Regeneration in the sponge *Sycon ciliatum* partly mimics postlarval development

Anael Soubigou, Ethan G. Ross, Yousef Touhami, Nathan Chrismas and Vengamanaidu Modepalli

DOI: 10.1242/dev.193714

Editor: Cassandra Extavour

Review timeline

Original submission:	6 June 2020
Editorial decision:	31 July 2020
First revision received:	26 August 2020
Editorial decision:	29 September 2020
Second revision received:	2 October 2020
Accepted:	13 October 2020

Original submission

First decision letter

MS ID#: DEVELOP/2020/193714

MS TITLE: Regeneration in sponge Sycon ciliatum mimics postlarval development

AUTHORS: Anael Soubigou, Ethan G Ross, Yousef Touhami, Nathan Chrismas, and vengamanaidu modepalli

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Sponges ability to regenerate, including reaggregation from dissociated cells, is a fascinating phenomenon. Very few other organisms (such as the cnidarian Hydra) can perform this trick. Therefore, understanding of molecular and cellular mechanisms of sponge regeneration is of great interest to biologists, and – in the very long run - might have implications for regenerative medicine.
Phylogenetic position of sponges is crucial to our understanding of animal origin and evolution, and it seems likely (but not sure!) that sponges ability to regenerate is reflecting regenerative abilities of early animals.
The current manuscript is presenting great progress in experimental protocols for regenerating calcareous sponges from dissociated cells. Huxley (1911) has shown this to be possible, but recent attempts, eg Eerkes-Medrano et al. 2014 have not been successful in this sponge lineage. The RNA-Seq data from the regeneration series produced by the authors will be of interest to many researches interested in animal regeneration.

Comments for the author

I would be delighted to see this report published, but I believe it requires major revisions.
My two major issues are: (1) a number of factual errors through the text; (2) lack of transcriptome data preventing future comparisons. I hope both can be addressed even in the current situation.

The following specific comments and suggestions are listed below in the order the issues appear in the manuscript:
l. 11: I would not call a reaggregate a juvenile, perhaps a juvenile-like sponge would be better? Or asconoid-grade sponge?
l. 14. Not sure if cell culture is the appropriate term – this might imply cell division, or at least maintenance of individual cells, but here the cells immediately aggregate.
l. 15 (and many times later, e.g. l. 143, 368) – the authors claim to compare regeneration to embryonic development, but the only datasets they use are those of postembryonic development.
l. 22-23 If sponges are the first branch of extant animals, then by definition they are a sister group to all other animals (not nearly all).
l. 28 It is an oversimplification to talk about gradient of regeneration - strong regenerative abilities are distributed throughout the animal kingdom (eg colonial ascidians).
l. 29 I would use plural form to talk about sponges as models.
l. 38 Was the term metamorphosis really coined for calcareous sponges?
l. 40 The references are incorrect – neither 8 nor 9 talk about regeneration; more importantly, 9 is on Sycon coactum, not Sycon ciliatum.
l. 41 reproduction, not embryogenesis, is viviparous Figure 1: The color scheme, in which the same color is used to depict completely unrelated cells types, is really misleading. Choanocyte chamber is not a developmental stage but a structure in a (juvenile or adult) sponge.
l. 62-63 Wilson did not use Sycon ciliatum, but a demosponge species Microciona. The appropriate reference here would be Huxley 1911.
l. 94 What are bilayer cells?
l. 98-101 It would be helpful to see illustration of the two types of primmorphs.
l. 127-128 Choanoderm is an epithelial cell layer, not a cavity.
l. 132 What is increase in complexity of spongocel? That would imply formation of syconoid body plan from asconoid, but this is not what the authors are showing.
l. 133 What are intermediate porocytes?
l. 139 How can stage V be choanoderm? The calcaronean juvenile is an ascon (asconoid grade); what is the difference between stage VI and VII?
l. 142- l. 147 How is regeneration described here similar to embryonic development? It has clear similarities to postembryonic development – which for S. ciliatum has been described in reference 17, not 8; note that the Eerkes-Medrano and Leys study is on S. coactum, not S. ciliatum.
l. 157 ostium should be plural ostia Table 1 inter cell mass should be inner cell mass. The color scheme is misleading – red color should not be used for macromeres in the larva and the inner cell

mass – the macromeres will become pinacocytes; it is the micromeres which will become inner cell mass.
l. 185-8 The datasets for S. ciliatum postembryonic development have been published alongside reference 17, not 8 (which was associated with embryonic development datasets). This reference did not use term “choanocyte chamber” to describe a stage of development. Why is PY used to indicate asconoid juvenile (this is also a question for Fig. 5)?
Figure 5 The colour code in A is misleading (as in the table above). For H, it would be good to see some statistical test to understand how much overlap would be expected by random.
l. 242-244 and Fig. 6. I do not understand on which basis the authors conclude that the clusters are overlapping.
l. 247 zygote?
l. 249 What is rudimentary embryonic and swimming larval morphology?
l. 266 How does GO enrichment analysis reveal gene network?
l. 268 How do we know that the transcription factors identified to be differentially expressed are tightly controlling the process?
L 269-270 Gene families are not expressed; genes or transcripts are.
Figure 7 (and all figures including gene/transcript names). Looking at the gene names, and reading the methods section reveal a serious issue. As far as I understand, the authors used the available S. ciliatum genome (although the source is not clearly indicated) to map RNA-seq reads, some of which they have generated in the current study, and some which were previously published along reference 17. The authors carried their own transcriptome assembly and annotation. But this new transcriptome assembly is not made available (unless I missed it), and the only insight the reader gets into identity of the differentially expressed genes is by looking at the short names embedded in the figures. Some of these names are the same, and yet the expression pattern is opposite (eg NOTC1 in suppl. Fig, 8). Another problem is that the authors say the genes were annotated based on UNIPROT database. But, for example, the names in the wnt family include WntQ (which has been found is S. ciliatum, but is not in the uniprot database, and Wnt7a – which is not present in S. ciliatum, but is found in this database). So the annotation could not have been done the way it is described, and the reader has no way to investigate identity of the transcripts any further. Minor points – why are some names of genes also on the left side? Why is smad expression not a part of the tgf-beta signalling panel?
l. 324-336 This section is rather clumsily written, and does not seem to contribute much to the manuscript.
l. 346 If the main focus was to compare regeneration to embryonic development, why were embryonic development datasets (which are also available for S. ciliatum) used?
l. 400-403 It would have been great to get insight into what is removed by the slow speed.
l. 467. Wouldn't excluding transcripts with expression in less than 25% samples result in excluding genes with high but specific expression?
l. 472 – please see the annotation related comment to fig. 7.

I hope these comments will be helpful in revising the manuscript.
With best regards,
Maja Adamska

Reviewer 2

Advance summary and potential significance to field

Overall, this is clearly-written manuscript about an important topic in developmental and regenerative biology: how sponges can undergo a regeneration program and how this process overlaps with, and differs from, sponge development via fertilization and embryogenesis. The authors present a combination of microscopy images, captured over time, as well as gene expression analyzed from bulk tissue RNA-seq. They are careful throughout to report their findings in a straightforward way rather than over-interpreting what they observed. The main conclusions are that the sponge regeneration program differs morphologically in the early stages from early embryonic sponge development. However, the morphologies eventually converge.
The global changes in gene expression seem to overlap a bit more than the morphological changes might suggest. However, they do seem to uncovered that there are some differences in the early stages, which parallels the differences in morphologies to get to the “ciliated chamber” stage.

They highlight what most people would suspect would have been the key players (canonical cell-cell signaling pathways as well as genes encoding molecules most likely involved in regulating cell adhesion). While there are no surprises here, the work is very well executed and crisply presented. Cell death pathways being active and PI-stained cells showing up in regenerating samples undergoing remodeling early in the regeneration process is interesting and data they present to support that finding is solid. Their goal of creating a standardized S. ciliatum regeneration protocol was achieved. This could be a foundational work in this field and certainly brings the study of sponge regeneration further into the molecular genetic age. The only question seems to be whether this contribution, which is an entirely descriptive work, should also include some hypothesis-driven experimentation to follow the results already presented. I think this could fall into the category of very-well-executed descriptive studies that Development does sometimes publish.

Comments for the author

Specific comments:
• I favor tweaking the title because the reader is going to think all that's been uncovered overlaps with embryogenesis, when in reality, they did find significant differences between regeneration (especially the early stages) and embryogenesis. This is a huge and old question in the field, and a title like the one they have here undersells and oversimplifies the work.

• In the abstract, I think they need another sentence, between the current first and second sentences, that is more overt. Right now, it's not that intuitive. I suggest something like this: "Somatic cells dissociated from an adult sponge can re-organize and develop into a juvenile. This phenomenon therefore represents an instance of regeneration. However, the extent to which regeneration . . . "

• The descriptive terminology is very clear, unusually so for a heavy embryology paper.

• The EM figures are outstanding.

• Figure 1: Can the authors please make the colors of ciliated micormeres and choanocytes a bit more different from one another?

• Figures 2-4: Can the authors please say in the legend or in the individual panels directly what the blue and green colors are? The blue is probably DAPI and the green, from the Methods, appears to be anti-tubulin (so just a counterstain to see the cells), but I did not see this information in the text, the legends, or the figure panels themselves.

• Table 1 is fantastic.

• The authors need to present more of the gene expression data, which could be supplementary tables. I appreciate that they have presented some very curated analyses, but it would be even better if they provided the full datasets so that others could mine information from this work. That seems impossible from the submission as-is, but perhaps I have missed it. For example, the genes in the clustering analysis from Figures 6A and 6B should all be deposited in supplementary files. If they made 14 supplementary files and dumped all the transcripts along with presumptive orthologs/protein names there (for those that are identifiable), with each row a transcript and each column a time point with expression value, these types of matrices would be very helpful. The only data they have deposited in a public database to support this manuscript appears to be the raw data as a GEO submission.
• The authors could try to block apoptosis and see if that interferes with the early remodeling processes and thereby is necessary for regeneration. This was recently done for Nematostella/sea anemone (Warner et al., bioRxiv, 2019) and, similar to what the prediction would be here in sponges, it did not interfere with embryogenesis, but it did block early stages of regeneration. I am hesitant to propose experimentation because of current Covid restrictions, so perhaps they can at least mention this work (which appears still not to be accepted in a peer-reviews format) in the Discussion as it is an interesting parallel.

First revision

Author response to reviewers' comments

Reviewer #1:
Reviewer 1 Advance summary and potential significance to field
Sponges ability to regenerate, including reaggregation from dissociated cells, is a fascinating phenomenon. Very few other organisms (such as the cnidarian Hydra) can perform this trick. Therefore, understanding of molecular and cellular mechanisms of sponge regeneration is of great interest to biologists, and – in the very long run - might have implications for regenerative medicine.
Phylogenetic position of sponges is crucial to our understanding of animal origin and evolution, and it seems likely (but not sure!) that sponges ability to regenerate is reflecting regenerative abilities of early animals.
The current manuscript is presenting great progress in experimental protocols for regenerating calcareous sponges from dissociated cells. Huxley (1911) has shown this to be possible, but recent attempts, eg Eerkes-Medrano et al. 2014 have not been successful in this sponge lineage. The RNA-Seq data from the regeneration series produced by the authors will be of interest to many researches interested in animal regeneration.

Reviewer 1 Comments for the author
I would be delighted to see this report published, but I believe it requires major revisions.
My two major issues are:
(1) a number of factual errors through the text;
(2) lack of transcriptome data preventing future comparisons. I hope both can be addressed even in the current situation.

The following specific comments and suggestions are listed below in the order the issues appear in the manuscript:
Comment 1: l. 11: I would not call a reaggregate a juvenile, perhaps a juvenile-like sponge would be better? Or asconoid-grade sponge?

Answer 1: L. 32-33: As the reviewer suggested we have changed the term “juvenile” to “juvenile-like sponge”

Comment 2: l. 14. Not sure if cell culture is the appropriate term – this might imply cell division, or at least maintenance of individual cells, but here the cells immediately aggregate.

Answer 2: L. 36: Thanks for the comment, we have removed the cell-culture term from the sentence.

Comment 3: l. 15 (and many times later, e.g. l. 143, 368) – the authors claim to compare regeneration to embryonic development, but the only datasets they use are those of postembryonic development.

Answer 3: L. 38: We agree with the reviewer, the regeneration data was only compared with postembryonic data. As suggested we replaced the term embryonic with postembryonic.

Comment 4: l. 22-23 If sponges are the first branch of extant animals, then by definition they are a sister group to all other animals (not nearly all).
Answer 4: L. 45-46: As suggested we removed the second part of the sentence “the sister lineage to nearly all animals”

Comment 5: l. 28 It is an oversimplification to talk about gradient of regeneration - strong regenerative abilities are distributed throughout the animal kingdom (eg colonial ascidians).

Answer 5: L. 50: We agree with the reviewer the term gradient is confusing, we therefore revised the text as follows. “The regenerative capacity widely varies in the animal kingdom”

Comment 6: l. 29 I would use plural form to talk about sponges as models.

Answer 6: L. 51: As suggested we made the changes to the text “The sponges are exceptional model organisms with an extraordinary regenerative ability”

Comment 7: l. 38 Was the term metamorphosis really coined for calcareous sponges?

Answer 7: L. 61: Thanks for raising this question, hence we could not find a direct reference to support the statement we have now removed the term metamorphosis from the sentence. Nevertheless, we noticed that HUXLEY, J. S often referred to the transition into functional young sponges (the Olynthus stage) as metamorphosis.

Comment 8: l. 40 The references are incorrect – neither 8 nor 9 talk about regeneration; more importantly, 9 is on Sycon coactum, not Sycon ciliatum.

Answer 8: L. 62: We have now provided an appropriate reference for regeneration in Sycon ciliatum (HUXLEY, J. S. 1921) and also added additional references related to embryogenesis and development (Fortunato, S., et al. EvoDevo, 2012. 3(1): p. 14. Leininger, S., et al., Nat Commun, 2014. 5: p. 3905. Franzen, W., Zoomorphology, 1988. 107(6): p. 349-357)

Comment 9: l. 41 reproduction, not embryogenesis, is viviparous
Answer 9: L. 64: Thanks for the correction, we made the changes to the text “In S. ciliatum, reproduction is viviparous.”

Comment 10: Figure 1: The color scheme, in which the same color is used to depict completely unrelated cells types, is really misleading. Choanocyte chamber is not a developmental stage but a structure in a (juvenile or adult) sponge.

Answer 10.1: As suggested, we now used different colours to distinguish ciliated micromeres and choanoflagellates.
Answer 10.2: We removed the Choanocyte chamber and renamed them as S4 juvenile and S5 juvenile as referred in publication Fortunato, S. A. V., et al. (2014).

Comment 11: l. 62-63 Wilson did not use Sycon ciliatum, but a demosponge species Microciona. The appropriate reference here would be Huxley 1911.
Answer 11: L. 79-81: Thanks for the correction, we have now updated the sentence as follows and provided appropriate references. “Many studies have been dedicated to sponge regeneration to understand their morphological signatures, starting from Wilson, H.V. 1907 [3] and Huxley, J.S. 1911 [4, 13], the pioneers of this topic.”

Comment 12: l. 94 What are bilayer cells?

Answer 12: L. 111-112: We apologize for the lack of clarity, we have amended the text as follows “Earlier studies coined this phenomenon of forming two distinctive cell layers (the inner cell mass surrounded by a single layer of outer cells) as redevelopment, or somatic development.”

Comment 13: l. 98-101 It would be helpful to see illustration of the two types of primmorphs.

Answer 13: L. 118 & Fig.S1: Thanks for the suggestion, we have now illustrated the second kind of primmorphs in the supplementary (Fig. S1) and added the details to the manuscript. Note that we were unable to provide SEM images in Fig. S1, as these primmorphs were extremely delicate and their distinctive bubble structure (blow-outs) collapsed during SEM sample preparation (as shown in the SEM image).

Comment 14: l. 127-128 Choanoderm is an epithelial cell layer, not a cavity.

Answer 14: L. 129: Thanks for the correction, we have changed the text as follows “Gradually, these choanocyte chambers fuse into a single epithelial cell layer of choanocytes (also known as choanoderm).”

Comment 15: l. 132 What is increase in complexity of spongocel? That would imply formation of syconoid body plan from asconoid, but this is not what the authors are showing.

Answer 15: L. 133-137: Thanks for raising this question, hence we never continued our observations beyond the experimentation period (~ 3-4 weeks) and we never observed a transition from asconoid to syconoid body plan. Now we added the following sentence to make it clearer to the readers. “In the current study, we maintained the asconoid juveniles for only 3-4 weeks and during this period we observed an increase in the size of spongocoel and number of porocytes with ostia.”

Comment 16: l. 133 What are intermediate porocytes?

Answer 16: L. 135: We apologize for the lack of clarity, we meant to say an increase in the number of porocytes. Hence the term “intermediate porocytes” is unclear, we now changed the text as follows: “number of porocytes with ostia.”

Comment 17: l. 139 How can stage V be choanoderm? The calcaronean juvenile is an ascon (asconoid grade); what is the difference between stage VI and VII?

Answer 17.1: L. 166-167: During regeneration, around 10-14 dpd a single layer of choanoderm is formed with flagellated choanocytes, hence we thought choanoderm is a suitable label for the stage V regeneration. However, if the reviewer suggests changing this with the more suitable term, we would be happy to amend this.
Answer 17.2: Table.1: By stage VI (~ 16-18 dpd) both osculum opening and porocytes with ostia are already formed, the only key difference in stage VII (~ 21- 24 dpd) is an increase in the size of ascon and number of porocytes. We have now updated this information in Table 1.

Comment 18: l. 142- l. 147 How is regeneration described here similar to embryonic development? It has clear similarities to postembryonic development – which for S. ciliatum has been described in reference 17, not 8; note that the Eerkes-Medrano and Leys study is on S. coactum, not S. ciliatum.

Answer 18: L. 144-148: We agree with the reviewer, the regeneration described here is similar to postembryonic development and not embryonic. We have now revised the sentence as follows and updated the references as suggested by the reviewer. “Remarkably these morphological signatures are vastly comparable to postembryonic development of S. ciliatum (Fig.1 and Table 1, 4th column) [1, 11]. To gain further insights into their morphological overlap, we compared regeneration with postembryonic development.”

Comment 19: l. 157 ostium should be plural ostia
Answer 19: L. 623: As suggested we have changed the text “ostium” to “ostia”

Comment 20: Table 1 inter cell mass should be inner cell mass. The color scheme is misleading – red color should not be used for macromeres in the larva and the inner cell mass – the macromeres will become pinacocytes; it is the micromeres which will become inner cell mass.

Answer 20: Table 1: We thank the reviewer for noticing that, we changed the text in the table. As suggested by the reviewer we have removed the red colour from the S3 larva.

Comment 21: l. 185-8 The datasets for S. ciliatum postembryonic development have been published alongside reference 17, not 8 (which was associated with embryonic development datasets). This reference did not use term “choanocyte chamber” to describe a stage of development. Why is PY used to indicate asconoid juvenile (this is also a question for Fig. 5)?

Answer 21.1: L. 167: We have now updated the reference as suggested.
Answer 21.2: L. 166: We agree with the reviewer, the term “choanocyte chamber” was not used in the publication. One of the key developmental changes during the stages S4 & S5 is the formation of choanoderm. Hence similar developmental changes were also observed during regeneration, for the comparative study and to make it more accessible for the readers we reflected to add the term “choanocyte chamber” to S4 & S5. To avoid any further confusion as the reviewer suggested we now removed the term “choanocyte chamber” and renamed the stages as S4 juvenile & S5 juvenile as referred to in Fortunato, S. A. V., et al. 2014.

Answer 21.3: L. 167 & fig.5 legends: Thank you for noticing this, the PY is a wrong text, now we corrected it as YS (Young syconoid) as referred in Fortunato, S. A. V., et al. 2014.

Comment 22: Figure 5 The color code in A is misleading (as in the table above). For H, it would be good to see some statistical test to understand how much overlap would be expected by random.

Answer 22.1: As suggested we have removed the red colour from the S3 larva in Figure 5A
Answer 22.2: Fig 5H & L. 662-665: As the reviewer suggested we have carried out a suitable statistical test using "GeneOverlap" an R package to test gene overlaps using Fisher's exact test to find the statistical significance of the overlap between two gene lists. The statistical test output is now presented in Fig. 5H as follow (Overlapping p-value=0e+00, Odds ratio=3.2, Overlap tested using Fisher's exact test (alternative=greater), Jaccard Index=0.3). Here the P-value is 0e+00, which means the overlap is highly significant. Fisher's exact test also gives an odds ratio which represents the strength of association. Here the odds ratio is much larger than 1, showing that the association is strong.
In addition to the statistical test, we also remade the Venn diagram and added the percentages to the chart. We are grateful for the reviewer's comment which enabled us to improve the analysis.

Comment 23: l. 242-244 and Fig. 6. I do not understand on which basis the authors conclude that the clusters are overlapping.

Answer 23: L. 198-208: We thank the reviewer for this remark and now elaborate on this result in the manuscript to make it clearer to the reader. "We subjected the candidates to Fuzzy c-means clustering [19] to group the genes based on the expression profiles and generated 7 clusters for each dataset (Fig.6 A&B). After clustering the genes, we analysed the correlation of these clusters among regeneration and PLD by combing all the 14 clusters and subjecting them to sample correlation analysis. As observed from the dendrogram, the gene clusters with similar expression patterns between regeneration and PLD likely form a single branch (fig 6 C). For example, regeneration cluster R6 and PLD cluster D5 are grouped together: notably, these clusters have comparable expression profiles (fig 6 A and B). Similar results were also reproduced through correlation analysis: the box including R6 and D5 has a relatively high correlation value (fig 6 D). We found that the majority of the regeneration gene clusters exhibited overlap with specific PLD clusters (Fig.6 C&D), suggesting that regeneration partly resembles PLD by displaying similar gene expression profiles." We hope the reviewer finds our explanation to be more comprehensive.

Comment 24: l. 247 zygote?
Answer 24: L. 212: We added the term zygote to the text. "Since the regeneration is not starting from a zygote or amphiblastula larva"

Comment 25: l. 249 What is rudimentary embryonic and swimming larval morphology?

Answer 25: L. 214-215: Thanks for pinpointing this, after aggregation during the transition of primmorphs stages, some of the morphological changes do resemble an amphiblastula-like or embryo-like morphology. However, we were a bit reluctant to describe it as embryo-like and termed it as "rudimentary embryonic and swimming larval morphology". Hence, this text is not clear enough and we removed the sentence.

Comment 26: l. 266 How does GO enrichment analysis reveal gene network?
Answer 26: L. 224-225: We meant to say, study genes associated with certain biological processes and molecular functions, hence we agree the term gene networking is misleading, we updated the sentence as following "We performed a GO-term enrichment analysis to reveal genes associated with various biological processes and molecular function during regeneration."

Comment 27: l. 268 How do we know that the transcription factors identified to be differentially expressed are tightly controlling the process?

Answer 27: L. 225-226: Since the statement solely relies on the current gene expression data, we agree the sentence is making over-assumptions, therefore we removed the text from the manuscript.
Comment 28.1: L 269-270 Gene families are not expressed; genes or transcripts are.

Answer 28.1: L. 227: We changed the text by removing the families.

Comment 28.2: Figure 7 (and all figures including gene/transcript names). Looking at the gene names, and reading the methods section reveal a serious issue. As far as I understand, the authors used the available S. ciliatum genome (although the source is not clearly indicated) to map RNA-seq reads, some of which they have generated in the current study, and some which were previously published along reference 17. The authors carried their own transcriptome assembly and annotation. But this new transcriptome assembly is not made available (unless I missed it), and the only insight the reader gets into identity of the differentially expressed genes is by looking at the short names embedded in the figures. Some of these names are the same, and yet the expression pattern is opposite (eg NOTC1 in suppl. Fig, 8). Another problem is that the authors say the genes were annotated based on UNIPROT database. But, for example, the names in the wnt family include WntQ (which has been found is S. ciliatum, but is not in the uniprot database, and Wnt7a – which is not present in S. ciliatum, but is found in this database). So the annotation could not have been done the way it is described, and the reader has no way to investigate identity of the transcripts any further.

Answer 28.2.1: We apologies for not providing the transcriptome data in our first submission. We made the following changes as suggested and provided the necessary transcriptome data in the supplementary (Table S1).
Answer 28.2.2: L. 401-402: The S. ciliatum genome was sourced from Marcin, A., et al. 2016 (https://dryad.figshare.com/articles/dataset/Sycon_ciliatum_genome/4090545/1), now we updated the reference under the material and method section.
Answer 28.2.3: One of our key aims is to understand the expression profiles of the genes that were previously studied in Sycon ciliatum development, hence we selected a list of candidates published in Leininger, S., et al. (2014) by Maja Adamska group. The reviewer is right in the heat-map analysis we have integrated our own transcriptome assembly along with the published Sycon ciliatum transcriptome data (Marcin, A., et al. 2016). We have updated the heatmap by integrating the gene names that were previously studied in Sycon ciliatum development by the Maja Adamska group, for example, WntQ is now renamed as Sci-WntQ. We are grateful for the reviewer's comment which enabled us to improve the data presentation.
Answer 28.2.4: The duplicate gene names were used according to BLASTP hits, we now recognize this issue, and thus provide the raw heatmap data in the supplementary (Table S3). We believe this will allow the readers to access the raw data information for further investigation.
Answer 28.2.5: As suggested we have included annotation GTF file detailing the coordinates of new transcriptome assembly, and rlog normalized counts with Blastp hits in the supplementary (Table S1).

Comment 28.3: Minor points – why are some names of genes also on the left side?

Answer 28.3: Thanks for pointing this out, those gene names were meant to represent a set of closely related genes, however, we agree the names might be misleading, so we removed those gene names from the left side.

Comment 28.4: Why is smad expression not a part of the tgf-beta signalling panel?

Answer 28.4: We changed the heatmap by moving smad genes to tgf-beta signalling panel.

Comment 29: l. 324-336 This section is rather clumsily written, and does not seem to contribute much to the manuscript.

Answer 29: L. 271-292: Thanks for the comment, as this is not the main message in our paper, we now revised the paragraph and removed irrelevant information.

Comment 30: l. 346 If the main focus was to compare regeneration to embryonic development, why were embryonic development datasets (which are also available for S. ciliatum) used?

Answer 30.1: L. 297: Thanks for the suggestion. In fact, our initial plan was to compare the regeneration data with both embryonic and postembryonic data sets, however after concluding the morphological analysis we understood the regeneration is majorly overlapping with postembryonic

stages rather than embryonic, hence we focused only on comparing regeneration data with postembryonic data.

Comment 31: l. 400-403 It would have been great to get insight into what is removed by the slow speed.

Answer 31: We appreciate the reviewers interest in this point, we also find this question interesting, in fact, we pursued this by observing the cell fractions collected at different centrifugation speeds. Based on our observations, In 2.5 rpm fraction, we have a clean single-cell suspension of diverse cell types such as choanocytes, amoeboid, and granulated cells. Interestingly similar cell types were also observed in the cell fraction collected at 1.2 rpm. However, one key difference was that the 1.2 rpm fraction still retained aggregates of choanocyte and sclerocytes with small spicules (as shown in the following figure). It seems the currently selected centrifugation speed and time has only managed to remove the aggregates and attained a clean single-cell suspension in 2.5 rpm. Probably the selected centrifugation conditions may have some effect on the proportion of cell types rather than completely separating specific cell types, as we perceived regeneration in both fractions.
Most likely those aggregates from 1.2 rpm might have some effect on the rate of regeneration. As suggested by Huxley, Julian S, often those initial aggregates form large restitution masses, “large size is associated with less viability”. Similar conclusions were also made by Wilson, H. V. from a study in Hydroids: “The size of the restitution masses was of great importance. Large masses almost invariably died early.” Hence the current explanation is generally hypothetical, we are not entirely confident to add these insights into the results.

Comment 32: l. 467. Wouldn't excluding transcripts with expression in less than 25% samples result in excluding genes with high but specific expression?

Answer 32: L. 409-410: Thank you for raising this comment, this sentence was mistakenly included from an additional analysis we carried out with edgeR in parallel to DESeq2, however, this was later removed from the final manuscript, as we came across the same question as the reviewer suggested. The current analysis present in the manuscript does include all gene-models, we apologies for this mistake and we removed this text from the manuscript.

Comment 33: l. 472 – please see the annotation related comment to fig. 7.
Answer 33: As mentioned in the previous response we have provided the raw heatmap data in the supplementary (Table S2).

I hope these comments will be helpful in revising the manuscript.
With best regards,
Maja Adamska

Reviewer 2 Advance summary and potential significance to field
Overall, this is clearly-written manuscript about an important topic in developmental and regenerative biology: how sponges can undergo a regeneration program and how this process overlaps with, and differs from, sponge development via fertilization and embryogenesis. The authors present a combination of microscopy images, captured over time, as well as gene expression analyzed from bulk tissue RNA-seq. They are careful throughout to report their findings in a straightforward way rather than over-interpreting what they observed. The main conclusions are that the sponge regeneration program differs morphologically in the early stages from early embryonic sponge development. However, the morphologies eventually converge.
The global changes in gene expression seem to overlap a bit more than the morphological changes might suggest. However, they do seem to uncovered that there are some differences in the early stages, which parallels the differences in morphologies to get to the “ciliated chamber” stage. They highlight what most people would suspect would have been the key players (canonical cell-cell signaling pathways as well as genes encoding molecules most likely involved in regulating cell adhesion). While there are no surprises here, the work is very well executed and crisply presented. Cell death pathways being active and PI-stained cells showing up in regenerating samples undergoing remodeling early in the regeneration process is interesting and data they present to support that finding is solid. Their goal of creating a standardized S.ciliatum regeneration protocol

was achieved. This could be a foundational work in this field and certainly brings the study of sponge regeneration further into the molecular genetic age. The only question seems to be whether this contribution, which is an entirely descriptive work, should also include some hypothesis-driven experimentation to follow the results already presented. I think this could fall into the category of very-well-executed descriptive studies that Development does sometimes publish.

Reviewer 2 Comments for the author
Specific comments:

Comment 1: I favor tweaking the title because the reader is going to think all that's been uncovered overlaps with embryogenesis, when in reality, they did find significant differences between regeneration (especially the early stages) and embryogenesis. This is a huge and old question in the field, and a title like the one they have here undersells and oversimplifies the work.
Answer 1: Dear reviewer, thanks for the suggestion, after a thoughtful discussion we amend the title as follows "Regeneration in sponge Sycon ciliatum partly mimics postlarval development". As observed from morphological and gene expression data the later stages from the ciliated chamber to juvenile converge with PLD as they progress through development. Whereas the early regeneration stages (day1-4) were distinctive from postlarval development, to highlight it we added word partly in the title. We hope the reviewer finds our new title more comprehensive.

Comment 2: In the abstract, I think they need another sentence, between the current first and second sentences, that is more overt. Right now, it's not that intuitive. I suggest something like this: "Somatic cells dissociated from an adult sponge can re-organize and develop into a juvenile. This phenomenon therefore represents an instance of regeneration. However, the extent to which regeneration . . . "
Answer 2: We thank the reviewer for this suggested and now added the text to the abstract. "Somatic cells dissociated from an adult sponge can re-organize and develop into a juvenile-like sponge, a remarkable phenomenon of regeneration."

Comment 3 & 4: The descriptive terminology is very clear, unusually so for a heavy embryology paper. • The EM figures are outstanding.

Answer 3&4: Many thanks for the compliment.

Comment 5: Figure 1: Can the authors please make the colors of ciliated micormeres and choanocytes a bit more different from one another?

Answer 5: We thank the reviewer for this helpful suggestion, we now used different colours to distinguish ciliated micromeres and choanoflagellates.

Comment 6: Figures 2-4: Can the authors please say in the legend or in the individual panels directly what the blue and green colors are? The blue is probably DAPI and the green, from the Methods, appears to be anti-tubulin (so just a counterstain to see the cells), but I did not see this information in the text, the legends, or the figure panels themselves.
Answer 6: Thanks for the suggestion, we now add the following information to figure legends 2, 3 & 4 "Anti-α-Tubulin (green) with the nuclei counterstained with DAPI (blue)."

Comment 7: Table 1 is fantastic.

Answer 7: Many thanks for the compliment.

Comment 8: The authors need to present more of the gene expression data, which could be supplementary tables. I appreciate that they have presented some very curated analyses, but it would be even better if they provided the full datasets so that others could mine information from this work. That seems impossible from the submission as-is, but perhaps I have missed it. For example, the genes in the clustering analysis from Figures 6A and 6B should all be deposited in supplementary files. If they made 14 supplementary files and dumped all the transcripts along with presumptive orthologs/protein names there (for those that are identifiable), with each row a transcript and each column a time point with expression value, these types of matrices would be

very helpful. The only data they have deposited in a public database to support this manuscript appears to be the raw data as a GEO submission.

Answer 8: We apologies for not providing the transcriptome data in our first submission. We followed the reviewer's suggestion and provided the necessary transcriptome data in the supplementary file, included annotation GTF file detailing the coordinates of new transcriptome assembly and rlog normalized counts (Table S1 & S2). As suggested we also provided the gene expression profiles for all clusters from Figures 6A and 6B, along with Blastp predicted protein names (Table S2).

Comment 9: The authors could try to block apoptosis and see if that interferes with the early remodeling processes and thereby is necessary for regeneration. This was recently done for Nematostella/sea anemone (Warner et al.,bioRxiv, 2019) and, similar to what the prediction would be here in sponges, it did not interfere with embryogenesis, but it did block early stages of regeneration. I am hesitant to propose experimentation because of current Covid restrictions, so perhaps they can at least mention this work (which appears still not to be accepted in a peer-reviews format) in the Discussion as it is an interesting parallel.

Answer 9: L 243-245: Dear reviewer many thanks for the suggestion and your kind concern towards the current situations. This is an interesting experiment and we would like to pursue this in a future study. As kindly suggested by the reviewer we have provided the reference and added the following sentence to the manuscript. "This coincides with a recent study in Nematostella vectensis showing at the functional level that apoptosis is required for the initiation of regeneration (Warner, J. F., et al. (2019).

Second decision letter

MS ID#: DEVELOP/2020/193714

MS TITLE: Regeneration in sponge Sycon ciliatum partly mimics postlarval development

AUTHORS: Anael Soubigou, Ethan G Ross, Yousef Touhami, Nathan Chrismas, and vengamanaidu modepalli

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. Please attend carefully to the specific and justified comments of Reviewer 1, and provide all data sources as highlighted by Reviewer 2.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

As in my original report.

Comments for the author

This is a greatly improved version of the manuscript, and I am happy with all the changes made in response to reviewers' comments. It seems however that in quite a few cases the authors made only some of the changes requested in the previous round.

For example:

Comment 3: l. 15 (and many times later, e.g. l. 143, 368) – the authors claim to compare regeneration to embryonic development, but the only datasets they use are those of postembryonic development.

Answer 3: L. 38: We agree with the reviewer, the regeneration data was only compared with postembryonic data. As suggested we replaced the term embryonic with postembryonic.

Yet, the revised text still has multiple references to the embryonic development, for example: "We performed high-throughput sequencing on regenerating samples and compared the data with regular embryonic and postlarval development." "Hence we aim to study 83 the regeneration of S. ciliatum and compare it to embryonic and postlarval development at 84 both morphological and transcriptional levels. We first carried out S. ciliatum regeneration to 85 acquire morphological data for comparison to regular embryonic and postlarval development 86 and classified the regeneration into seven key stages. Then, we collected the regenerating 87 aggregates at defined time points and performed high-throughput sequencing to compare the transcriptional profile with those of normal embryonic and postlarval development."

I suggest the authors carefully re-read their manuscript and make sure that use of the term "embryonic" is justified in all instances - of course it should remain in some.

Comment 2: l. 14. Not sure if cell culture is the appropriate term – this might imply cell division, or at least maintenance of individual cells, but here the cells immediately aggregate.

Answer 2: L. 36: Thanks for the comment, we have removed the cell-culture term from the sentence.

Current version:
"93 First, we standardized and established a S. ciliatum regeneration protocol to achieve 94 consistent sponge regeneration in cell culture."

The manuscripts still suggests that Eerkes-Medrano and Leys 2006 study used S. ciliatum - but it was done on S. coactum.

The appropriate reference the Sycon ciliatum genome is Fortunato, Sofia A. V. et al. (2014), Calcisponges have a ParaHox gene and dynamic expression of dispersed NK homeobox genes, Nature, Article-journal, https://doi.org/10.1038/nature13881, this genome assembly is avaialable at https://datadryad.org/stash/dataset/doi:10.5061/dryad.tn0f3 (this link is included in the above reference). The link (https://dryad.figshare.com/articles/dataset/Sycon_ciliatum_genome/4090545/1) does not work, ref. [48] should be removed (Marcin is the first name of the second author of the Fortunato et al. paper).

It also seems that the gene list associated with Wnt pathway is a bit broader than generally used (including non-canonical cadherins FAT3 and FAT4, which are again shown in cadherin-centered figure 8.

Finally, and I do feel like might be overly picky over a relatively minor issue, but I am still not convinced by the color scheme of the schematic figures. In particular:
In Fig. 1 and Fig. 5 the oocyte and early blastomeres are the same color as the macromeres in later steps - I would use another colour for the macromeres, as the current scheme appears to indicate that macromeres are somehow more similar/related to the early blastomeres. Similarly, in the PL (postalarval) stages all cells are blue, until choanocytes form. Blue is also used for pinacocytes - but the inner cell mass are not "related" to pinacocytes... It would be good to indicate continuity between macromeres and pinacocytes (which are not part of the inner cell mass) on the one hand, and the micromeres and inner cell mass on the other. Finally, what are the greay, epithelial-like cells in the centre of the larva? The grey mesenchymal cells are maternal cells.

Reviewer 2

Advance summary and potential significance to field

This remains unchanged from the previous submission, though the authors have now clarified may issues raised by reviewers.

Comments for the author

Thank you for addressing most all my my concerns, some of which overlapped with the (very thorough) first reviewer. Perhaps I have missed it, but I believe we were both asking for the actual assembled transcriptome data (the sequences attributed to each transcript) to be accessible to the reader. You have now included important annotation and gene expression files that were also asked for. However, I cannot find a file with the assembled sequences for each contig. This information is critical for the issue Reviewer 1 raised regarding readers having the ability to work with the sequence data itself when, for instance, examining homology to other species. The reader needs to be able to simply recover the sequence for a specific transcript to do anything else with it. Was this file included? The GTF file produced by StringTie has important details, but I do not see the base sequence.

Second revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field As in my original report.
Reviewer 1 Comments for the author
This is a greatly improved version of the manuscript, and I am happy with all the changes made in response to reviewers' comments. It seems however that in quite a few cases the authors made only some of the changes requested in the previous round.
For example:

Comment 1: Comment 3: l. 15 (and many times later, e.g. l. 143, 368) – the authors claim to compare regeneration to embryonic development, but the only datasets they use are those of postembryonic development.
Answer 3: L. 38: We agree with the reviewer, the regeneration data was only compared with postembryonic data. As suggested we replaced the term embryonic with postembryonic.
Yet, the revised text still has multiple references to the embryonic development, for example: "We performed high-throughput sequencing on regenerating samples and compared the data with regular embryonic and postlarval development." "Hence we aim to study the regeneration of S. ciliatum and compare it to embryonic and postlarval development at both morphological and transcriptional levels. We first carried out S. ciliatum regeneration to acquire morphological data for comparison to regular embryonic and postlarval development and classified the regeneration into seven key stages. Then, we collected the regenerating aggregates at defined time points and

performed high-throughput sequencing to compare the transcriptional profile with those of normal embryonic and postlarval development."
I suggest the authors carefully re-read their manuscript and make sure that use of the term "embryonic" is justified in all instances - of course it should remain in some.

Answer 1: Dear reviewer, we apologies for that, now we have carefully revised the manuscript and removed the text embryonic, wherever it's necessary (Line 39, Line 82, Line 84, Line 87, Line 91, Line 146 & Line 206).
Nevertheless, as the reviewer suggested we used the term embryonic in some instance as follow and we consider these sentences are justifiable.

- Line 40-41: "We find that sponge regeneration is orchestrated by recruiting pathways like those utilized in embryonic development."
- Line 219-220: "Surprisingly, the majority of listed genes were previously identified in S. ciliatum during embryonic and postlarval development."
- Line 294-295: "The S. ciliatum dissociated cells thus deploy a similar set of genes as their embryonic and larval development to assist regeneration in cell culture."

Comment 2: Comment 2: l. 14. Not sure if cell culture is the appropriate term – this might imply cell division, or at least maintenance of individual cells, but here the cells immediately aggregate.
Answer 2: L. 36: Thanks for the comment, we have removed the cell-culture term from the sentence.
Current version:
"93 First, we standardized and established a S. ciliatum regeneration protocol to achieve
94 consistent sponge regeneration in cell culture."

Answer 2: As the reviewer suggested, we removed the term cell culture and amended text in Line 94, Line 295 & Line 311-312.

Comment 3: The manuscripts still suggests that Eerkes-Medrano and Leys 2006 study used S. ciliatum - but it was done on S. coactum.

Answer 3: Line 146 & Line 565: We now removed the reference Eerkes-Medrano and Leys 2006.

Comment 4: The appropriate reference the Sycon ciliatum genome is Fortunato, Sofia A. V. et al. (2014), Calcisponges have a ParaHox gene and dynamic expression of dispersed NK homeobox genes, Nature, Article-journal, https://doi.org/10.1038/nature13881, this genome assembly is avaialable at https://datadryad.org/stash/dataset/doi:10.5061/dryad.tn0f3 (this link is included in the above reference). The link (https://dryad.figshare.com/articles/dataset/Sycon_ciliatum_genome/4090545/1) does not work, ref. [48] should be removed (Marcin is the first name of the second author of the Fortunato et al. paper).

Answer 4: Line 379-380: We thank the reviewer for providing the relevant reference details, now we updated the reference under the material and method section.

Comment 5: It also seems that the gene list associated with Wnt pathway is a bit broader than generally used (including non-canonical cadherins FAT3 and FAT4, which are again shown in cadherin-centered figure 8.

Answer 5: Thanks for raising this point, we now remade the heatmap (figure 7A) and removed the FAT3, FAT4 and PCDBG genes from Wnt pathway.

Comment 6: Finally, and I do feel like might be overly picky over a relatively minor issue, but I am still not convinced by the color scheme of the schematic figures. In particular: In Fig. 1 and Fig. 5 the oocyte and early blastomeres are the same color as the macromeres in later steps - I would use another colour for the macromeres, as the current scheme appears to indicate that macromeres are somehow more similar/related to the early blastomeres. Similarly, in the PL (postalarval) stages all cells are blue, until choanocytes form. Blue is also used for pinacocytes - but the inner cell mass are not "related" to pinacocytes... It would be good to indicate continuity between macromeres and pinacocytes (which are not part of the inner cell mass) on the one hand, and the micromeres and

inner cell mass on the other. Finally, what are the greay, epithelial-like cells in the centre of the larva? The grey mesenchymal cells are maternal cells.

Answer 6: We appreciate the reviewer suggestion, now we made the following edits to Fig. 1 and Fig. 5.

- We changed the colour of oocyte and early blastomeres.
- We used different colours for pinacocytes and inner cell mass.
- We agree with the reviewer it would be good to indicate continuity between macromeres and pinacocytes. However, we decided to use different colours for macromeres and pinacocytes to allow the reader to distinguish them.
- The grey cells are maternal cells, as suggested in Leininger, S., et al. (2014).

Reviewer 2 Advance summary and potential significance to field
This remains unchanged from the previous submission, though the authors have now clarified may issues raised by reviewers.

Reviewer 2 Comments for the author
Thank you for addressing most all my my concerns, some of which overlapped with the (very thorough) first reviewer. Perhaps I have missed it, but I believe we were both asking for the actual assembled transcriptome data (the sequences attributed to each transcript) to be accessible to the reader. You have now included important annotation and gene expression files that were also asked for. However, I cannot find a file with the assembled sequences for each contig. This information is critical for the issue Reviewer 1 raised regarding readers having the ability to work with the sequence data itself when, for instance, examining homology to other species. The reader needs to be able to simply recover the sequence for a specific transcript to do anything else with it. Was this file included? The GTF file produced by StringTie has important details, but I do not see the base sequence.

Answer: Line 382-383: We apologies for not providing the assembled sequences in our previous submission. We now followed the reviewer's suggestion and provided the assembled sequences for each contig were made available at DOI - https://doi.org/10.17031/1669. We updated this information under the material and method section.

Additional edits:
Table 1: Dear editor and reviewers, in the current version, we moved the Table 1 to supplementary (Table S1). As per the journal requirement, the tables in the main manuscript are required to be in word format; hence Table 1 is partially figure/ word, we decied to move it to supplementary. Thank you.

Third decision letter

MS ID#: DEVELOP/2020/193714

MS TITLE: Regeneration in sponge Sycon ciliatum partly mimics postlarval development

AUTHORS: Anael Soubigou, Ethan G Ross, Yousef Touhami, Nathan Chrismas, and vengamanaidu modepalli
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.