Wnt1/βcatenin injury response activates the epicardium and cardiac fibroblasts to promote cardiac repair

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1st Editorial Decision 21 June 2011

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees appreciate the findings reported, but also find that further experiments and data are needed to fully support the conclusions reported. I suspect that you should be able to address most of the concerns raised. Referee #1 requests loss of function data to show the importance of Wnt1 in cardiac injury using conditional Wnt1 knockout mice. I don't know if you have access to such mice, otherwise we can discuss further how this issue can be resolved. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely
In the present study, the authors showed the association between Wnt1/b-catenin signaling and activation of epicardium and cardiac fibroblast during ischemia-reperfusion (IR) injury of the heart. The authors found induced expression of Wnt1 in the area of IR and epicardium. Using reporter systems, such as Wnt1-Cre/R26RlacZ, TOPGAL-Tg, Col1a2-CreER(T)/R26RlacZ, Wt1-Cre/R26RlacZ, the authors showed lacZ expressing was increased in epicardial cells and cardiac fibroblasts in the area of IR. Wnt1 induced cultured epicardial cells to activate their proliferation and to express pro-fibrotic gene, which suggest EMT in vitro. Deletion of b-catenin by Wt1-Cre or Col1a2-CreER(T) induced rapid LV dilatation and cardiac dysfunction after 8 days of IR, which may associate with deficiency of EMT and lack of collagen deposition. From these results, the authors concluded that Wnt1/b-catenin injury response is critically required for preserving cardiac function after IR.

In general the study is interesting, but premature lacking important data supporting their conclusion. This study would represent an important contribution if a number of concerns could be properly addressed.

1) Although the authors detected the responsive transactivation of Wnt1 in epicardial cells and cardiac fibroblasts during IR injury of the heart. They also identified the importance of the b-catenin pathway by loss-of-function of b-catenin. However, the authors did not show the critical requirement of Wnt1 using loss-of-function. Moreover, although the authors showed the RT-PCR results of Wnt family in Figure S1A, B, and C, these results never exclude the possibility of Wnts contribution other than Wnt1. To clarify the importance of Wnt1 in the IR injury response as in the title and the authors’ conclusion, it is necessary to analyze inducible and/or conditional Wnt1 knockout mice.

2) In Figure 1, how about protein level of Wnt1? The authors should present Western blotting for Wnt1 during cardiac injury.

3) Figure 1Dii and iii have some dust (?) in the field. Please replace to clean pictures.

4) In Figure 4, the authors showed EMT by the administration of Wnt1. Does b-catenin knockdown by siRNA block the EMT induced by Wnt1?

5) Procedure of IR is irregular and resultant cardiac function depends on infarct size. It is necessary to show infarct size and infarct area/area at risk (IA/AAR) ratio to verify the procedure. The authors should show infarct size and IA/AAR ratio, and representative figure, such as TTC staining and Evans blue staining, for Figure 5 and 6.

6) The authors showed representative M-mode pictures, cardiac function (%FS) and LV end systolic volume in Figure 5 and 6. Although anesthesia and heart rate influence the cardiac function, the authors did not mention animal condition during the echocardiography. For example, M-mode picture of tamoxifen injected Day8 IR in Figure 6C (lower left panel) showed very slow heart rate compared to the others. The authors should describe the anesthesia and the number of mice for the echocardiographic analysis in Experimental Procedure, and show echocardiographic parameters including heart rate on a table in the supplement. And please replace the picture of Tamoxifen injected Day8 IR to equal HR level in Figure 6C.

7) Cardiac functions after IR in Wt1-Cre/b-cateninfl/fl and Col1a2-CreER(T)/b-cateninfl/fl were evaluated at only one point (day8 after IR). Reduced fibrosis can often be associated with better cardiac function. To evaluate long term effects of deletion of b-catenin, please perform late IR (day 14 or 28).

8) The authors showed deletion of b-catenin by Western blotting in FigureS4E, but not in Figure S5.
Please show deletion of b-catenin by Col1a2-CreER(T) in the heart.

9) Measurement of epicardial thickness is tricky. Because, in Figure 1E at day10 after IR, Masson's staining showed no thickened epicardium in this picture, in Figure 2C, epicardium does not increased in this picture, while day2 image in Figure 1C showed thickened epicardium. Assuming that epicardium thickness depends on the observed section, please explain more detail about the measurement of epicardial thickness, and show representative sections to measure it.

10) Pictures of IR heart in this manuscript showed limited area. For better understanding, please add picture of macro or low magnification with staining (LacZ and Masson's) in the Supplemental data.

Referee #2

Adult epicardium has been shown to differentiate into cardiomyocytes, fibroblast and vascular cells in response to myocardial injury. Several signaling pathways involved in this process have also been identified, such as Wnt/b-catenin signaling. In this article, Duan et al. demonstrate a dynamic role for Wnt1/b-catenin signaling in promoting cardiac repair by activating epicardium and cardiac fibroblast. The authors show that Wnt1 was particularly activated in the epicardium and fibroblasts in the region of the injury. Following injury, the epicardium is activated in a Wnt/b-catenin dependent manner, expands, undergoes epithelial-mesenchymal transition to generate cardiac fibroblasts. These fibroblasts are shown to respond to b-cat/TCF signaling in vivo using the TOPGAL reporter mice that express lacZ driven by TCF4 response elements. Disruption of downstream b-catenin expression in epicardium or fibroblasts following acute myocardial injury (ischemia reperfusion protocol) impairs the injury response and leads to decreased cardiac function.

Overall, this is a very interesting study demonstrating a positive role for Wnt/b-catenin signaling in cardiac repair that should be of great interest to the field. The authors effectively utilize different in vitro and in vivo approaches to support their hypothesis.

Specific Comments:

The authors conclude that Wnt/b-catenin signaling is important for cardiac repair, however it is not clear from the data presented whether the initial response to the injury was similar between the b-catenin mutants and control (i.e. is the infarct size the same between the two groups?).

Are epicardial-derived fibroblasts dependent on b-catenin signaling in vivo? The authors should look at the Wt-1/Cre; b-cat fl/fl; R26R hearts following acute injury. Using the animal model, do the authors see b-catenin negative, b-gal positive fibroblasts in the heart after injury?

In addition to its signaling function, b-catenin plays a structural role as part of the cadherin/catenin adhesion complex. There is a modest decrease in fractional shortening and an increase in LV systolic volume in pre-IR Wt-1/Cre; b-cat fl/fl animals. It is possible that loss of b-catenin affects the structural integrity of the epicardium leading to a more severe response to acute injury. The authors should check expression of E-cadherin and N-cadherin in the epicardium after deleting b-catenin. Although difficult to tease out, the signaling versus structural role for b-catenin should be mentioned in the discussion. It was previously shown that N-cadherin is important for epicardial-myocardial cell-cell interactions (Luo et al., Dev Biol. 2006, 299:517).

The Masson Trichrome staining did not work very well in Fig 1 C,E. I don't see any blue compared to Fig 6D. Better images of the fibrosis should be provided.

Figure 6D(i) right panel, Col1a2CreER/b-catenin fl/fl section post IR, shows increased cellularity in the subepicardium. What are these cells? Are they infiltrating lymphocytes? Beta-catenin appears responsible for the collagen deposition (Figure 6D(ii) right panel), however there appears to be less fibroblasts. Does b-catenin affect fibroblast proliferation and/or survival in vivo?

Minor comments:

p. 7 concentration of hydrogen peroxide should be in molar.
Referee #3

The manuscript by Duan et al demonstrates that a pro-fibrotic wnt/b-catenin injury response is required for preserving cardiac function after acute ischemic cardiac injury. While the findings are interesting, there are several questions/concerns for the authors to address.

Major comments

1. In Figure 2-D the authors show that following IR injury numerous cells express Wnt1. The authors claim that these cells are epicardial-originated fibroblasts based on gene expression analysis. The likelihood that infiltrating inflammatory cells could also express Wnt1 is not addressed. It is possible that inflammatory cells are also expressing Wnt1 following cardiac injury, if so, this would change the interpretation of the data.

2. The authors demonstrate that b-catenin KO mice have a significant decrease in their systolic function after IR in Fig.5-D. However, the same animals show a slight decrease in their cardiac function prior to injury. It would useful to employ 2-way ANOVA analysis for this set of data in order to check for pre-injury differences among the animal groups. Additionally, the authors should consider presenting LV diastolic dimensions following IR in addition to systolic volume as a marker for cardiac remodeling / performance.

3. The authors showed that heart function was reduced in b-catenin KO mice after IR injury. It is critical to provide some molecular mechanisms for the observed cardiac dysfunction. The authors show that Wnt signaling affects the proliferation of fibroblasts and thus it might affects cardiac fibrosis and cardiac remodeling. Did the b-catenin KO mice had less fibrotic areas compared to control mice? Did the b-catenin KO mice show higher mortality rate? Did they see any cardiac rupture in mice after RI? Was the size of area at risk after IR injury similar is all groups?

4. The authors chose to perform experiments on embryonic epicardial cells in Fig-2. It is known that Wnt signaling has a time and/or context dependent role in various models. It is likely that Wnt signaling mediates different effects on embryonic and adult epicardial cells. Thus, the authors need to confirm these results in adult epicardial cells.

Minor comments

1. The authors should provide statistic methods in the context.
important contribution to the field.

1) Although the authors detected the responsive transactivation of Wnt1 in epicardial cells and cardiac fibroblasts during IR injury of the heart. They also identified the importance of the b-catenin pathway by loss-of-function of b-catenin. However, the authors did not show the critical requirement of Wnt1 using loss-of-function. Moreover, although the authors showed the RT-PCR results of Wnt family in Figure S1A, B, and C, these results never exclude the possibility of Wnts contribution other than Wnt1. To clarify the importance of Wnt1 in the IR injury response as in the title and the authors' conclusion, it is necessary to analyze inducible and/or conditional Wnt1 knockout mice. We agree with the reviewer that possessing a floxed Wnt1 mouse to generate a fibroblast/epicardial specific conditional Wnt1 knockout would further confirm our findings. However a floxed Wnt1 mouse is not currently available and although our laboratory is actively engaged in constructing one, this typically involves a lengthy span of time. Several groups have demonstrated that Wnt1 acts through the canonical β-catenin pathway. We provide further evidence in this paper that Wnt1 increases β-catenin in cardiac fibroblasts (Fig S5E) and demonstrate in some newly performed experiments that silencing β-catenin with siRNA significantly decreases Wnt1 induced epicardial EMT by approximately 60% (New data shown in Fig S3A,B). Although a potential contribution of other Wnts to increased β-catenin in cardiac fibroblasts cannot be ruled out, Wnt1 is the only Wnt that is elevated early following cardiac injury (i.e when fibroblast specific β-catenin deletion was performed). Nevertheless, to assuage this reviewer’s concerns, we have gone through the manuscript in detail and have been careful to always couple “Wnt1 injury response” with “Wnt1/β-catenin” injury response as appropriate. Moreover, we have briefly discussed the potential effects of other Wnts in contributing to the repair process (First paragraph in Discussion section, Page 15).

2) In Figure 1, how about protein level of Wnt1? The authors should present Western blotting for Wnt1 during cardiac injury. Specificity of antibodies of various Wnts is a recognized problem amongst Wnt researchers (Wnt Home page http://www.stanford.edu/group/nusselab/cgi-bin/wnt). We tried several commercially available antibodies and while they were sensitive to detect cells expressing Wnt1 (in-vitro), they were not sensitive to detect Wnt1 expression in the heart. It is for this reason that we confirmed our observations of Wnt1 up-regulation in the injured heart with two independent methods; i.e using in situ hybridization and a Wnt1Cre/R26RlacZ reporter mouse. To address the reviewer’s concerns, as well as overcome problems with low sensitivity of Wnt antibodies for in vivo detection, we dissected out the area of injury after harvesting the heart 4 days post injury and performed Western Blotting for Wnt1. As shown in (Fig 1 above and Fig. S1F in revised manuscript), we observed upregulation of Wnt1 by Western blotting. The text of the manuscript has been changed accordingly to describe this piece of data. (Page 6 in revised manuscript)
3) Figure 1Dii and iii have some dust (?) in the field. Please replace to clean pictures. We apologize for this oversight and the revised manuscript has been updated to replace Fig1Dii and Fig1Diii.

4) In Figure 4, the authors showed EMT by the administration of Wnt1. Does b-catenin knockdown by siRNA block the EMT induced by Wnt1?
As recommended by this reviewer, we have performed shRNA experiments targeting β-catenin to determine whether Wnt1 mediates epicardial EMT via β-catenin. Epicardial cells were harvested from Col1a2CreER(T)/R26RtdTomato mice using techniques described in the manuscript. We infected epicardial cells with a lentiviral β-catenin shRNA (Santa Cruz) and subsequently treated them with Wnt1 for 7 days. Epicardial cells treated with a scrambled shRNA virus served as controls. Tamoxifen was concomitantly added for the duration of Wnt1 treatment. Wnt1 mediated EMT will induce collagen 1 expression in these cells which then should express tomato fluorescence. We obtained approximately 98% transduction efficiency and observed approximately 60% reduction in the number of cells expressing tomato fluorescence, when treated with β-catenin shRNA compared to scrambled shRNA treated cells (Page 9, last paragraph of revised manuscript and Fig S3A,B in revised manuscript). These experiments thus demonstrate that Wnt1 predominantly acts via the β-catenin canonical pathway in mediating epicardial EMT.

5) Procedure of IR is irregular and resultant cardiac function depends on infarct size. It is necessary to show infarct size and infarct area/area at risk (IA/AAR) ratio to verify the procedure. The authors should show infarct size and IA/AAR ratio, and representative figure, such as TTC staining and Evans blue staining, for Figure 5 and 6
As recommended by our reviewer, we performed TTC/Evans blue staining using described protocols and have enclosed this new data in the revised manuscript. There was no difference in the infarct area/area at risk ratio in either the Wt1 Cre or Col1Cre:β-catenin mutant mice compared to their respective controls. Image J software was used to estimate infarct area and the area at risk. This data has been included in the revised manuscript (Fig S4G and Fig S5B).

6) The authors showed representative M-mode pictures, cardiac function (%FS) and LV end systolic volume in Figure 5 and 6. Although anesthesia and heart rate influence the cardiac function, the authors did not mention animal condition during the echocardiography. For example, M-mode picture of tamoxifen injected Day8 IR in Figure 6C (lower left panel) showed very slow heart rate compared to the others. The authors should describe the anesthesia and the number of mice for the echocardiographic analysis in Experimental Procedure, and show echocardiographic parameters including heart rate on a table in the supplement. And please replace the picture of Tamoxifen injected Day8 IR to equal HR level in Figure 6C.
All animals were subjected to Echocardiography in a conscious state; thus there was no influence of anesthesia on cardiac function during echocardiography. We have provided more representative echocardiographic images of the Tamoxifen injected D8 IR (Fig 6D in revised manuscript) as requested by the reviewer and mentioned procedural details of Echocardiography under the Methods section (Page 20 of revised manuscript). We also provide a table with the echocardiographic parameters including heart rates at which the acquisition of images was done and the number of animals in each group (Table S1 and Table S2 of revised manuscript).

7) Cardiac functions after IR in Wt1-Cre/b-cateninfl/fl and Col1a2-CreER(T)/b-cateninfl/fl were evaluated at only one point (day8 after IR). Reduced fibrosis can often be associated with better cardiac function. To evaluate long term effects of deletion of β-catenin, please perform late IR (day 14 or 28).
The cardiac function between day 8 and day 14 did not significantly change in both Wt1-Cre and Col1Cre β-catenin knockout mice. Shown below (Fig 2 in this response, next page) are the complete echocardiographic parameters for Wt1-Cre/β-catenin knockout mice at D7 and D14 after ischemia reperfusion injury. Similarly the fractional shortening and LV volumes at D7 and D14 in Col1Cre/β-catenin knockout mice were not significantly different. It is for this reason, we have kept the Day 8 data unchanged in the revised manuscript and have opted not to present the D14 data as it is redundant.
Fig3. Western blotting for β-catenin in cardiac fibroblasts following tamoxifen injection in Col1Cre;β-catenin<sup>fl/fl</sup> mice with corresponding densitometry analysis demonstrates approximately 75-80% decrease in β-catenin levels compared to cardiac fibroblasts isolated from tamoxifen injected Cre negative mice. (data shown in revised manuscript as Fig S5F)

8) The authors showed deletion of β-catenin by Western blotting in Figure S4E, but not in Figure S5. Please show deletion of β-catenin by Col1α2-CreER(T) in the heart.

We have included Western blotting data (Fig3 in this response, Fig S5F and Page 14 of revised manuscript) to demonstrate successful deletion of β-catenin in cardiac fibroblasts of tamoxifen injected Col1α2CreERT;β-catenin<sup>fl/fl</sup> mice. Briefly, we isolated cardiac fibroblasts from mice 10 days following injection of tamoxifen and performed Western blotting for β-catenin, with identical littermates not carrying the Cre transgene serving as controls. As demonstrated we observed 75-80% reduction in β-catenin in cardiac fibroblasts followed tamoxifen injection compared to Cre negative control mice. The persistence of β-catenin (approximately 20%) in cardiac fibroblasts likely arises from the fact that all cardiac fibroblasts do not express type 1 collagen.
Measurement of epicardial thickness is tricky. Because, in Figure 1E at day 10 after IR, Masson's staining showed no thickened epicardium in this picture, in Figure 2C, epicardium does not increase in this picture, while day 2 image in Figure 1C showed thickened epicardium. Assuming that epicardium thickness depends on the observed section, please explain more detail about the measurement of epicardial thickness, and show representative sections to measure it.

We agree with the reviewer that thickening of the epicardium and subepicardial regions may not be uniform in all sections. The epicardium circumferentially envelops the heart and epicardial thickness differed within the same section as well as between different sections of the heart. To circumvent this issue, and obtain a true representation of differences in epicardial activation following injury, we did the following: a) measured epicardial thickness in sections where we observed injury; we did this as we observed epicardial and subepicardial thickening to be prominent in heart sections with greater injury. b) measured maximal epicardial thickness in 3-5 regions around the heart (as shown in diagram below) c) Averaged maximal epicardial thickness in 3 different heart regions (i.e close to base, mid ventricle and apex, as shown in diagram below). We have included these experimental details in the Methods Section (Page 22 of revised manuscript) and provide representative sections (Fig 4, below) to further illustrate our methodology as requested by the reviewer. We thus believe that the mean epicardial thickness as shown in Fig 5C integrates maximal epicardial thickness in 5 different sectors of the heart from the base to the apex.
The thickness of the epicardium was measured in sections with injury. Separate measurements were made at heart sections close to the base of the heart (A), mid ventricle (B) and apex (C). In each section, epicardial thickness was measured in 3-5 regions depending upon the circumferential thickening of the epicardium. Measurements were made by drawing a line perpendicular from the epicardial thickness to the outer edge of the epicardium as shown by black lines in (D, boxed area highlighted in C). Arrows demonstrate thickening of the epicardium. All epicardial measurements taken at the base, mid and apex were averaged to determine the “mean” epicardial thickness.

10) Pictures of IR heart in this manuscript showed limited area. For better understanding, please add picture of macro or low magnification with staining (LacZ and Masson’s) in the Supplemental data.

We presume that the reviewer wants a low magnification image to visualize the entire heart section. We have provided representative pictures of the heart (Masson’s trichrome and lac Z staining) of Wt-1Cre/R26RlacZ as well as Col1a2Cre:R26RlacZ mice following IR injury. These data have been shown in Fig S4B, S5A in the revised manuscript.

Reviewer 2

Adult epicardium has been shown to differentiate into cardiomyocytes, fibroblast and vascular cells in response to myocardial injury. Several signaling pathways involved in this process have also been identified, such as Wnt/b-catenin signaling. In this article, Duan et al. demonstrate a dynamic role for Wnt1/b-catenin signaling in promoting cardiac repair by activating epicardium and cardiac fibroblast.....Overall, this is a very interesting study demonstrating a positive role for Wnt/b-catenin signaling in cardiac repair that should be of great interest to the field. The authors effectively utilize different in vitro and in vivo approaches to support their hypothesis.

We thank the reviewer for finding this study interesting and appreciating the use of different approaches to prove our hypothesis.

Specific Comments:

The authors conclude that Wnt/b-catenin signaling is important for cardiac repair, however it is not clear from the data presented whether the initial response to the injury was similar between the b-catenin mutants and control (i.e. is the infarct size the same between the two groups?).

Triphenyl-tetrazolium chloride (TTC ) combined with Evans blue staining is a standard approach for quantitating injury area and infarct size to area at risk ratios (IA/AAR) following injury. Briefly, hearts of animals were subjected to TTC/Evans blue staining 24 hours after initial ischemic injury and infarct size/area at risk ratio assessed. The initial response to injury was similar
between the two groups and no differences in infarct size/area at risk ratios were observed between the Col1 and the Wt-1 β-catenin mutant mice and their respective controls. Representative images along with quantitative data have been added to the revised Supplemental data (Fig S4G and Fig S5B in revised manuscript).

Are epicardial-derived fibroblasts dependent on b-catenin signaling in vivo? The authors should look at the Wt-1/Cre: b-cat fl/fl, R26R hearts following acute injury. Using the animal model, do the authors see b-catenin negative, b-gal positive fibroblasts in the heart after injury?

We subjected Wt-1Cre:R26R LacZ;βcateninfl/fl mice to acute ischemic cardiac injury and isolated lac Z expressing cells. As shown below we found rare (less than 1%) of Cre positive cells to express the fibroblast marker DDR2 (Fig 5A in this response and Fig S4D in revised manuscript). To confirm this observation, we also performed immunostaining against β-catenin and observed that all DDR2 expressing cells co-expressed β-catenin (Fig 5B in this response). These observations would support the notion that epicardium derived cardiac fibroblasts require β-catenin to be present. The data shown below in Fig 4A has been added to the revised manuscript as Fig S4D and the results described on Page 12.

In addition to its signaling function, b-catenin plays a structural role as part of the cadherin/catenin adhesion complex. There is a modest decrease in fractional shortening and an increase in LV systolic volume in pre-IR Wt-1/Cre; b-cat fl/fl animals. It is possible that loss of b-catenin affects the structural integrity of the epicardium leading to a more severe response to acute injury. The authors should check expression of E-cadherin and N-cadherin in the epicardium after deleting b-catenin. Although difficult to tease out, the signaling versus structural role for b-catenin should be mentioned in the discussion. It was previously shown that N-cadherin is important for epicardial-myocardial cell-cell interactions (Luo et al., Dev Biol. 2006, 299:517).

We thank the reviewer for mentioning the potential functional importance of β-catenin as a structural molecule bound to E cadherin in our study. Following deletion of β-catenin in the epicardium, we did not see any changes in E-cadherin or N-cadherin expression in epicardial cells. As shown in (Fig 6 in this response), E-cadherin was expressed in the epicardium in wild
type littermates as well as the Wt-1βcatenin KO mice. Ecadherin was also expressed in junctions between myocytes as described (Luo & Radice, 2003). Ncadherin was not expressed in the epicardium but strongly expressed in the myocardium (Soler & Knudsen, 1994) of both Wt-1βcatenin KO and wild type littermates. As the reviewer suggests, this does not however disprove the notion that the structural function of βcatenin contributed to epicardial dysfunction after injury. We have discussed this issue in the revised manuscript and acknowledged that although we did not see changes in epicardial N-cadherin expression after βcatenin deletion, disruption of cell-cell adhesion complexes secondary to βcatenin deletion could have partially contributed to cardiac dysfunction. (Page 13 of revised manuscript).

The Masson Trichrome staining did not work very well in Fig 1 C,E. I don't see any blue compared to Fig 6D. Better images of the fibrosis should be provided.

We have replaced these images with images where the masson trichrome staining demonstrates the fibrotic area as “blue”. Note the blue stain is not as strong at D2 post injury compared to D10 as collagen deposition is minimal 2 days post injury. Higher magnification demonstrates typical collagen expressing interstitial fibroblasts, that stain blue at 2 days post injury (Fig 7, in this response). As per the reviewer’s recommendations, Fig 1C and Fig 1E have been replaced with better images.
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**Fig 7. Masson-trichrome staining of heart sections 2 days after injury** (Sections corresponds to section shown in Fig 1C in manuscript). Arrowheads point to masson trichrome staining. Fig 1C shows the same section in lower magnification.

*Figure 6D(i) right panel, Col1a2CreER/bcatenin fl/fl section post IR, shows increased cellularity in the subepicardium. What are these cells? Are they infiltrating lymphocytes? Beta-catenin appears responsible for the collagen deposition (Figure 6D(ii) right panel), however there appears to be less fibroblasts. Does b-catenin affect fibroblast proliferation and/or survival in vivo?*  
Undoubtedly the cell population in the subepicardium is a heterogeneous population comprising inflammatory cells, epicardial derived cells (EDC) and fibroblasts (Zhou et al, 2011). As suggested by the reviewer, we performed staining for inflammatory marker Cd11b in the subepicardial region and observed that a portion of the subepicardial cells were inflammatory in origin (*Fig 8 in this response*). To directly address the reviewer’s questions about the effects of β-catenin on cardiac fibroblast proliferation in vivo, we isolated cardiac fibroblasts from Col1Cre;bcateninfl/fl (after tamoxifen injection as described in manuscript) mice 7 days after cardiac injury. Cardiac fibroblasts immediately following harvest were seeded onto 10 cm dishes and fibroblast numbers in 6 random fields counted 48 hours later. As shown in (*Fig S6A,B of revised manuscript*), cardiac fibroblasts were fewer by approximately 80%, when compared to those isolated from oil injected mice after injury at similar time point. To confirm this further, we performed immunohistochemistry for DDR2 and observed substantial reduction in DDR2.

**Fig 8. Immunohistochemistry for inflammatory marker CD11b on heart sections of Col1CreER(T)/bcateninfl/fl isolated 8 days following cardiac injury.** Subepicardial cellular infiltrate comprising CD11b positive cells (arrows). Magnification 400X.
expression in subepicardial regions of Col1Cre: β-cateninfl/fl mutant mice compared to oil injected controls (Fig. S6C). Finally, immunohistochemistry for Ki67 (marker of proliferation) demonstrated decreased Ki67 expression in subepicardial and region of injury of Col1Cre: β-catenin mutant mice compared to control mice (Fig. S6D,E). These findings suggest that fibroblast numbers and proliferation in the injured heart are Wnt/β-catenin dependent and are in agreement with data shown in Fig5C and Fig6E demonstrating β-catenin mutant mice have reduced collagen deposition. These observations have also been discussed (Page 15 and Page 17 of revised manuscript) with regard to the physiological mechanisms of cardiac worsening...i.e. β-catenin dependent profibrotic repair response is impaired early in the heart with consequent worsening of post injury cardiac function.

Minor comments:

p. 7 concentration of hydrogen peroxide should be in molar.
We have corrected this.

p. 8 Gessert and Kuhl reference is missing year.
We have updated the reference (Page 9, line 2).

p. 9 Wilms tumor (Wt-1) abbreviation should be used first time Wt-1 is introduced.
We have corrected this (Page 9, line 13).

p. 11 Fig S5D should read Fig S4E.
We have corrected this and the figure numbering has changed owing to new data being added.

Reviewer 3

The manuscript by Duan et al demonstrates that a pro-fibrotic wnt/β-catenin injury response is required for preserving cardiac function after acute ischemic cardiac injury. While the findings are interesting, there are several questions/concerns for the authors to address.

1. In Figure 2-D the authors show that following IR injury numerous cells express Wnt1. The authors claim that these cells are epicardial-originated fibroblasts based on gene expression analysis. The likelihood that infiltrating inflammatory cells could also express Wnt1 is not addressed. It is possible that inflammatory cells are also expressing Wnt1 following cardiac injury, if so, this would change the interpretation of the data.
in injured are of heart demonstrates Wnt1 expressing cells (red, white arrow) and CD11b(green, white arrowhead) (Fig 9A is shown in the revised manuscript).

To directly answer this reviewer’s question as to whether inflammatory cells recruited to the injured heart are a source of Wnt1, we subjected Wnt1Cre:R26RlacZ mice to ischemic cardiac injury and harvested their hearts 48 hours later when their hearts are maximally infiltrated with a mononuclear cellular infiltrate. We performed double immunostaining using βgal and CD11b antibody, CD11b being a commonly used marker of inflammatory cells. As shown in Fig9 in this response and in Fig S2C of revised manuscript, we did not see CD11b positive cells to coexpress Wnt1.

2. The authors demonstrate that β-catenin KO mice have a significant decrease in their systolic function after IR in Fig.5-D. However, the same animals show a slight decrease in their cardiac function prior to injury. It would useful to employ 2-way ANOVA analysis for this set of data in order to check for pre-injury differences among the animal groups. Additionally, the authors should consider presenting LV diastolic dimensions following IR in addition to systolic volume as a marker for cardiac remodeling / performance.

We have employed 2 way anova to determine differences in these groups and the figure legends and statistical methods have been modified to demonstrate this. Analysis using 2 way Anova with Bonferroni’s post test demonstrated that cardiac function and both systolic and diastolic left ventricular dimensions were significantly worse in the Wt-1/βcatenin KO mice at 8 days post injury compared to mice with intact epicardial βcatenin. This analysis and diastolic ventricular dimensions have been added to the revised manuscript (Fig 5D). In addition, we have presented all relevant echocardiographic parameters in a tabular format in the supplemental data (Table S1 and Table S2 of revised manuscript).

3. The authors showed that heart function was reduced in b-catenin KO mice after IR injury. It is critical to provide some molecular mechanisms for the observed cardiac dysfunction. The authors show that Wnt signaling affects the proliferation of fibroblasts and thus it might affects cardiac fibrosis and cardiac remodeling. Did the b-catenin KO mice had less fibrotic areas compared to control mice? Did the b-catenin KO mice show higher mortality rate? Did they see any cardiac rupture in mice after RI? Was the size of area at risk after IR injury similar is all groups?

The reviewer has essentially asked for a molecular explanation underlying the worsening of cardiac function in βcatenin knockout animals compared to control animals after cardiac injury. We provide in-vitro data to demonstrate that Wnt1/βcatenin signaling enhances cardiac fibroblast proliferation (Fig4G). Cardiac fibroblast proliferation is an essential component of cardiac repair and peak cardiac fibroblast proliferation occurs within the first 7 days after injury (Sun & Weber, 2000). We thus hypothesized that disruption of epicardial and fibroblast βcatenin significantly decreases cardiac fibroblast proliferation after injury, impairs wound healing and leads to worsening cardiac function.

To investigate this hypothesis, we isolated cardiac fibroblasts from Col1Cre:βcateninfl/fl (after tamoxifen injection as described in manuscript) mice 8 days after cardiac injury. Cardiac fibroblasts immediately following harvest were seeded onto 10 cm dishes and fibroblast numbers in 6 random fields counted 48 hours later. As shown in (Fig S6A,B of revised manuscript), cardiac fibroblasts were fewer by approximately 80%, when compared to those isolated from oil injected animals after injury at similar time point. As recommended by this reviewer, we confirmed decreased fibroblast numbers in-vivo as well. We performed immunohistochemistry for DDR2 and observed substantial reduction in DDR2 expression in subepicardial regions of βcatenin mutant mice compared to oil injected controls. Finally to determine whether decreased fibroblast numbers were secondary to decreased proliferation, we performed immunohistochemistry and observed decreased Ki67 expression (marker of proliferation) in subepicardial region as well as in region of injury of Col1Cre:βcatenin mutant mice compared to control mice (Fig S6D). Although we did not see any cardiac rupture in these animals, we observed markedly reduced collagen (Masson trichrome staining) in Col1Cre:βcateninfl/fl as well as Wt-1Cre:βcateninfl/fl mice hearts at 7 days following injury (Shown in original manuscript and Fig5C and Fig6E in revised manuscript). Observations on decreased fibroblast proliferation, decreased fibroblast numbers and decreased DDR2 expression have been included as new data in the revised manuscript (Fig S6 of revised manuscript) and strongly suggest that interruption of βcatenin signaling markedly reduces...
fibroblast proliferation and the fibroblast response to injury within the first few days after cardiac injury. This results in impaired wound healing that contributes to cardiac dysfunction. Based on these observations we have also included a discussion on mechanisms in the revised manuscript (Page 15 and Page 17 of revised manuscript).

4. The authors chose to perform experiments on embryonic epicardial cells in Fig-2. It is known that Wnt signaling has a time and/or context dependent role in various models. It is likely that Wnt signaling mediates different effects on embryonic and adult epicardial cells. Thus, the authors need to confirm these results in adult epicardial cells.

We have elected to perform studies with embryonic epicardial cells as in our hands they are more robust for expansion and are easily viable in tissue culture for one-two weeks (Dong et al, 2008). Primary epicardial cells isolated from embryonic and neonatal hearts can be maintained for approximately 4 passages and retain typical cobblestone morphology and epithelial characteristics (Chen et al, 2002). In contrast, epicardial cell outgrowths from the hearts of postnatal Day 1 mice are only about 10% of the epicardial outgrowths from E10.5 dpf hearts (Chen et al, 2002). The trophic and migratory abilities of the epicardium rapidly decline between E12dpf and post natal D4 (Smart et al, 2007b). Protocols for successful epicardial cell mobilization from the adult heart use agents such as ThymosinB4 for outgrowth (Smart & Riley, 2009; Smart et al, 2007a) from explanted hearts and we did not want Thymosin B4 to interfere with Wnt1 in our studies. In fact, in the absence of ThymosinB4, Smart et al reported that no epicardial cell outgrowth was seen from adult heart explants (Smart et al, 2007a).

As requested by the reviewer, we repeated these experiments and observed very few epicardial outgrowths of adult explanted hearts in the absence of ThymosinB4. Moreover, from a technical standpoint, isolation of embryonic epicardial cells is associated with minimal cardiac manipulation ex vivo and leads to a pure and homogeneous population of cells. Isolation of a homogeneous population of adult epicardial cells is challenging. It involves cutting the adult heart into pieces and at least in our hands is inherently associated with fibroblast contamination (Smart & Riley, 2009).

Given the above mentioned technical hurdles with fibroblast contamination, relatively poor yields from adult hearts, inability of the epicardium isolated from the adult heart to express trophic or migratory properties and requirement for ThymosinB4 to boost adult primary epicardial cells, we elected to work with embryonic epicardial cells. We do agree with the reviewer that the physiological effects of Wnt signaling are context dependent. However, the in vivo effects observed by us in the epicardium of adult hearts (EMT & proliferation) was faithfully recapitulated in embryonic epicardial cells ex vivo and thus from this standpoint, there is no conflict between our in vivo data generated in adult mouse hearts and its demonstration ex vivo in embryonic epicardial cells.

Minor comments

1. The authors should provide statistic methods in the context.
We apologize for this oversight and have included a section on statistical techniques under the “Methods” section. We used the Student’s test, one way and 2 way anova as appropriate with Bonferroni’s post test correction. All statistical values were computed using Graph Pad prism software.

Bibliography
2nd Editorial Decision 13 October 2011

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by the original three referees and their comments are provided below.

As you can see, the referees appreciate the introduced changes and support publication here. They raise a few minor issues, that shouldn't involve too much additional work to resolve. I would therefore like to ask you to respond to the last issues in a final revision.

Best

Editor
The EMBO Journal

REFEEREE REPORTS

Referee #1

The authors responded to reviewers' comments with some additional experiments and edited text, which improve the manuscript. However, a couple of minor concerns are still in their manuscripts.

1) Figure 4 in "Response to Reviewers" clearly illustrates their measurement of epicardial thickening after the I/R. I want to add this Figure 4 to the supplemental data for better understanding.

2) The authors replaced D8 Tam IR M-mode in Figure6C, but the replaced M-mode view still has some arrhythmia. Furthermore, data of Table S2 indicate significantly enlarged LV volume in D8 Tam IR, however LVdD and Ds in Figure 6D (ii) did not so enlarge as compared to (i), (iii), and (iv). Please show appropriate figures and adjust scale of these M-mode figures.

3) The authors should express %FS, LVESV and LVEDV in Table S1 and S2 as mean ± S.D. In Table S1 and S2, wall thicknesses (intraventricular septum and posterior wall) and LV dimensions (LVEDd and LVEDs) should be added as markers for cardiac remodeling and function to avoid misunderstanding such as 2).

Referee #2

The authors have adequately address my concerns in this revised manuscript.
Referee #3

The authors provided satisfactory answers to several of this reviewer's comments; however, there are couple points that require attention, as outlined below:

1. The authors claim that following IR injury numerous epicardial-derived cells express Wnt1. In a recent report by Aisagbonhi et al (Aisagbonhi et al, 2011) it was shown that in the infarcted area Wnt signaling is activated in various types of cells including endothelial cell, smooth muscle cells, fibroblasts and macrophages. Have the authors examined the activation status of Wnt signaling in these cell populations in their system? Moreover, in the same paper, Aisagbonhi et al (Aisagbonhi et al, 2011) suggest that in sham animals there is basal Wnt activity in major vessels in the heart. Are there potential mechanisms that may underlie the discrepancy between this prior report and Fig 3C, in which no baseline Wnt activity was observed?

2. The same paper by Aisagbonhi et al (Aisagbonhi et al, 2011) demonstrated that following injury there is formation of myofibroblasts from activated endothelial cells and this phenomenon is stimulated by Wnt signals. Have the authors examined the possibility that the origin of myofibroblasts following cardiac injury is not exclusively from an epicardial origin, and that alternatively Wnt signaling might be involved in formation of fibroblasts from alternative sources?

3. The authors show that deletion of β-catenin in epicardial (Wt-1+) cells and in fibroblasts (Col1a2+ cells) reduced cardiac performance and suggest that this is due to reduced fibrosis following cardiac injury. Typically, decreased cardiac performance is associated with either reduced number or decreased contractile function of cardiomyocytes in the injured heart. What are the effects of genetically altering β-catenin on cardiomyocytes? Have the authors detected increased cell death in the hearts of the β-catenin null mice?
MANUSCRIPT: Wnt1/β-catenin injury response activates the epicardium and cardiac fibroblasts to promote cardiac repair (EMBOJ-2011-78179R)

Response to Reviewers: We provide a step by step response to all reviewer comments with reviewer critiques in bold followed by our response. Changes in the revised manuscript including figures are mentioned in our response.

Reviewer 1

The authors responded to reviewers' comments with some additional experiments and edited text, which improve the manuscript. However, a couple of minor concerns are still in their manuscripts.

We thank the reviewer for his/her critique and appreciating the revised manuscript.

1) Figure 4 in “Response to Reviewers” clearly illustrates their measurement of epicardial thickening after the I/R. I want to add this Figure 4 to the supplemental data for better understanding.

As per the reviewer’s request, we have added this data to a new Supplemental Figure (Fig S7) and added the figure legend accordingly. The methods of the manuscript has also been revised to reflect this.

2) The authors replaced D8 Tam IR M-mode in Figure 6C, but the replaced M-mode view still has some arrhythmia. Furthermore, data of Table S2 indicate significantly enlarged LV volume in D8 Tam IR, however LVDd and Ds in Figure 6D (ii) did not so enlarge as compared to (i), (iii), and (iv). Please show appropriate figures and adjust scale of these M-mode figures.

We have changed the D8 Tam IR M Mode to include a M mode figure without any arrhythmia. It is also worthwhile to mention that measurements were made in only those beats that were NOT a result of arrhythmia. So for instance, we performed measurements made on regular beats (Fig 1 in this response, white asterisk) and avoided the ones where the rhythm is not regular (Fig1 in this response, red crosses). We have also adjusted the scale of the M mode figures to remove the visual discrepancy that the reviewer mentions.

3) The authors should express %FS, LVESV and LVEDV in Table S1 and S2 as mean {plus minus} S.D. In Table S1 and S2, wall thicknesses (intraventricular septum and posterior wall) and LV dimensions (LVEDd and LVEDs) should be added as markers for cardiac remodeling and function to avoid misunderstanding such as 2).

We have revised Tables S1 and S2 as per the reviewer’s recommendations and now contain the data represented as Mean ± S.D. We have also added data regarding LVEDd and LVEDs as suggested by the reviewer.

Reviewer 2

The authors have adequately address my concerns in this revised manuscript.

We thank the reviewer for his/her critique and appreciating the revised manuscript.
Reviewer 3

The authors provided satisfactory answers to several of this reviewer's comments; however, there are couple points that require attention, as outlined below:

1. The authors claim that following IR injury numerous epicardial-derived cells express Wnt1. In a recent report by Aisagbonhi et al (Aisagbonhi et al, 2011) it was shown that in the infarcted area Wnt signaling is activated in various types of cells including endothelial cell, smooth muscle cells, fibroblasts and macrophages. Have the authors examined the activation status of Wnt signaling in these cell populations in their system? Moreover, in the same paper, Aisagbonhi et al (Aisagbonhi et al, 2011) suggest that in sham animals there is basal Wnt activity in major vessels in the heart. Are there potential mechanisms that may underlie the discrepancy between this prior report and Fig 3C, in which no baseline Wnt activity was observed?

We thank the reviewer for pointing out this paper to us and we have cited this paper in the revised manuscript (Page 8 in revised manuscript). Aisagbonhi induce myocardial infarction in TOPGAL mice and observed Wnt activity (βgal expression) in the infarcted area. They performed immunostaining and demonstrate that Wnt activity was present in several types of cells including endothelial cells in the peri-infarct region. However upon closer inspection of their manuscript, the authors clearly note that predominant βgalactosidase expression in post injury hearts was “mainly due to the de novo appearance of large numbers of subepicardial mesenchymal cells”. In this regard, we would argue that our observations are similar as we observed intense Wnt activity in the epicardium and subepicardium and we go on in our paper to demonstrate the physiological importance of Wnt signaling in subepicardial fibroblasts.

Aisagbonhi et al. observed a significant fraction of SMA positive cells in the peri-infarct area that are Wnt responsive. Again, our observations are concordant as a large number of fibroblasts in the peri-infarct area are known to express SMA. We did not see macrophages to be Wnt responsive and as we have shown in the previous response to reviewers, we observed that macrophages at the infarct site did not express Wnt1 either. We did not see endothelial cells in the peri-infarct area to be Wnt responsive but such differences could also be secondary to different animal models used by us (ischemia-reperfusion injury) versus permanent ligation by Aisagbonhi’s group. In the sham uninjured animal, we observed rare cells to be Wnt responsive and observed rare perivascular cells to be Wnt activated after cardiac injury (Fig 2 in this response). Aisagbonhi et al observed a significant number of perivascular cells to be Wnt responsive in the sham injured TOPGAL heart but did not observe any appreciable change in Wnt activity in these cells following injury. Thus the contribution of Wnt responsive perivascular cells to the injury response is uncertain.

Our study cannot definitively exclude small populations of endothelial cells or macrophages in the peri-infarct area that could have been Wnt responsive, as one would have to use an exhaustive panoply of cell surface markers for endothelial cells and macrophages to definitively answer this question. Notwithstanding these caveats, we would argue that the overarching conclusions from Aisagbonhi’s study are concordant with some of the observations in this study i.e. post injury, the epicardial/subepicardial as well as the peri-infarct areas are Wnt responsive and peri-infarct fibroblasts as well as subepicardial fibroblasts are activated by Wnt signaling.

2. The same paper by Aisagbonhi et al (Aisagbonhi et al, 2011) demonstrated that following injury there is formation of myofibroblasts from activated endothelial cells and this phenomenon is stimulated by Wnt signals. Have the authors examined the possibility that the origin of myofibroblasts following cardiac injury is not exclusively from an epicardial origin, and that alternatively Wnt signaling might be involved in formation of fibroblasts from alternative sources?

Cardiac fibrosis is a complex spatio-temporal process with different populations reported in the literature to
contribute directly or indirectly to cardiac fibrosis including resident cardiac fibroblasts (i.e fibroblasts natively present in the heart in the uninjured state), circulating bone marrow cells and endothelial cells by endothelial to mesenchymal transition\(^2\)-\(^6\). The novelty of our current work arises in pointing to the epicardium as a source of cardiac fibroblasts and demonstrating the critical physiological importance of this process. Our study was not designed to interrogate potentially other cell populations contributing to cardiac fibroblasts. In general, what the author points out here is a drawback of all lineage tracing studies i.e the fate of the genetically labeled cell can be effectively described but other cell populations potentially contributing to the “same fate” cannot be reported by the Cre. This can be only done using different cell specific Cre reporters and falls beyond the scope of this study.

However to answer this reviewer’s specific query, we looked at cardiac fibroblasts isolated from hearts of Wt-1\textsuperscript{Cre}/R26\textsuperscript{R\lacZ} mice 8 days following injury when epicardial expansion peaks. We only observed rare β\textsuperscript{gal} negative (i.e of non-epicardial origin) fibroblasts (DDR2\textsuperscript{+}) cells. While this does not answer the reviewer’s question of the cell source contributing to non-epicardial derived cardiac fibroblasts, it at least suggests that a large majority of cardiac fibroblasts are predominantly of epicardial origin.

3. The authors show that deletion of beta-catenin in epicardial (Wt-1+) cells and in fibroblasts (Col1a2+ cells) reduced cardiac performance and suggest that this is due to reduced fibrosis following cardiac injury. Typically, decreased cardiac performance is associated with either reduced number or decreased contractile function of cardiomyocytes in the injured heart. What are the effects of genetically altering beta-catenin on cardiomyocytes? Have the authors detected increased cell death in the hearts of the beta-catenin null mice?

Bergmann’s group has previously reported that deletion of β\textsuperscript{catenin} specifically in cardiomyocytes enhanced cardiac function and repair after ischemic cardiac injury via indirect effects on a cardiac progenitor population\(^7\). To answer the reviewer’s question about cell death, we performed TUNEL staining (Fig 4 in this response) and did not observe any differences in number of apoptotic myocyte nuclei between mice lacking epicardial β\textsuperscript{catenin} (Wt-1\textsuperscript{Cre}/β\textsuperscript{catenin\textsuperscript{fl/fl}}) and mice with intact epicardial β\textsuperscript{catenin}. This piece of data in conjunction with TTC/Evans blue staining (Shown in revised manuscript) would suggest that deletion of β\textsuperscript{catenin} in epicardial cells or fibroblasts did not directly affect myocyte viability.

**Fig 3. Fibroblasts isolated from Wt-1Cre/R26R\textsuperscript{lacZ} mice hearts 8 days following cardiac injury stained with β\textsuperscript{gal}(green) and DDR2(red) antibodies showing rare DDR2 expressing cells not expressing β\textsuperscript{gal} (white arrows).**

**Fig 4. TUNEL staining of peri-infarct region of (A) wild type and (B) mice lacking epicardial β\textsuperscript{catenin} 8 days following cardiac injury demonstrating similar numbers of TUNNEL positive nuclei (dark brown color, black arrows). Nuclei counter stained with methyl green.**


