Reviewer Comments 22 June, 2015

**Reviewer 1**

This manuscript from McLennan et al. describes a new model for how VEGF signaling regulates NCC guidance using extensive transcriptional profiling, computational modelling and functional experiments in the chick embryo. By performing qRT-PCR profiling using a previously characterized set of markers to define cellular phenotypes, the authors show that VEGF induces lead character in NCCs in vivo, and in so doing serves as a chemoattractant to "lead cells", or to "trailer cells" that are confronted with ectopic VEGF. Whereas the authors have previously shown that knockdown of VEGF leads to defects in NCC migration, they now show that trailing NCC migration does not depend on VEGF. Interestingly, though NCCs cultured in vitro do not exhibit lead/trailer identities, the addition of VEGF can endow a partial lead character in this situation. The authors also show that trailer NCCs are competent to change their molecular profile to one more similar to a lead profile in correlation with their chemotaxis toward a VEGF source. These findings allow the authors to make modifications to their mathematical model that help to define how lead and trailer cell identities lead to their migratory behavior within a NC stream. The experiments in this manuscript are well-conceived and well-executed and contribute to an advance in our understanding of cell migratory control that will be of significant broad interest. There are some concerns that could be addressed to improve this manuscript.

1-1) One major concern is why the results of Np1-Fc inhibition of VEGF did not affect lead NCC profile, whereas Np1 siRNA did. Although the authors point out that "knockdown of VEGF signaling by distinct methods altered the expression profile in lead neural crest cells in distinct ways suggesting that there are different cell responses to loss of VEGF signaling" there is no explanation offered for why this might be the case. If one method Np1 siRNA has off-target effects, or Np1-Fc is incompletely inhibiting, it is important to know which one represents true loss of VEGF as this is central to the model. This discrepancy needs to be addressed, possibly by controls in embryos or cells that determine which of these is the more reliable method for blocking VEGF signaling.

*An obvious difference is that Np1-Fc represents sequestration of the ligand, while Np1-siRNA actively perturbs expression of the receptor. The possibility that Np1-Fc doesn’t completely remove VEGF could be a valid point? One would expect differences in the two perturbations if Np1 has uses other than serving as a receptor for VEGF (which would be affected by the siRNA but not the Fc)*

1-2)  In experiments related to Fig. 3, it is surprising to me that the authors observe transcriptional decreases so rapidly (within 4 minutes of withdrawal of VEGF).  What are the typical mRNA half-lives for a few of these transcripts? 4 minutes seems to be a very short time to see decreases in transcript in response to withdrawal of a signal. On a related note, are the decay rates for the mRNAs analyzed sufficiently short that the 90 minute withdrawal was sufficient to return cells to a truly VEGF-free gene expression profile before reinduction of VEGF at 90 minutes? If the gene expression profile at 90 minutes does not really reflect a VEGF-free state, then post 90 minute induction might also represent blunted transcriptional changes compared to what would be expected from a truly VEGF naïve cell. The authors should discuss these issues.  
*I have encountered the concern about short decay times before at a poster session. I think these are all valid points. Can we look up decay rate estimates in the literature? We could mention cell-behavioral evidence that shows response to VEGF within 60 minutes (I seem to remember Bec mentioning this).*

1-3) In experiments associated with Figure 9, it is interesting that knockdown/inhibition of VEGF does not have an indirect effect on the trailer NCC gene expression profile. Since Np1 siRNA leads to large effects on the ability of all NCCs to reach the second branchial arch, trailers should be indirectly affected by the loss of lead cells. In other words, how do the authors think that loss of leader identity with loss of VEGF signaling results in defective trailer migration? Are these changes in behavior not related to transcriptional profile?   
*I think the reviewer misunderstood. There is an effect of global kd/inh of VEGF on the trailer profile, but no effect on migration when targeting this to the trailing portion only (Figure 8).*

1-4) It would be of interest to include the data mentioned regarding filopodial protrusions towards ectopic VEGF described on Page 17. Do the filopodia extending toward the adjacent VEGF source make and break contact? This is particularly interesting since most trailer cells are still somewhat confined to their streams.

*Do we have the data to show?*

1-5) If lead/trailer cell phenotypes are not required in vitro, how does directional cell migration away from the neural tube explant occur?

*Do we know if the cells migrate as far in the same time in vitro as in vivo?*

*We should state what the scale-bar in figure 1A is. I could also provide a simulation of undirected migration, if we think that is needed.*

***Suggested response****: Migration from the neural tube does not have to be directed, but could simply result from undirected dispersal. It is important to note that the substrate is substantially in vitro and in vivo, and migration may occur at different speeds as a result. The focus of this paper was to investigate the effect of the microenvironment on neural crest cell migration, hence we think the in vitro migratory behavior, which is not representative of migration in the embryo, is beyond the scope of the current work.*

1-6) There are a few instances where complex transcriptional analysis is not explained as precisely as it could be, and in general this data was very difficult to deduce. I would encourage the authors to look carefully at their text describing transcriptional changes to make sure that it is as clear and precise as possible. For example: Text on page 12 "there were 11/58 genes that were upregulated in the lead for both in vitro and in vivo" is confusing since both data sets exhibit differences in 11/58 genes. Maybe instead of "both" use the word "each" or "For each in vivo and in vitro, there were 11/58 genes upregulated in the lead, but only one that was common…"

1-7) On page 13, text reads "…induced significant expression of 14 genes examined (Fig2D). Of these, 9/14 were up- and 5/14 were down-regulated compared to in vitro lead (distal) neural crest cells not exposed to VEGF(Fig 2D)"  Are these 14 genes "induced" in vitro, or are 9 induced and 5 suppressed? Also, whereas text above states "compared to in vitro lead (distal)" Fig2D shows relative to "in-vivo distal" Please clarify.

1-8) In Figure 3B, what are gene expression levels relative to? Presumably, these were normalized to some timepoint, but it is difficult for me to see what timepoint that might be.

*These are just relative to the normalization of “housekeeping genes”. I was working off the data I got from Jason in “intergrate & switch for Linus.xlsx”. Feel free to label the axes differently (in whatever the correct units of the expression data are). I did not normalize the data further, so the data are not normalized to any time-point.*

***Suggested response:*** *We did not normalize the RTqPCR expression data to the baseline (t=-120 minutes) or t=0 time-point, as we felt that this would distort the data for those genes that start out a low expression, and for which the normalized values would hence be artificially large.*

**Reviewer 2**

In this paper, McLennan et al. continue their analysis of subpopulations within migrating chick cranial neural crest. Here, they revisit their identification of VEGF as a neural crest chemoattractant to evaluate the impact of this cue on lead/trail identity as a test of the hypothesis that signals in the microenvironment trigger the distinct molecular signatures of leading and trailing neural crest cells. There is a lot in this paper, and it represents an interesting synthesis of their previous work. However, it is disappointing that there is no consistent response to VEGF across experiments (different genes are up/down-regulated in vitro and in vivo). Moreover, two different methods of blocking neuropilin 1 function give different molecular outcomes. While these issues need to be acknowledged, discussed, and explanations considered, outside of this I think that, for the most part (except see below), their conclusions are reasonable and interesting given their data, with mainly issues of clarity/presentation that need to be addressed.

2-1) Switching between use of leader/trailer and proximal/distal is confusing.  In general one refers to embryonic and the other to culture, but "in vitro trail" and "in vitro lead" are also used (as well as in vivo proximal/distal).  Please pick one and stick to it—it would be easiest to just define distal as lead and proximal as trailing, so that the same nomenclature can be used for both in vitro and in vivo.

2-2) The Results describe "very low" HAND2 and BAMBI expression throughout migrating NCC.  Very low relative to what?  The HCR expression shown in Fig. 1 looks reasonable enough on the high resolution image.  Also a detail: in Fig. 1G HAND2 is blue not yellow as indicated in the text (would be best to match the color in F).  
*Relative to FoxD3?*

2-3) The title to Fig. 2D states it is a comparison of in vitro + VEGF with in vivo distal, but according to the text this is just a representation of genes up- or down-regulated by the VEGF treatment, with trailblazer signature genes highlighted—if it is a comparison, please use a venn diagram as in 1D,E; if the text is correct, please adjust the title on the figure.

2-4) On p.14, it is not fair to make a general statement that, "lead and trailer neural crest cells molecular signatures are an emergent property after exposure to signals within the embryonic neural crest microenvironment, one of which is VEGF" when they specifically note that "VEGF lead and trailer gene profiles in vitro were still very different to the lead and trail molecular signatures measured in vivo" and they only test the response to one factor. It is noteworthy that some trailblazer signature genes are upregulated by VEGF, and this should be indicated as consistent with the idea that the microenvironment impacts lead/trail identity, but they can't say much more than that and need to be careful not to over-interpret.  This happens again in the first paragraph of the discussion, when they conclude that, "VEGF and other microenvironmental signals" establish lead cells, when they only tested VEGF.

2-5) Fig. 3 is difficult to take in.  In A, the time marks are not all placed to scale, and the arrangement is very cluttered so that it is hard to keep track of what is happening at each timepoint.  Perhaps + or - VEGF could be indicated by coloration or shading on the time vector. In B, the graphs are marked as "relative expression" of "all genes." In the text, it is stated that 96 total genes were analyzed, but the ratios are all out of 25. What is the 25?  And in B are "all genes" all 25 (whatever they are) or all 96 (which it does not appear to be based upon the number of lines)? Points on the lines in B presumably correspond to timepoints assayed in A, but which ones?  Is this the 90 min time course after VEGF removal? And lastly for B, what is the difference between the pink and the green graph? Based upon the x-axis green may be a continuation of pink, but the color change is not explained (in text or legend).  In C, the y axis contains timepoints relative to the start of the experiment, but is labeled as being time in min after VEGF addition (which would be 2, 4, 8 min, etc). This representation shows the number of genes responding within a certain timeframe, but does not indicate what "first response" means (up or down regulation?).   
*A: I think shading of the timecourse based on experimental conditions is a good idea. Originally these were pink and green, which is why these colours were chosen in B. Please let me know what colours you choose, so that I can coordinate panel B accordingly.*

*B: The label “all genes” was incorrect. Shown here are only the genes with differential expression (p<0.1) that is consistent under all three changes in experimental conditions (13 genes). I changed the axis ticks for panel B (omitting only 92 min to make it less crowded), see pdf in e-mail.*

*C: The 25 genes were all genes that showed differential expression (p<0.1) after both removal and re-addition to VEGF. We did not consider changes after 2 hours of initial exposure to VEGF, as we did not have samples during period to identify first response times.*

***Suggested response:*** *We thank the reviewer for the helpful suggestions on how to improve the clarity of this figure, and have implemented these. The two panels in B correspond to the time-course of expression after removal and readdition of VEGF, respectively. “First response” indicates the earliest time-point at which differential expression was detected.*

2-6) Fig. 4 computationally explores the effects of switching time on migration efficiency and cell number within a domain. One of the observations in Fig. 3 is that the molecular response to a changed environment ("switching") is not always the same with repetition, however, this computational exercise does not seem to take this into account.

*Briefly, I think that particular comment is as much about the interpretation of fig. 3 as it is about the modelling. One could also say that only the changes we observe that are consistent upon repeated exposure to vegf are the relevant ones, and the others occur by chance.*

*Ruth and I briefly discussed using a distribution of switching times in the model, but did not think it was worth it: I'm not sure there is the motivation a distribution of switching times, as we don't have evidence for the same gene responding not at different times in repeats (not that it doesn't happen, but the data don't allow us to conclude that). Also, I would expect it to give the same results with more variability, and requiring much higher N. Let me know what you think.*

***Suggested response:*** *The reviewer raises an interesting point about the interpretation of the results in Fig. 3. We interpret this not as a changing response to repeated change in conditions. Rather, we suggest that only the changes consistent under both removal and readdition of VEGF can be attributed to the changing environment. The other, non-consistent changes in expression could occur by chance, or due to other factors such as time since explantation. Thus, we tested the hypothesis that changes between leader- and follower-states occur at a defined rate upon exposure to VEGF. We don’t model the molecular response explicitly, and it is yet fully understood how different molecular signals, such as VEGF and potentially other microenvironmental cues, are integrated to give observed cell-level behaviors. This means our model is not necessarily inaccurate, just that it tests a particular aspect of the observations reflected in our hypotheses. The result of our computational experiment was that migration is more efficient and less variable when the response to addition and removal of VEGF occurs on the same time-scale, rather than at different rates. Finally, please note that the effective switching times will vary from cell to cell, as the local chemoattractant profile is a) different in the local environment of each cell and b) sampled stochastically by each cell. Thus, the particular state-switching behavior of each cell will depend on its recent history of exposure to a chemoattractant profile (and lack thereof), and this is captured in our simulations since we implemented hysteresis in the integrate-and-switch mechanism.*

2-7) Fig. 5: VEGF non-expressing cells (the same cell line used in B and C) must be implanted for this to be a proper control.  I realize that they have previously shown that NCC are not attracted to these cells, but when they are measuring the stream width, cell number, etc, using control cells will control for the surgical manipulation and other aspects of the procedure that could impact these measurements. 5G needs more informative labels (include something about r3). 5I is not entirely clear (what is a "migration profile"? How does this show, "break-up of the stream into a distal-moving subpopulation"? Why are there are leaders 100 µm along the stream, mixed in with followers?). The conclusion of this section is that, "trailer neural crest cells can respond to VEGF, but prefer to remain within their zone of the migratory stream if possible."  I am not sure how this data shows this?  More explanation is needed.

*The reviewer clearly misunderstood the simulations here. I previously suggested using panels E&F were the transplant regions are highlighted, and I will attach these again in the e-mail.*  
*I: Migration profile is just the histogram of cell number vs distance migrated.* ***Suggested response:*** *There are leaders 100um along the stream in the simulations representing the tissue transplants (see E&F). As the local production of chemoattractant was increased, some cells switched to a leader state, who were then followed by some of the surrounding cells. Thus we get a break-up of the stream of cells into a group that moves towards (or stays close to) the region representing the VEGF transplant.*

2-8) It would be informative to have access to the movies used to prepare Fig. 6.  
*Can we make these available?*

2-9) It is implied, but not indicated explicitly (that I could find), that single cells are profiled in Fig. 7. Please clarify.  
*I didn’t think we profiled single cells here, did we? Perhaps we could clarify this in the corresponding text section.*

2-10) In Fig. 9A and B, we are instructed to compare Euclidian clustering plots that look almost nothing like each other (particularly in B), and then told that in fact only 13 or 9 genes are different.  The dissimilarity matrix plots are definitely helpful, and it also helps when specific genes are highlighted, but these data are not obvious. Also, why do Np1 siRNA and Fc give such different results?? No reasonable explanation is provided. Given two different methods of disrupting VEGF were likely done in order to support the findings, disparate outcomes are an issue and possible explanations should be provided.