Ccn2a is an injury-induced matricellular factor that promotes cardiac regeneration in zebrafish

Debanjan Mukherjee, Ganesh Wagh, Mayssa H. Mokalled, Zacharias Kontarakis, Amy L. Dickson, Amey Rayrikar, Stefan Günther, Kenneth D. Poss, Didier Y. R. Stainier and Chinmoy Patra
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First decision letter

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MS TITLE: Ccn2a is an injury-induced matricellular factor that promotes cardiac regeneration in zebrafish

AUTHORS: Debanjan Mukherjee, Mayssa Mokalled, Zacharias Kontarakis, Ganesh Wagh, Amy L. Dickson, Amey Rayrikar, Stefan Günther, Kenneth D. Poss, Didier Stainier, and Chinmoy Patra

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.

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

In the submitted manuscript, Mukherjee and colleagues link Ccn2a to ameliorated cardiac regeneration in zebrafish. The authors provide several lines of evidence to suggest that TGF-beta modulation by Ccn2a is the main mechanism of their findings.

Overall, the manuscript has a logical flow and presents the clear results thoroughly and with good figure support. The results are clearly of interest to the regeneration community and extend the authors' previous work on spinal cord injury and regenerative mechanisms.
Throughout the manuscript, several points do require the attention of the authors to clarify the interpretations and the used reagents to render the work acceptable for publication.

Comments for the author

* Major points:

1) p8, line 11, the authors say approximately 60% of injured hearts in WT show vigorous at 60dpci, while 50% of hets fail to regenerate.
a) Can the authors provide more information to explain the absence of fully penetrant regenerative phenotypes? Is this typical and are there other examples in the literature to back this observation as regular occurence?
b) p8, line 14-15 What percentage of hets and homos developed this persistent collagenous scar?

2) Does the ctgfabns50 (referred to as ctgfa-) allele make a truncated protein or is it a null allele? This does not seem to have been investigated in the authors' previous work (Mokalled et al., 2016). Is the effect observed in hets due to haploinsufficiency or possibly dominant-negative function? Further while the heterozygous phenotypes are brought up, heterozygous animals are not mentioned in the majority of experiments throughout the text, such as:
- CM infiltration
- CM proliferation
- pSmad3 levels

Related, the authors state “TGF harbors four protein interaction domains and a protease domain that self-cleaves CTGF into profibrotic N-terminal and proliferative C-terminal peptides (11, 12).” Although their previous work has shown the C-terminus is responsible for pro-regenerative activity, this was tested in the spinal cord (Mokalled et al., 2016). Could a functional N-terminus from a truncated protein in mutants conbtribute differently to cardiac regeneration, i.e. through the IGFB domain as per protein schematic? These points should be clarified to properly interpret the results, i.e. describe het phenotypes in settings above, measure heterozygous transcript levels, possibly assess formation of truncated protein.

3) The authors chemically perturb Smad3 activity throughout the animals, which might lead to an impact beyond the heart itself, i.e. immune system modulation vascular properties, etc. Do the authors have any non-cardiac parameters they could show to indicate that the observed effects are predominantly autonomous to the heart, i.e. impact on collagen gene expression.

4) Can the authors provide more context on the differences between the injury models used, including
- Why is ccn2b expressed after cryoinjury but not amputation?
- Why do hets and homo show a much more similar “regenerative profile” with ventricular resection vs. cryoinjury? (complete, moderate, poor)
- Why is CM proliferation different?

5) The LUTs in the figures are not particularly amenable to color-impaired readers and should be revisited for alternatives (yellow magenta, or similar).
Further, arrowheads in several figure panels are miniscule and should be enlarged to render them more visible.

6) The authors link Ccna2 proteins to the proper signaling of TGF-beta ligands through Smads in cardiac injury. While definitely of interest to cardiac regeneration and likely an exciting future avenue of research, the link between Ccna2 and TGF-beta is hardly new and has been described in other tissues and cell types, i.e. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3132735/ https://www.nature.com/articles/s41598-018-25370-3 The authors are encouraged to revisit the description of this interplay and to provide references to previous work linking the two, including in the discussion where the authors speculate about modes of function. This does not impact any novelty as the authors provide ample other data in their paper.

* Minor points:

* Throughout the manuscript, the authors should revisit the official nomenclature conventions for individual genes or their generalizations, i.e. SMAD3.

* In the introduction, the authors glance over the existing data of human fetal and neonatal regeneration that is quite capable and would deserve a mention here.
* In the introduction, the authours may want to rephrase two consequtive uses of "belief" (p3 line11, line 15)

* The role of the ECM in signaling is known since quite a while beyond what the authors state, especially from work in Drosphila, and should be cited more thoroughly.
* The screening for ECM molecules should be rephrased to ECM genes.

* In the introduction, p4, the authors introduce ccn2a also known as ctgfa. A couple of sentences later ccn2 is introduced as ctgfa, which becomes redundant.

* In the introduction, the authors state that ccn2a is expressed in injured zebrafish cardiac tissue, followed by stating "it is interesting that" the expression of ccn2 is incresed in regenertaing heart. This expression seems rather expected, than interesting, based on the known data.

* In p4, line 11, the authors state that CCN2 expression is related to the progression of fibrosis. The uninitated reader would certainly appreciate if the authors could specify in what way the "progression" during fibrosis is observed (increased vs decreased, etc.).

* The initial descriptions of ccn2a and b expression (p5, first results paragraph) are confusing and seemginly inconsistently described. First, the expression levels were measured on homogenized bulk tissue without any information of cellular composition, so any quantification relative to other genes seems moot. The description of wildtype expression (or lack thereof) is confusing and should be reworded.

* The observation that ccn2a is induced in injured endocardial cells is described as being made first with the BAC reporter. Did the authors possibly see this also with the in situs earlier?

* p10, the stage of the observation should be specified (i.e. line 13/14). The absence of CMs at this stage, shortly after onset of vessel sprouting, is then hardly surprising given the sequence of events.
Related, the phenotype description on p11, line 10 should be better specified (wt, mutant, etc.)

* p18, the authors introduce a next transcriptomics analysis but the input material is not well described. How was the tissue composition and how would this affect or bias the genes to be found?

* p18, the authors show that they found additional genes that had decreased expression. Why were these not picked up in the original transcriptome analysis that started the manuscript?

* p19: col1a1, and col1a2 are unaffected in ccn2 mutants, are these unaffected in ccn2 overexpression experiments?

* p21: serpine1 and mixL are decreased in ccn2 mutants, are these increased in ccn2 overexpression experiments?

* Typos:
Page 4, line 22 – CCN2 should be Ccn2 since it's zebrafish protein
Page 5, line 16 – "is upregulated upon cardiac injury", should be "are upregulated…"
Page 7, line 10 – injure should be injury
Page 27, line 8 - typo: damage should be damaged
Page 27, line 9 - the authors talk about a striking difference but do not describe it.
Page 28, line 5 - typo, gene should be genes?

Reviewer 2

Advance summary and potential significance to field

In this article Mukherjee et al study the role of the gene ccna2 also known as ctgf, during heart regeneration in the zebrafish.
Ctgf had previously been shown to be essential for spinal cord regeneration in the zebrafish (Mokalled et al 2016 Science), here the authors explore its role in the heart.
For this they use null mutant lines as well as heat shock inducible lines.
They observe that loss of function impairs cardiomyocyte proliferation while overexpression enhances cardiomyocyte proliferation.
Ccna2 mutant heart revealed changes in ECM proteins such as fibronectin previously shown to be involved in heart regeneration. It also leads to reduced levels of pSmad staining in cardiomyocytes. This observation promoted authors to assess if there is an interplay between tgfb signaling and ccn2a. They found that pharmacological inhibition of Tgfb also affected expression of ECM genes as found in ccna2 mutants and cardiomyocyte proliferation was also reduced confirming previous results (Chablais and Jazwinska 2012 Development).

Overall, it is an interesting study with a quite complete characterization of the role of cnna2 on heart regeneration. I made few suggestion to improve the quality of the figures and data presented. However, the link to tgfb signaling is in my view still quite preliminar and the proposed model is not fully sustained by the data provided.

Comments for the author

Major comment:
The relation between ccna2 and tgfb signaling is very weak. The authors would need to provide more evidence functionally linking both pathways. For example they could assess what happens in cases of pharmacological inhibition of Tgfb and overexpression of ccna2, compared to ccna2 gain of function alone. They could also perform a qPCR for tgfb in cnn2a to support immunostaining shown in Fig 8A.

Minor comments: Some of the Figures need to be revised and include separate channels for better visualization of co-stainings and revision of scale bars. I would also recommend the use of sd and not sem when replicate numbers are small.

Figure 1
Figure 1D-E: scale bars do not match each other. In E, a higher magnification is shown but scale bars remain same size as in D.
Figure 1D-E: It would be nice to compare D and E with the same magnification, as it could inform about expression dynamics on ccn2a and ccn2b in the whole heart upon heart resection, not only in the injury area.
Figure 1F-G: it would be nice to show a merged + single channels in gray scale in order to better determine the collocalization.
Figure 2
The authors use a semi-quantitative method to estimate the regeneration of the heart. Nevertheless, no information regarding the exact criteria to classify the regeneration is explained neither in the main text or material and methods. In many studies addressing fibrosis regression in the zebrafish, a % of injured ventricle is quantified as: total fibrotic area/(total myocardium area+total fibrotic area) in all the sections of the heart. Please include also quantification of the fibrotic area, not only the ratio.

Figure 3
Figure 3A, D: define how thick the maximum intensity projection of each panel is in figure legend.
Figure 3B-F: unit of area is not defined.
Figure legend: “total number of CMs in the injured tissue of each sample was considered as 100%”. Do the authors refer that the total number of CMs was averaged and this value used for normalizing the rest of values to 100%?
Figure 4
Figure 4A, C: the authors show images of cardiomyocyte proliferation stained by EdU incorporation at 4 dpci. Please also include images at 7 dpi, as this corresponds to the timepoint where cardiomyocytes proliferation peaks.
Additionally, as described in Figure S4, no differences in terms of cardiomyocytes infiltration into the injury area via coronary arteries are observed. Would it be better to show images from 7 dpi hearts?
Is the effect on proliferation specific to cardiomyocytes? Authors could provide also number of non-cardiomyocytes.
Figure 5
Is there is higher cardiomyocytes infiltration into the injury area in ccn2a overexpressing animals?
Figure 5D, E: addressing regeneration as % injured ventricle would be a better parameter than the semi-quantitative method used.

Material and methods: kdrl:HRAS-mCherry is not included in the zebrafish lines used in the paper.
Figure S1 Figure S1A: revise scale bars in B and D.
Figure 1D-E: it would be nice to see overview of the hearts to check the expression of ccn2a in remote parts of the heart upon resection.
Legend: dpci not defined in abbreviations.

Figure S3
Figure S3D appears stretched.

First revision

Author response to reviewers' comments

POINT-BY-POINT RESPONSES TO THE REVIEWERS' COMMENTS

We thank both the reviewers for their time to go through our manuscript thoroughly and for their insightful comments. Please find reviewers comments address point by point (in blue font) in our reply below. Alterations to the original manuscript are displayed in red font in the revised marked version of our manuscript.

Reviewer 1

* Major points:

1) p8, line 11, the authors say approximately 60% of injured hearts in WT show vigorous at 60dpci, while 50% of hets fail to regenerate.

a) Can the authors provide more information to explain the absence of fully penetrant regenerative phenotypes? Is this typical and are there other examples in the literature to back this observation as regular occurrence?

b) p8, line 14-15 What percentage of hets and homos developed this persistent collagenous scar?

We thank the reviewer for pointing this out and apologize for not clearly describing the analysis.

We have used below criteria to classify the regeneration process. Essentially, we have measured

the area of the collagenous scar that lack CMs invasion on the tissue section of 60 and150 dpci hearts. If the highest collagenous scar area without any visible invading muscle is < 2000 μ^2, then that heart was scored as 'vigorously regenerating heart'. A scar area > 15000 μ^2 was scored as 'failed to regenerate' or 'having persistent collagenous scar' at that time point.

Thus, when we refer to '60% vigorous regeneration' it indicates that 60% wild type animals show scar area <2000 μ^2. However, none of the wild type animals show persistent scar at 60 dpci. Unlike the wild type animals, around 45% heterozygous and all mutant animals show persistent scar at 60 dpci. This variability in the rates of regeneration among the animals from a specific genotype is likely to be due to difference in the amount of collagenous scar generated which arises due to the manual injury process and variation among the biological replicates.

The variability in scar tissue has also been reported in a study by Chablais et al (PMID: 21473762). They have shown variability in scar volume among injured wild type hearts at a specific time point after injury.

Based on the above criteria, regeneration in the different genotypes at 60 and 150 dpci are as follows:

At 60 dpci
Wild-type: None of the hearts show persistent scar.
Heterozygous: ~45% hearts show persistent scar.
Mutant: 100% hearts show persistent scar.

At 150 dpci
Wild-type: None of the hearts show persistent scar.
Heterozygous: ~14% hearts show persistent scar.
Mutant: ~60% hearts show persistent scar.
We have also clarified this in the text

Changes to the manuscript:
a. Addition to MATERIALS AND METHODS: 'Degree of heart regeneration was analyzed based on the presence of collagenous scar on the 60 dpci or 150 dpci heart tissue sections. For each heart, the section showing the highest area covered by scar tissue was considered for analysis. If the highest area covered by the collagenous scar without any visible invading muscle on the tissue section was < 2000 μ^2, then that heart was considered as 'vigorously regenerating heart' and if the scar area was > 15000 μ^2, then that heart was considered as 'poorly regenerating heart' or having persistent collagenous scar at that time point. To group the hearts based on degree of regeneration, we considered complete regeneration when highest scar area was <2000 μ^2, moderate regeneration when highest scar area was in between 2000-15000 μ^2, and very poor regeneration when highest scar area was >15000 μ^2.' (Page 19, Line# 16-25).

b. Addition to Figure: New analysis data provided in Panel C and D of Figure 2 (New panels showing highest scar area of each heart).

c. Addition to Results: 'approximately 14%' and '60%'. (Page 6, Line# 19).

2) Does the ctgfabns50 (referred to as ctgfa-) allele make a truncated protein or is it a null allele? This does not seem to have been investigated in the authors' previous work (Mokalled et al., 2016). Is the effect observed in hets due to haploinsufficiency or possibly dominant-negative function? Further, while the heterozygous phenotypes are brought up, heterozygous animals are not mentioned in the majority of experiments throughout the text, such as:

-CM infiltration

-CM proliferation

-pSmad3 levels

Related, the authors state "TGF harbors four protein interaction domains and a protease domain

that self-cleaves CTGF into profibrotic N-terminal and proliferative C-terminal peptides (11, 12)." Although their previous work has shown the C-terminus is responsible for pro-regenerative activity, this was tested in the spinal cord (Mokalled et al., 2016). Could a functional N-terminus from a truncated protein in mutants conbtribute differently to cardiac regeneration, i.e. through the IGFB domain as per protein schematic? These points should be clarified to properly interpret the results, i.e. describe het phenotypes in settings above, measure heterozygous transcript levels, possibly assess formation of truncated protein.

To evaluate whether ctgfa bns50 allele is a null allele, we have checked the *ccn2a* transcript level in wild type, *ccn2a*$^{+/-}$, and *ccn2a*$^{-/-}$ embryos at 48 hpf. Our qPCR analysis shows that *ccn2a* transcripts are reduced by approximately 40% in het and approximately 66% in *ccn2a* mutants relative to wild-type embryos, suggesting that the mutated *ccn2a* transcript is unstable. Thus the effect observed in *ccn2a*$^{+/-}$ is likely due to haploinsufficiency.

We have included the heterozygous phenotype data in the revised version of the manuscript.

Changes to the manuscript:

a. Addition to MATERIALS AND METHODS: 'For *ccn2a* transcripts stability analysis, total RNA was isolated from 48 hpf *ccn2a*$^{+/+}$, *ccn2a*$^{+/-}$, and *ccn2a*$^{-/-}$ embryos using Trizol reagent (Invitrogen) according to the manufacturer's instructions. 40 embryos were pooled per biological replicate, and 2 µg of total RNA was reverse transcribed into cDNA with MMLV reverse transcriptase (Invitrogen).' (Page 16, Line# 19-23).

b. Addition to Figure: New analysis data provided as Panel G in Figure 2 (showing quantitative analysis of *ccn2a* transcripts in wild-type, het and mutant embryos).
New analysis data provided as Panel C in Figure 7 (showing quantification of nuclear pSmad3 in 4 dpci wildtype het and mutant hearts).

c. Addition to Results:
'Since heterozygous animals also showed poor heart regeneration, we sought to explore whether the heart regeneration phenotype is due to the formation of a functional, N-terminal-truncated Ccn2a protein in mutants, which contributes differently to the regeneration process or due to the haploinsufficiency of Ccn2a. We measured expression levels of *ccn2a* transcripts in *ccn2a*$^{+/+}$, *ccn2a*$^{+/-}$, and *ccn2a*$^{-/-}$ embryos at 48 hpf, qPCR analysis showed that *ccn2a* transcript level is reduced by approximately 40% in *ccn2a*$^{+/-}$ and approximately 66% in *ccn2a*$^{-/-}$ relative to wild- type embryos (Fig. 2G), suggesting that mutated *ccn2a* transcript is unstable and the cardiac phenotype in *ccn2a*$^{+/-}$ is likely due to haploinsufficiency.' (Page 6, Line# 22-30).

'CM infiltration was also impaired in *ccn2a*$^{+/-}$ animals (Fig. S3B)'. (Page 7, Line# 20).

'Although CM proliferation was not affected in 4 dci *ccn2a*$^{+/-}$ hearts, an approximately 65% reduction in CM proliferation was detected in *ccn2a*$^{+/-}$ hearts relative to wild-type hearts at 7 and 12 dpci (Fig. 4A,B and Fig. S6). Non-CM proliferation in 7 dpci hearts remains indistinguishable among the three genotypes (Fig. 4C).' (Page 8, Line# 17-20).

'Moreover, statistical analysis indicated a trend toward a decreasing number of nuclear pSmad3 positive cells in injured *ccn2a* heterozygous hearts compared to wild-type controls (Fig. 7C).' (Page 11, Line# 6-8).

3) The authors chemically perturb Smad3 activity throughout the animals, which might lead to an impact beyond the heart itself, i.e. immune system modulation, vascular properties, etc. Do the authors have any non-cardiac parameters they could show to indicate that the observed effects are predominantly autonomous to the heart, i.e. impact on collagen gene expression.

We have analyzed the expression of collagen and fibronectin genes in other organs like eyes, brain and vertebral tissue after 3 days of treatment with the SB431542. We don't see significant effect on the expression of collagen and fibronectin genes in the above mentioned organs upon SB431542 treatment in compared to the DMSO treated control. This suggests, observed effects in the 4 dpci wild-type heart upon pharmacological inhibition of Tgfß pathway has context- specific targets.

Changes to the manuscript:

a. Addition to Figure: New analysis data provided as Figure S10 (showing relative expression of collagen and fibronectin genes in no-cardiac tissue).
b. Addition to Results:

'Next, we explored whether global pharmacological perturbation of Tgfß signaling has an impact on the Tgfß mediated functions in other healthy tissues. We have analyzed the expression of *col1a1a*, *col11a1*, *col15a1b*, *fn1b*, and *fn1b*, in non-cardiac tissue upon pharmacological inhibition of Tgfß pathway by SB431542. Decreased expression of *col11a1,* and *col1a1a* transcripts were observed in the healthy vertebral tissue, and eyes, respectively upon the inhibition of Tgfß pathway (Fig. S10). However, the expression of other collagen and fibronectin genes remained unaltered in these tissues. Moreover, in the brain, inhibition of Tgfß pathway had no detectable effect on the expression of these genes (Fig. S10). Taken together, these results indicate that pharmacological inhibition of Tgfß pathway has context-specific targets.' (Page 12, Line# 12-21).

) Can the authors provide more context on the differences between the injury models used, including Why is ccn2b expressed after cryoinjury but not amputation?

One of the critical differences between the cryoinjury and resection models is the amount of scar tissue produced. After cryoinjury, massive transient scar appears in place of the dead tissue mass; in contrast, in resection-injured hearts, a smaller amount of scar appears mostly superficially on the cut surface of the ventricle. Thus, it is possible that the expression level of *ccn2b* was much less in amputated heart compared to in cryoinjured heart. We didn't detect *ccn2b* in amputated heart; presumably expression is below detection limits by ISH method.

- Why do hets and homo show a much more similar "regenerative profile" with ventricular resection vs. cryoinjury? (complete, moderate, poor)

When we compare heterozygous and homozygous mutant hearts at 60 and 150 dpci, it is clear that homozygous mutants show increased severity in the phenotype (Figure 2).

At 60 dpci, where 100% mutants shown persistent scar, only 45% hets shown persistent scar at this stage. Similarly, at 150 dpci, where 60% mutants shown persistent scar, only 14% hets shown persistent scar (Figure 2). Suggesting, *ccn2a*$^{+/-}$ heart has better regenerative capacity in compare to *ccn2a*$^{-/-}$.

- Why is CM proliferation different?

As discussed in the manuscript, one of the critical differences between the cryoinjury and resection models is the amount of scar tissue produced. After cryoinjury, massive transient collagen appears in place of the dead tissue mass; in contrast, in resection-injured hearts, a smaller amount of collagen localizes mostly superficially on the cut surface of the ventricle. Indicates, scar composition, scar amount, and mechanical properties of the scar tissue are different between this two injury models. It is possible that the influence of Ccn2a on CM proliferation is dependent on the molecular and mechanical properties of the scar. Thus, CM proliferation phenotype is different between the two injury models.

5) The LUTs in the figures are not particularly amenable to color-impaired readers and should be revisited for alternatives (yellow magenta, or similar). Further, arrowheads in several figure panels are miniscule and should be enlarged to render them more visible.

As suggested, we have replaced red and green with magenta and yellow, respectively throughout the manuscript. We have also enlarged arrows and arrowheads throughout the manuscript.

6) The authors link Ccna2 proteins to the proper signaling of TGF-beta ligands through Smads in cardiac injury. While definitely of interest to cardiac regeneration and likely an exciting future avenue of research, the link between Ccna2 and TGF-beta is hardly new and has been described in other tissues and cell types, i.e.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3132735/

https://www.nature.com/articles/s41598-018-25370-3

The authors are encouraged to revisit the description of this interplay and to provide references to previous work linking the two, including in the discussion where the authors speculate about modes of function. This does not impact any novelty as the authors provide ample other data in their paper.

We have included the information and made the below changes to the manuscript.

Changes to the manuscript:

a. Addition to Results: 'It has been described that CCN2 regulates TGFß1-induced expression of fibronectin in Graves' orbital fibroblasts (Tsai et al., 2018). (Page 10, Line# 29-30).

b. Addition to Discussion: 'and CCN2 regulates TGFß1-induced expression of fibronectin *in vitro* (Tsai et al., 2018).' (Page 13, Line# 23).

'Another study using human fibroblasts suggested that CCN2 regulates TGFß-induced phosphorylation of Smad1 through the integrin $\alpha_V\beta_3$ receptor (Nakerakanti et al., 2011).' (Page 14, Line# 7-9).

* Minor points:

* Throughout the manuscript, the authors should revisit the official nomenclature conventions for individual genes or their generalizations, i.e. SMAD3.

We made suggested corrections throughout the manuscript.

* In the introduction, the authors glance over the existing data of human fetal and neonatal regeneration that is quite capable and would deserve a mention here.

* In the introduction, the authours may want to rephrase two consequtive uses of "belief" (p3 line11, line 15)

We have rephrased.

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We have cited old articles about ECM in signaling.

* The screening for ECM molecules should be rephrased to ECM genes.

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* In p4, line 11, the authors state that CCN2 expression is related to the progression of fibrosis. The uninitated reader would certainly appreciate if the authors could specify in what way the "progression" during fibrosis is observed (increased vs decreased, etc.).

We have edited.

* The initial descriptions of ccn2a and b expression (p5, first results paragraph) are confusing and seemginly inconsistently described. First, the expression levels were measured on homogenized bulk tissue without any information of cellular composition, so any quantification relative to other genes seems moot. The description of wildtype expression (or lack thereof) is confusing and should be reworded.

We are sorry for this confusion. The initial qPCR analysis was carried out using total RNA isolated from cardiac ventricles. The expression of genes was normalized to *ef1a*. Subsequently, we have performed RNA *in situs* on paraffin section using *ccn2a* and *ccn2a* probes to explore the tissue specific expression. These details have now been included in the first paragraph of the results section and we hope that this will bring more clarity to the reading. (Page 5, Line# 3-12).

* The observation that ccn2a is induced in injured endocardial cells is described as being made first with the BAC reporter. Did the authors possibly see this also with the in situs earlier?

We have not performed double *in situs* to see if *ccn2a* is expressed in endocardial cells upon injury. However, as presented in the manuscript both the *in situ*- and reporter line based analysis shown similar domain of *ccn2a* expression in healthy and injured hearts.

* p10, the stage of the observation should be specified (i.e. line 13/14). The absence of CMs at this stage, shortly after onset of vessel sprouting, is then hardly surprising given the sequence of events. Related, the phenotype description on p11, line 10 should be better specified (wt, mutant, etc.).

We have made the suggested changes to the text.

* p18, the authors introduce a next transcriptomics analysis but the input material is not well described. How was the tissue composition and how would this affect or bias the genes to be found?

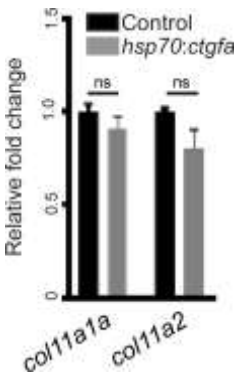
For the transcriptomics analysis described in Fig 6A, the input material was RNA isolated from 4 dpci wild type and *ccn2a*$^{-/-}$ cardiac ventricles (bulbus arteriosus and atrium were dissected out from each heart). We have clarified this in the revised manuscript 'Materials and Methods'. (Page 20, Line# 9-11).

* p18, the authors show that they found additional genes that had decreased expression. Why were these not picked up in the original transcriptome analysis that started the manuscript?

The additional genes showed decreased expression in RT-PCR analysis also shown the same tendency in the RNAseq analysis, but the counts had a high variation between two replicates. Hence they were not selected.

* p19: col1a1, and col1a2 are unaffected in ccn2 mutants, are these unaffected in ccn2 overexpression experiments?

May be reviewer means *col11a1a* and *col11a2*. We have tested the expression level of the two genes and our data suggests that both genes remained unaffected upon Ccn2a overexpression (see below).



* p21: serpine1 and mixL are decreased in ccn2 mutants, are these increased in ccn2 overexpression experiments?

We have performed the experiment and data suggest that transcripts levels of *serpine1* and *mixl1* are increased upon Ccn2a overexpression. We have included this result in the revised manuscript.

Changes to the manuscript:

a. Addition to Figure: New analysis data provided as Panel G Figure 7.

b. Addition to Results: 'and expression of *serpine1* and *mixl1*, was increased by approximately 8- and 1.6 fold, respectively'. (Page 11, Line# 12-13).

* Typos:

Page 4, line 22 – CCN2 should be Ccn2 since it's zebrafish protein

Corrected

Page 5, line 16 – "is upregulated upon cardiac injury", should be "are upregulated…"

Corrected

Page 7, line 10 – injure should be injury

Corrected

Page 27, line 8 - typo: damage should be damaged

Corrected

Page 27, line 9 - the authors talk about a striking difference but do not describe it.

We have rephrased.

Page 28, line 5 - typo, gene should be genes?

Corrected

Reviewer 2

Reviewer 2 Comments for the Author:

Major comment:

The relation between ccna2 and tgfb signaling is very weak. The authors would need to provide more evidence functionally linking both pathways. For example, they could assess what happens in

cases of pharmacological inhibition of Tgfb and overexpression of ccna2, compared to ccna2 gain of function alone.

We thank the reviewer for this suggestion. We have compared the nuclear localization of pSmad3 among the wild type, Ccn2a overexpressing heart and upon pharmacological inhibition of TgfB/pSmad3 signaling in Ccn2a gain-of-function condition. Our analysis suggests that pharmacological inhibition of TgfB/pSmad3 signaling suppress the Ccn2a gain-of-function mediated activation of pSmad3 nuclear localization. These results have now been incorporated as part of the manuscript as given below.

Changes to the manuscript:

a. Addition to Figure: New data provided as Panel H-J in Figure 8.

b. **Addition to Results:** 'Next, we investigated whether Ccn2a-mediated activation of nuclear pSmad3 localization acts through the TgfB/pSmad3 pathway. We found that pharmacological inhibition of TgfB signaling cascade suppresses the Ccn2a gain-of-function mediated activation of pSmad3 nuclear localization (Fig. 7H-J); consistent with a mechanism in which Ccn2a positively regulates nuclear pSmad3 localization through the TgfB/pSmad3 signaling cascade.. (Page 11, Line# 15-19).

They could also perform a qPCR for tgfb in cnn2a to support immunostaining shown in Fig 8A.
We have analyzed *tgfB1*, *tgfB2*, and *tgfB3* transcript level in wild-type, *ccn2a*$^{-/-}$, Ccn2a gain-of-function situation. Both loss- and gain-of-function data indicates that Ccn2a does not regulate expression of *tgfB* genes in regenerating heart (Fig. S8 in the earlier version of the manuscript and Fig. S9 in the present manuscript). (Page 11, Line# 24-28).

Minor comments: Some of the Figures need to be revised and include separate channels for better visualization of co-stainings and revision of scale bars. I would also recommend the use of sd and not sem when replicate numbers are small.

As suggested we have used mean ± SD wherever n≤4.

Figure 1

Figure 1D-E: scale bars do not match each other. In E, a higher magnification is shown but scale bars remain same size as in D.

Figure 1D-E: It would be nice to compare D and E with the same magnification, as it could inform about expression dynamics on ccn2a and ccn2b in the whole heart upon heart resection, not only in the injury area.
As suggested, we have replaced the images of Figure 1E.

Figure 1F-G: it would be nice to show a merged + single channels in gray scale in order to better determine the collocalization.

We have provided single channels and merged images as suggested.

Figure 2

The authors use a semi-quantitative method to estimate the regeneration of the heart. Nevertheless, no information regarding the exact criteria to classify the regeneration is explained neither in the main text or material and methods. In many studies addressing fibrosis regression in the zebrafish, a % of injured ventricle is quantified as: total fibrotic area/(total myocardium area+total fibrotic area) in all the sections of the heart. Please include also quantification of the fibrotic area, not only the ratio.

We thank the reviewer for this suggestion. We have added information regarding the used criteria to classify the regeneration in the material and methods and included quantification of the fibrotic area for cryoinjury model.

For the amputation model we have used only the semi-quantitative method in our papers. Because the injury is small and on the periphery, quantifying collagen is prone to error or misinformation.

Changes to the manuscript:

a. Addition to MATERIALS AND METHODS: 'Degree of heart regeneration was analyzed based on the presence of collagenous scar on the 60 dpci or 150 dpci heart tissue sections. For each heart, the section showing the highest area covered by scar tissue was considered for analysis. If the highest area covered by the collagenous scar without any visible invading muscle on the tissue section was < 2000 μ^2, then that heart was considered as 'vigorously regenerating heart' and if the scar area was > 15000 μ^2, then that heart was considered as 'poorly regenerating heart' or having persistent collagenous scar at that time point. To group the hearts based on degree of regeneration, we considered complete regeneration when highest scar area was <2000 μ^2, moderate regeneration when highest scar area was in between 2000-15000 μ^2, and very poor regeneration when highest scar area was >15000 μ^2.' (Page 19, Line# 16-25).

b. Addition to Figure: New analysis data provided in Panel C and D of Figure 2 and Panel F of Figure 5.

Figure 3

Figure 3A, D: define how thick the maximum intensity projection of each panel is in figure legend.

The thickness of maximum intensity projection is in-between 25-35 µm. We have mentioned the thickness of maximum intensity projection in figure legend.

Figure 3B-F: unit of area is not defined.
In the revised manuscript we have defined the Unit (µm^2).

Figure legend: "total number of CMs in the injured tissue of each sample was considered as 100%". Do the authors refer that the total number of CMs was averaged and this value used for normalizing the rest of values to 100%?

In Fig.3B and E, total number of infiltrating CMs for each individual heart was considered as 100%. Based on this, we calculated the % of CMs that infiltrate along the coronaries, and away from coronaries. Thus each dot in the graph reflects % of infiltrating CMs in a given injured heart either along the coronaries or away from the coronaries.

For Fig.3C and F, we counted total number of CMs in the injured tissue of each wild-type heart and averaged. The average value of the wild-type hearts was considered as 100% and this value used for normalizing the rest of the values.

Figure 4

Figure 4A, C: the authors show images of cardiomyocyte proliferation stained by EdU incorporation at 4 dpci. Please also include images at 7 dpi, as this corresponds to the time point where cardiomyocytes proliferation peaks. Additionally, as described in Figure S4, no differences in terms of cardiomyocytes infiltration into the injury area via coronary arteries are observed. Would it be better to show images from 7 dpi hearts?

We thank the reviewer for this suggestion. We have included images of proliferation at 7 dpci (Figure S6)

In Figure S4, we have shown there is no CM infiltration at 4 days post amputation (dpa), however new coronary vessels covered the entire apical wound at 4 dpa. This data suggest that coronary angiogenesis precedes the appearance of CMs in the injured tissue.

We have shown images of CM infiltration at 7 days post cryoinjury (dpci) and at 7 days post amputation (dpa) in Figure 3.

Is the effect on proliferation specific to cardiomyocytes? Authors could provide also number of non-cardiomyocytes.

We have analyzed the degree of non-cardiomyocytes proliferation in whole mount 7 dpci hearts.

Data suggest that non-cardiomyocytes proliferation remains unaffected in *ccn2a*$^{+/-}$ and *ccn2a*$^{-/-}$ hearts.

Changes to the manuscript:

a. Addition to Figure: New analysis data provided in Panel C of Figure 4

b. Addition to Results: 'Non-CM proliferation in 7 dpci hearts remains indistinguishable among the three genotypes (Fig. 4C).' (Page 8, Line# 19-20).

Figure 5

Is there is higher cardiomyocytes infiltration into the injury area in ccn2a overexpressing animals?

We have analyzed CMs infiltration in the wound at 4 dpci upon Ccn2a overexpression. We do see higher CM infiltration into the wound in Ccn2a overexpressing animals (See below). Arrows indicate infiltrated CMs.

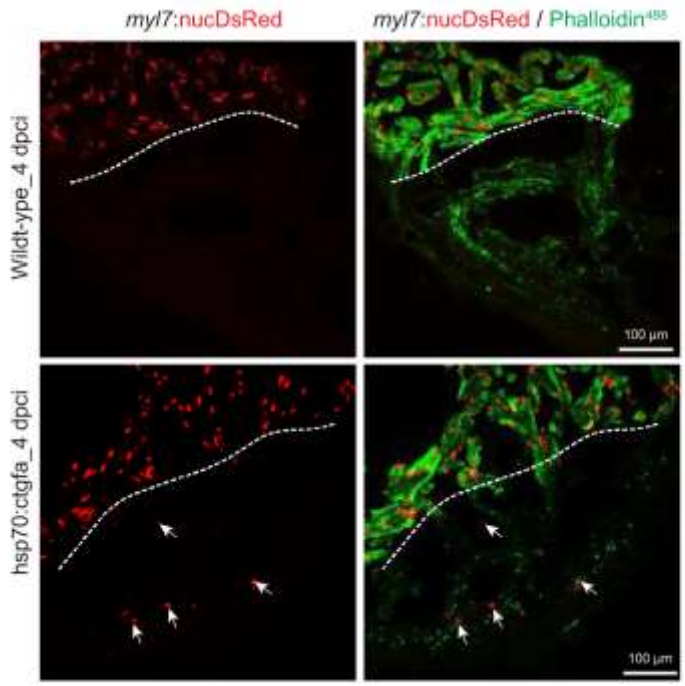


Figure 5D, E: addressing regeneration as % injured ventricle would be a better parameter than the semi-quantitative method used. Material and methods: kdrl:HRAS-mCherry is not included in the zebrafish lines used in the paper.

We thank the reviewer for this suggestion. We have analyzed scar area and the new analysis provided in Panel F of Figure 5.

We have also included *kdrl:HRAS-mCherry* line in 'Material and methods'. (Page 16, Line# 5).

Figure S1

Figure S1A: revise scale bars in B and D.

We have made corrections.

Figure 1D-E: it would be nice to see overview of the hearts to check the expression of ccn2a in remote parts of the heart upon resection.

We have provided new images as suggested

Legend: dpci not defined in abbreviations.

We have included the information.

Figure S3

Figure S3D appears stretched. Replaced with a new dot plot

As suggested, we have provided a new dot plot (mean±s.d.).

Second decision letter

MS ID#: DEVELOP/2020/193219

MS TITLE: Ccn2a/Ctgfa is an injury-induced matricellular factor that promotes cardiac regeneration in zebrafish

AUTHORS: Debanjan Mukherjee, Ganesh Wagh, Mayssa Mokalled, Zacharias Kontarakis, Amy L. Dickson, Amey Rayrikar, Stefan GÃ¼nther, Kenneth D. Poss, Didier Stainier, and Chinmoy Patra

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. I am onlysuggesting text revisions. An additional comment was made that the figures are heterogeneous in resolution within individual figure panels (i.e. bar diagrams with low resolution); please ensure that you upload high resolution figures.

Reviewer 1

Advance summary and potential significance to field

The authors have added substantial and significant information to the manuscript that has rendered the work easier to understand and to follow. The authors should be applauded for their responsiveness to the provided reviewer input, and only a few minor points remain to be addressed.

Comments for the author

1) In line 13 of the introduction, the authors still refer to "molecules", which should rather be genes or gene products.

2) The authors did not address the criticism that the human fetal heart has considerable regenerative capacity that should be provided for context in the introduction of their work.

3) The still most-significant issue remains with the ccn2a allele used and the authors newly proposed data for haploinsufficiency of the used allele.
Defining a null allele in genetic terms requires cross to a deletion allele that cannot lead to any gene product, the phenotypes of this trans-heterozygosity then being compared to homozygous alleles in question. The criticism raised by the reviewer also referred to the nature of the used allele that could lead to a truncated protein. While the authors might not have an antibody, this detail should be pointed out.
Instead, the authors investigate mRNA levels - interesting, but not sure this addresses the underlying issue. They find that homozygous mutants still have 34%
mRNA compared to wildtype, while heterozygous animals are at 60%. Without knowing expression levels off each wildtype allele (which are not necessarily 50% each), drawing any conclusions about mRNA stability, etc. seem preliminary.
The information is nonetheless interesting for the reader to make up their mind about the nature of the underlying allele. The authors should clarify their findings as final expression percentage relative to wildtype (i.e. 34% mRNA for homozygous mutants, instead of 66% reduction). These data could indicate that heterozygous mutants have a phenotype-relevant reduction in gene expression and/or protein levels.

Reviewer 2

Advance summary and potential significance to field

see review 1

Comments for the author

Overall the suggestions were followed. I just have two remaining requests:

1) Figure 8I: the model is misleading since this work is not analyzing the role of ccn2a in stabilizing tgfb or modulating its activity nor are there to my understanding experiments assessing the effect on cxcr3 positive cells.
2) The title of Panel Figure 1A should be changed as overall inflammatory response is not well presented, the panel focusses on the fibrotic response.

Minor grammar mistakes:

1) Page 30 Line 14 ,and

2) Page 32 line 22 ccn2a+/-,and

Second revision

Author response to reviewers' comments

POINT-BY-POINT RESPONSES TO THE REVIEWERS' COMMENTS

We thank both the reviewers for their time to go through our revised manuscript thoroughly and for their insightful comments. Please find the address to the reviewers' comments in a point by point (in blue font) format in our reply below. Alterations to the original manuscript are displayed in red font in the revised marked version of our manuscript.

Reviewer 1:
* In line 13 of the introduction, the authors still refer to "molecules", which should rather be genes or gene products.

As suggested, we have replaced ‘molecules’ with ‘genes’ (Page 3, Line# 17).

* The authors did not address the criticism that the human fetal heart has considerable regenerative capacity that should be provided for context in the introduction of their work.

We have included the published information about the regenerative capacity of the human fetal hearts and the possibility to induce cell proliferation in post-mitotic human cardiomyocytes. Below changes have been made to the manuscript.

Changes to the manuscript:

Addition to Introduction: Another study has provided evidence that in humans, a high level of CM cytokinesis can be detected in infants, which was not visible after 20 years of age (Mollova et al., 2013). Importantly, Mohamed et al, (2018) have reported that overexpression of a combination of cell cycle regulators can induce cell division in post-mitotic human cardiomyocytes. (Page 3, Line# 10-14).

* The still most-significant issue remains with the ccn2a allele used and the authors newly proposed data for haploinsufficiency of the used allele. Defining a null allele in genetic terms requires cross to a deletion allele that cannot lead to any gene product, the phenotypes of this trans-heterozygosity then being compared to homozygous alleles in question. The criticism raised by the reviewer also referred to the nature of the used allele that could lead to a truncated protein. While the authors might not have an antibody, this detail should be pointed out. Instead, the authors investigate mRNA levels - interesting, but not sure this addresses the underlying issue. They find that homozygous mutants still have 34% mRNA compared to wildtype, while heterozygous animals are at 60%. Without knowing expression levels off each wildtype allele (which are not necessarily 50% each), drawing any conclusions about mRNA stability, etc. seem preliminary. The information is nonetheless interesting for the reader to make up their mind about the nature of the underlying allele. The authors should clarify their findings as final expression percentage relative to wildtype (i.e. 34% mRNA for homozygous mutants, instead of 66% reduction). These data could indicate that heterozygous mutants have a phenotype-relevant reduction in gene expression and/or protein levels.

We agree that the invoking haploinsufficiency may not be appropriate given that we are not absolutely certain about the nature of the allele. The possibility that the mutant allele gives rise to a truncated gene product cannot be ruled out and in the absence of an antibody this cannot be tested. However, heterozygous animals (*ccn2a*$^{+/-}$) shown less severe cardiac phenotype compared to homozygous mutants (*ccn2a*$^{-/-}$) indicates that the defect in regeneration is indeed due to loss of Ccn2a. We have edited the text as suggested and made the below changes to the manuscript.

Changes to the manuscript:
Addition to Results: Since heterozygous animals also showed poor heart regeneration, we measured expression levels of *ccn2a* transcripts in *ccn2a*$^{+/+}$, *ccn2a*$^{+/-}$, and *ccn2a*$^{-/-}$ embryos at 48 hpf. Our qPCR analysis showed approximately 60% and 34% transcripts are present in *ccn2a*$^{+/-}$ and *ccn2a*$^{-/-}$ animals, respectively relative to the wild-type embryos (Fig. 2G). Given the nature of the mutation, we cannot formally rule out the possibility that the mutant allele gives rise to a truncated protein. Nevertheless, more severe cardiac phenotype in *ccn2a*$^{-/-}$ animals compared to *ccn2a*$^{+/-}$ siblings and the decrease in transcript level in the heterozygous and mutants suggest that the phenotype is likely to be associated with loss of gene function. (Page 6, Line# 25-31 and Page 7, Line# 1-2).

Reviewer 2:

4) Figure 8I: the model is misleading since this work is not analyzing the role of ccn2a in stabilizing tgfb or modulating its activity nor are there to my understanding experiments assessing the effect on cxcr3 positive cells.

Yes, the role of Ccn2a in stabilizing Tgfß or modulating its activity is speculative at this stage. As suggested, we have removed this information from the model (Fig. 8I).

We have not assessed the role of Ccn2a on Cxcr3.1 positive cells infiltration and in the working model (Fig. 8I), we didn't claim about the role of Ccn2a on the appearance of Cxcr3.1 positive cells in the injured heart tissue.
However, similar to collagen, fibronectin and other ECM genes, we have analysed the *cxcr3.1* gene expression level in injured hearts from *ccn2a*$^{-/-}$, Ccn2a gain-of-function animals relative to wild-type control. In addition, we have analysed the *cxcr3.1* gene expression upon pharmacological inhibition of TGFβ pathway. Altogether, our data suggest that Ccn2a and TGFβ signalling pathway positively regulates collagen and other ECM genes expression and negatively regulates the *cxcr3.1* gene expression in injured cardiac ventricle. The model is aimed at highlighting this point.

5) The title of Panel Figure 1A should be changed as overall inflammatory response is not well presented, the panel focusses on the fibrotic response.

Sorry for the confusion. In the Panel Figure 1A, we have depicted the known sequential events of the heart regeneration in zebrafish. This scheme is not highlighting findings of the present study. To make it more clear we have inserted 'known' in the figure legend of Fig. 1A (Page 28, Line#4).

Minor grammar mistakes:

1) Page 30 Line 14 ,and

2) Page 32 line 22 ccn2a+/-,and

These have been corrected.

Third decision letter

MS ID#: DEVELOP/2020/193219

MS TITLE: Ccn2a/Ctgfa is an injury-induced matricellular factor that promotes cardiac regeneration in zebrafish

AUTHORS: Debanjan Mukherjee, Ganesh Wagh, Mayssa Mokalled, Zacharias Kontarakis, Amy L. Dickson, Amey Rayrikar, Stefan Günther, Kenneth D. Poss, Didier Stainier, and Chinmoy Patra
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.