-renewal (e.g. Broihier et al, 1998; Leatherman and Dinardo, 2008; Postigo, 2003). As previous shown aberrant activation of Notch pathway via its ligand Delta and at less extent Ser (Dominguez et al, 2004) causes eye growth without dissemination (i.e. benign growth). Hence, we assayed whether the concurrent overexpression of Ser (or Delta) with Zfh1/ZEB1 was capable to initiate tumor metastasis.

By co-expressing Ser with zfh1 flies with distant metastases were seen in 10,41 % (5/48), the secondary eye-derived outgrowth were 3-5 time bigger than the micrometastasis found in flies only expressing zfh1 (2/82 flies showed micrometastasis and all animals showed inhibition of endogenous eye growth; data not shown). Massive metastases were also found when the Notch ligand Dl was co-expressed with zfh1 (57,77% of flies with distant macrometastases n=87; Figure 6F and G), resulting in 100% late pupa lethality.

I would really appreciate your feedback (if you have any question please do not hesitate to contact me) before I submit a revised manuscript with the details of the modification (and with or without the tumor metastasis, hopefully with) and hopefully to have a final decision on it so we can all move forward.

Acceptance letter 20 December 2010

Many thanks for submitting this final version of your manuscript EMBOJ-2010-75232. I have now had the chance to read through the modified text and I find it to be much improved - thanks for all the effort you put in to the re-write!
I'm therefore pleased to be able to tell you that we can now accept the manuscript for publication in the EMBO Journal - you should receive the formal acceptance email shortly.

We will be scheduling your paper for back-to-back publication with Thomas Brabletz's,
and I think the two complement each other really well.

 