Genetic developmental timing revealed by inter-species transplantations in fish

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Original submission

Note: this manuscript was transferred to Development after peer review at another journal. The referee reports from the other journal were used to inform the decision made at Development

First decision letter

MS ID#: DEVELOP/2020/192500
MS TITLE: Genetic Developmental Timing Revealed by Inter-Species Transplantations in Fish
AUTHORS: Jana F Fuhrmann, Lorena Buono, Juan Martinez-Morales, and Lazaro Centanin

Thank you for transferring your study to Development. I have considered the manuscript along with the referees' comments and I have discussed the paper with other editors.

We are interested in your work, but recommend a substantial revision of your manuscript before we can consider publication. As suggested by Referee 1, it seems important to include data about the later patterning and organisation of cell types in the transplanted retina. In addition, please address Referee 2's questions about availability and analysis of the RNAseq data. Finally, to place the work in context it would be helpful to briefly introduce the classic studies from Ross Harrison and Victor Twitty that documented the growth of transplanted optic cups in different species. I also think the study of the developmental tempo of transplanted human neurons in the mouse brain by Linaro et al., (2019, Neuron) is relevant for your Discussion.

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. 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.

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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.

First revision

Author response to reviewers' comments

Please find enclosed our revised version of the manuscript "*Genetic Developmental Timing Revealed by Inter-Species Transplantations in Fish*", by Jana Fuhrmann and colleagues. We have addressed the suggested revisions and previous concerns, and you will find a version with all changes **highlighted in color** - Manuscript_Highlighted_Changes. In brief, we have expanded the Introduction and the Discussion to better frame our findings in the context of previous works, and included new Figures, Supplementary Figure and Tables to illustrate in more detail the histological and cellular organisation of the ectopic retinae. We are confident that the revisions improved the message of the manuscript and that the present version meets the standards required by the editors of *Development* and by the broad community of *Development* readers.

In addition, we include some images to be considered as Cover Picture in the event of acceptance. We hope you find them aesthetically pleasant and reflecting the message of the manuscript.

Answers to the Referees

The color code for addressing the referee's concerns is: Questions and Concerns / Answers

Points raised by James Briscoe.

JB1: We are interested in your work, but recommend a substantial revision of your manuscript before we can consider publication. As suggested by Referee 1, it seems important to include data about the later patterning and organisation of cell types in the transplanted retina.

Answer 1: *We have addressed this and the other points raised by* ***Referee 1****. The new data about the pattering and layering of the transplanted retinae can be found in the* ***New Section*** **"Molecular confirmation and morphological characterisation of ectopic retinae"**, in the ***New Figure 4 and in the New Figure 5.***
*This is the extract from our response to Referee 1, Answer R1_3: Briefly, we report a partial layering and the presence of diverse cell types in the transplanted retinae in both zebrakas and medrafish. There is a clear layer containing retinal ganglion cells (which in a wild type retina corresponds to the ganglion cell layer, GCL), another layer containing photoreceptors (which in a wild type retina corresponds to the outer nuclear layer, ONL) and in the case of medrafish, another containing retinal pigmented cells. The GCL is recognised by using an Atoh7:*EGFP *that highlight cell bodies but also nerve projections navigating to the endogenous optic tecta. The ONL is recognised by the nuclear morphology, organisation (a monolayer of cells that is limited by a plexiform layer) and the use of and antibody recognising photoreceptors in both medaka and zebrafish. The retinal pigmented cells are recognised by their unique, elongated nuclear morphology, their relative position in the ectopic retina and in cases the presence of pigments. We could not detect a clear organisation in the INL, nor cell types that are found in this layer. The emerging picture is that the early cell types are indeed generated in the ectopic retinae, but the programs responsible for later cells types are not properly run.*

JB2: In addition, please address Referee 2's questions about availability and analysis of the RNAseq data.

Answer 2 - related to Answer_R2_4: *We have now provided the proper link to a GEO site where readers can find all the raw data coming from the RNA-seq that we performed on medaka, zebrafish*

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*and zebrakas. We have expanded as well the M&M section explaining how the data was analysed, including the **New Supplementary Dataset 1 & 2**. Finally, we have performed further analysis on the data to extend our finding to all retinal genes - complementing our previous analysis where we focused on a handful of examples. The new results and new analysis can be found in the **New Figure 6A,B**, and the **New Supplementary Figure 2**. We have addressed this and the additional points below - Answers to Referee 2.*

JB3: Finally, to place the work in context it would be helpful to briefly introduce the classic studies from Ross Harrison and Victor Twitty that documented the growth of transplanted optic cups in different species. I also think the study of the developmental tempo of transplanted human neurons in the mouse brain by Linaro et al., (2019, Neuron) is relevant for your Discussion.

Answer 3: *We have expanded our Introduction and Discussion including the mentioned work. The Revised Discussion (**New First Two Paragraphs**) contains now a more complete scenario on inter-species transplantations and their relevance at the time. We use this to frame our work in context and to highlight what is unique about our approach. We appreciate the suggestion by the referee to expand in this aspect.*

Answers to Previous Referees
The color code for addressing the referee's concerns is: Questions and Concerns / Answers

Referee #1:

Fuhrmann et al explore a very interesting question in the field of developmental biology - to what extent organogenesis is an 'autonomous' process versus one controlled by exogenous sources.

The authors approach this question by using blastomere transplantation between two distantly related teleost species, zebrafish and medaka, to investigate retinogenesis. By doing so the authors exploit the differences in timing of embryogenesis between the two species to ask whether the ectopic retina develops according to the schedule of the host or its own 'endogenous' schedule. They find that the latter is the case - that for example an ectopic zebrafish retina developing in a medaka host follows its own intrinsic program. This is a very clever approach that allows for investigating developmental timing in an in vivo, physiological context. Investigations of the retinotectal projections and lens induction powerfully reveal how endogenous programs and extrinsic cues interact.

To determine developmental timing of retinogenesis the authors use transcriptome analyses, taking advantage of the fact that homologous genes in the two species have low sequence similarity. They also used a transgenic reporter line, specifically one that reports atoh7 expression to follow the onset of retinal ganglion cell genesis. It is unclear to me however why the authors do their analysis at 26 hpf (Fig 3B). Literature suggests that ganglion cell genesis in the zebrafish retina starts a little later than 26 hpf (Hu and Easter, 1999). So while atoh 7 expression may come on by 26 hpf it does not necessarily reflect ganglion cell genesis then but probably atoh7+ progenitors that are poised to generate ganglion cells (see Poggi et al 2005).

Answer_R1_1: *We have changed the text accordingly to reflect this point. We have also stressed the difference between hpf and hpb - since many of the observations were done using blastula stage as the t = 0 to use the same starting point for both species (**whole section** "Excessive arborisation from zebrafish RGCs in the medaka optic tectum", page 8). We have also stressed that we compare EGFP (and not RGC formation) in all cases, i.e., zebrafish, medaka, zebrakas. In addition, we have confirmed the presence of RGCs by imaging the retinae later and revealing the organisation of atoh7 positive cell in pseudo layers **(New Figure 4)**.*

The experiments investigating the ability of putative ganglion cell axons from the ectopic retina to innervate the host optic tectum are remarkable. Curiously, in this set of experiments the authors choose to use the ubiquitous transgenic reporter (ß-actin2) in zebrakas, where expression of the fluorescent protein would not be limited to the ganglion cells alone. Thus it remains possible that other retinal neurons project (erroneously) out of the retina - could the authors comment on this. Why don't they use the atoh7 reporter in zebrakas? The atoh7 reporter is appropriately used in medrafish when similar investigations of the retino-tectal projections are made.

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Answer_R1_2: *The reviewer raises a valid point, and we have complemented the manuscript by including the suggested transplantations **(Updated Supplementary Table 1)**. We have observed the very same behavior as the reported with the previous reporter. These are displayed in the **New Figure 4C,D and 6D**. Our motivation to include the data using the βactin reporter is that this Tg line harbours a membrane-tagged EGFP (CAAX-EGFP) that facilitates the observation of long projections as the ones observed to the optic tectum and the miss-routing axons reaching the caudal fin of the medaka host.*

Overall I think this is a well-executed study using a combination of classical embryology and more modern approaches (RNA-seq) to address an important developmental biology question.
The one aspect I found significantly missing is a more in-depth investigation of the retina itself:Is the ectopic retina laminated? Are its cell types correctly located in their stereotypic locations? Are retinal cells generated in the correct sequence?

Answer_R1_3: *This is a fair point that we have overlooked in our initial submission. We have addressed it in the revised version by performing additional transplantations **(Updated Supplementary Table 1 & 2)** followed by DAPI and antibody staining to characterise the morphology, layering ad cellular content of the ectopic retina **(New Figures 4 & 5)**.*

Briefly, we report a partial layering and the presence of diverse cell types in the transplanted retinae in both zebrakas and medrafish. There is a clear layer containing retinal ganglion cells (which in a wild type retina corresponds to the ganglion cell layer, GCL), another layer containing photoreceptors (which in a wild type retina corresponds to the outer nuclear layer, ONL) and in the case of medrafish, another containing retinal pigmented cells. The GCL is recognised by using an Atoh7:EGFP that highlight cell bodies but also nerve projections navigating to the endogenous optic tecta. The ONL is recognised by the nuclear morphology, organisation (a monolayer of cells that is limited by a plexiform layer) and the use of and antibody recognising photoreceptors in both medaka and zebrafish. The retinal pigmented cells are recognised by their unique, elongated nuclear morphology, their relative position in the ectopic retina and in cases the presence of pigments. We could not detect a clear organisation in the INL, nor cell types that are found in this layer. The emerging picture is that the early cell types are indeed generated in the ectopic retinae, but the programs responsible for later cells types are not properly run. We are confident that the newly generated data provides a better characterisation of the ectopic retinae and reveals features and caveats of retinogenesis in trans-species.

Additionally I found a number of small inconsistencies, oversights and typos, some of which I will outline below:

1) Central nervous system is incorrectly abbreviated in the introduction
2) I think it is more appropriate to refer to ganglion cell axon innervation of the optic tectum rather than optic nerve innervation.
3) In Figure 1A can the authors label some structures in the panels to help orient a reader.
4) D and H in the figure panels are not explained in the figure legends as Donor and Host respectively.
5) What are the orange labeled structures in Fig 3B and 3D?
6) The asterisks in Fig 3 are not explained in the figure legend.
7) It was unclear to me what 'Binocular' images were - do the authors mean images taken using a stereomicroscope?
8) Supplementary Fig 4 - panel A and B are never referred to in the figure legend.

Answer_R1_4: *we have included all the suggested modifications in the revised manuscript. We thank the reviewer for noticing the inconsistencies and typos.*

Referee #2:

An important question in development and evolution is how organs and tissues develop in synchronized manner, despite being independent entities and how this is regulated molecularly and throughout evolution. This paper develops the fish retina as a model to address this question.

The authors use zebrafish and medaka as model systems because their developmental timing differs considerably with zebrafish developing faster than medaka. Using cross-species transplantation they find that transplanted blastomeres do not disperse in the host environment and form coherent groups of cells.

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Near the endogenous eye, these transplants often develop into an ectopic retina, and they demonstrate their donor origin using transgenic lines and RNAseq. The authors show that developmental timing is retained with cell differentiation in the ectopic retina occurring according to donor, but not host timing. Likewise, retinal ganglion cells innervate the visual cortex, and do so with cell intrinsic timing. Finally, they show that while zebrafish ectopic retinae in medaka recruit medaka lens tissue, this is not the case vice versa presumably because lens competence is lost by the time ectopic medaka retinae develop.

This paper provides a framework to investigate the mechanisms of developmental timing. However, it does not provide any insight into the mechanisms themselves.

Answer_R2_1: *The molecular mechanisms of retinogenesis and the interactions between eye tissues are very well described from decades of research in many different animal models, and are not the subject of this article. What we show here is that these well-known mechanisms have an endogenous, species specific developmental timing. The mechanism by which a new, trans- species retina is formed in an alien host is by reproducing the dynamics of the whole transcriptional cascades responsible for retinogenesis in the host species which we show by in vivo imaging and transcriptome analysis.*

The paper therefore remains descriptive and somewhat preliminary. Other systems have already been developed to study these questions (e.g. chick/duck, comparison of stem cell differentiation, etc).

Answer_R2_2: *This is a conceptual mistake. There are other systems to study inter-species chimeras, which we extensively mention in the introduction, the results and the discussion. These systems rely mainly in grafting pieces of tissues that have already gone through gastrulation, patterning, morphogenesis and in many cases even differentiation. The work combining neural crest from chick & quail, which we refer to in the manuscript, is a clear example of grafting a piece of tissue with a defined fate. Our manuscript refers to a completely different case, where the entire developmental history unfolds in an alien species - from a blastomere to a retinal neuron! The model we report can be used to study developmental timing of organs just because of that.*

The only other case in which blastomeres develop into an organ in a foreign species (as we mention in our ms) is a particular rat/mouse transplantation using hosts that are impaired to form the endogenous organ. The intra-uterine development of mammals prevents in vivo imaging of organogenesis, and the topic of developmental timing has not been addressed using this model.

Our case is the first one in which host and donors species are not genetically modified to facilitate or induce grafting.

We have stressed this point further in the ***New Last Paragraph of the Introduction*** *to make the novelty of our approach more evident to the reader, and* ***we have expanded the Discussion*** *to put our approach in perspective, mentioning the most relevant cases of inter-species transplantations and highlighting the differences, caveats and advantages when compared to our system.*

The authors do not explain the advantage the retina as a system offers or how it differs as a system to study developmental timing.

Answer_R2_3: *We have mentioned this in our previous version (transcriptional network defined, interaction with different lineages to recruit lens and navigate axons, stereotypical generation of retinal cell types). We have extended this aspect in a revised version to stress the point suggested by the reviewer* ***(Last paragraph of introduction & new Discussion section)***

The authors present RNAseq data to show that the ectopic retinae are donor derived. However, there appear to be no repeats and therefore no statistical analysis performed; it is therefore difficult to assess how reproducible these data are. There are no specifics on the data analysis and parameters used; although a fairly standard process and scripts should be available on github and freely accessible for anybody to repeat the analysis reproducibly. There is no statistical analysis when comparing the ratio of differentiation to progenitor markers (Sup Fig. 3), and most experiments lack n numbers.

Answer_R2_4: *This is a fair point and has been our mistake to not have the data uploaded to a*

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freely database in time. We thank the reviewer for noticing our mistake.

We have added the coordinates to find all raw data in the revised version: GEO GSE15000, The following secure token has been created to allow its review while it remains in private status: qxgzwgeifperfyv. Additionally, ***we have included the Supplementary Dataset 1 & 2*** *to present the reader a more general view on the RNA-seq data.*

RNA experiments were done in duplicates (for zebrafish and medaka) and in triplicates for zebrakas. We have stated this clearly and incorporated the details of these reads in the ***New Supplementary Table 3****. In addition, we have expanded the analysis of the transcriptomes beyond the ones mentioned by this referee and expand it to all retinal genes. These analysis are explained in the new M&M Section and shown in the* ***New Figure 6 A, B*** *and the* ***New Supplementary Figure 2****.*

Referee #3:

This manuscript attempts to address an interesting piece of biology, namely what controls the speed of developmental processes. They make use of interspecies transplantation between the zebrafish embryo which has a relatively fast development schedule and the medaka embryo that has a relatively slow schedule of development. They claim their results demonstrate that developmental schedules of (at least) retinal development are genetically controlled rather than being influenced by environmental signals that coordinate locally or across the whole embryo.

I'm afraid I don't find this work very compelling because the transplanted cells aggregate and self- organise into an ectopic embryonic eye that is composed only of the transplanted cells. Thus these cells are creating their own mini donor environment and are not really being exposed to the local environment of the host.

Answer_R3_1: *This is a profound misunderstanding of the objectives of the study. Precisely because the cells do not integrate (they are coming from an evolutionary distant species) we can follow the alien module unfolding its genetic program through development. In addition, we show in our work that the donor cells are indeed exposed to the local environment of the host. Our experiments show that retinal identity is defined by the position in which the donor cells are located, since an ectopic retina forms only if adjacent to an endogenous retina. So, our entire analysis is done on cells that were able to read cues from the host to trigger the retinogenic program. Since the donor cells do not intermingle with the host cells, we can address developmental modules (and not chimeric organs). We have stressed this point in our revised version to make it even clearer* ***(last paragraph of the introduction; third paragraph of the New Discussion)***

I would find the work much more interesting and the results more compelling if the donor cells integrated with the host cells, so cells from the different species are immediately next to one another, and then ask whether this alters the developmental schedule of the donor cells.

Answer_R3_2: *We have clarified that cells from the host and the donor are indeed adjacent to each other, but forming different retinae. The referee wants to see whether an individual cell, surrounded entirely by host cells, does also maintain its developmental timing. Although we have observed, and report in the manuscript, that this is the case for other cells types (pigmented cells, blood vessels) our analysis focuses in the only organ that can be formed exclusively by donor cells - the only module. The question that the referee is interested in is better approached in Linaro et al, 2019 (single/few human neurons developing in a mouse brain).* ***We have included this reference in the revised Discussion*** *and highlight the differences and complementarity to our own approach.*

I guess the authors could argue their results rule out a global signal that ubiquitously coordinates organogenesis throughout the embryo, but that possibility seems so unlikely that I don't think their result would be of broad interest. If the authors could eliminate local community effects that could operate on a neighbouring cell basis that would make a much more compelling argument for the dominance of genetically controlled developmental timing.

Answer_R3_3: *Again, we feel that by eliminating the local community effects we will miss all interactions occurring at the supra cellular scale - the retina as a developmental module. The*

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objectives of our work are quite different from those pointed by the referee (a genetic timing of development for the module, and a coordination among cells within to guarantee the proper program execution), and so it was the experimental design.

Second decision letter

MS ID#: DEVELOP/2020/192500
MS TITLE: Genetic Developmental Timing Revealed by Inter-Species Transplantations in Fish
AUTHORS: Jana F Fuhrmann, Lorena Buono, Leonie Adelmann, Juan Martinez-Morales, and Lazaro Centanin

Thank you for submitting your revisions of the above manuscript. To access the manuscript and associated files please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

I have read your revision and consulted with another editor and I am pleased to say that our overall evaluation is positive and we would like to publish a revised manuscript in Development. The additional data on retinal organisation is helpful. As you mention in the rebuttal, there appears to be some disorganisation with patches of laminations and clumps of differentiated cells. It would be useful for readers if this was clearly pointed out in the text. In addition, we would request that you carefully edit the text and legends to make sure that numbers are given for all observations (for instance, the numbers for the key observation that ath5:GFP is activated earlier in ectopic zebrafish retinae). Figure legends should describe what is shown in the figure panels (with more labelling and guidance to the reader) and the more generic results statements should remain in the Results sections. Finally, we request that you keep the description of the results balanced and objective: for example, “excessive arborisation from zebrafish RGCs….” - “excessive” is an interpretation to fit your conclusion, a better descriptor would be "robust" or "extensive". The more speculative statements should be kept for in the Discussion.

Please detail your changes in a point-by-point response. If you do not agree with any of these criticisms or suggestions explain clearly why this is so.

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.

Second revision

Author response to reviewers' comments

Please find enclosed our second revised version of the manuscript “*Genetic Developmental Timing Revealed by Inter-Species Transplantations in Fish*”, by Jana Fuhrmann and colleagues. We have addressed the suggested modifications, which you will find **highlighted in color**.

In addition to the suggested modification, we have actualised the reference “Rayon *et al*” from the previous Rayon *et al*, Biorxiv 2019 to Rayon *et al*, Science 2020. We have also included an additional reference to the accompanying paper by Matsuda *et al*, Science 2020 and have changed a sentence in the Discussion accordingly (first sentence, page 14).

When uploading the manuscript, I noticed the “Outstanding Paper Prize” option, which I opted for.

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I want to let you know that the first author of the manuscript was a Master student at the time of doing the experiments - not a Ph.D. student. I could not find whether a Master student was eligible for the prize, and ticked the box in case she can be considered.

We are confident that the suggested changes had a positive impact on the manuscript and are looking forward to hear from you.

Point - by - point answers

1) I have read your revision and consulted with another editor and I am pleased to say that our overall evaluation is positive and we would like to publish a revised manuscript in Development.
We are excited about the positive evaluation, and have included yours and the other editor's suggestions in the revised version of the manuscript.

2) The additional data on retinal organisation is helpful. As you mention in the rebuttal, there appears to be some disorganisation with patches of laminations and clumps of differentiated cells. It would be useful for readers if this was clearly pointed out in the text.
We have highlighted this observation in the section where we report the histological and molecular analysis of the ectopic retinae in chimeras - page 7.

3) In addition, we would request that you carefully edit the text and legends to make sure that numbers are given for all observations (for instance, the numbers for the key observation that ath5:GFP is activated earlier in ectopic zebrafish retinae).
We have now updated all numbers for the different panels in the main figures and in the supplementary figures. We have also included references to the Supplementary Tables, where every transplantation event is described, and have clarified the concept of a transplantation event - Legend of Figure 2, and main text - page 5.

4) Figure legends should describe what is shown in the figure panels (with more labelling and guidance to the reader) and the more generic results statements should remain in the Results sections.
*We have changed the Figure legends according to these suggestions, an have added coordinates within the panels to assist the reader. Changes are **highlighted** as in the main text.*

5) Finally, we request that you keep the description of the results balanced and objective: for example, "excessive arborisation from zebrafish RGCs…." - "excessive" is an interpretation to fit your conclusion, a better descriptor would be "robust" or "extensive". The more speculative statements should be kept for in the Discussion.
*We have changed the wording in cases where we recognised subjective words - this is three cases in page 9, **highlighted**.*

Third decision letter

MS ID#: DEVELOP/2020/192500
MS TITLE: Genetic Developmental Timing Revealed by Inter-Species Transplantations in Fish
AUTHORS: Jana F Fuhrmann, Lorena Buono, Leonie Adelmann, Juan Martinez-Morales, and Lazaro Centanin 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. Where referee reports on this version are available, they are appended below.

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