Ctnna3 Deficiency Promotes Heart Regeneration by Enhancing Cardiomyocyte Proliferation in Neonatal Mice

sha zou (✉ zousha21@163.com)

Fudan University - Handan Campus: Fudan University

Wuhou Dai

Fudan University - Handan Campus: Fudan University

Jifen Li

Thomas Jefferson University - Center City Campus: Thomas Jefferson University

Hongyan Wang

Fudan University - Handan Campus: Fudan University

Wufan Tao

Fudan University - Handan Campus: Fudan University

Research Article

Keywords: Ctnna3, α T-catenin, cardiomyocyte proliferation, heart regeneration

Posted Date: June 21st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-625734/v1>

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Abstract

Heart regeneration requires renewal of lost cardiomyocytes. However, the mammalian heart loses its proliferative capacity soon after birth, and the molecular signaling underlying the loss of cardiac proliferation postnatally is not fully understood. Here we report that ablation of *Ctnna3*, coding for an α T-catenin protein and highly expressed in hearts, accelerated heart regeneration following heart apex resection in neonatal mice. Our results show that *Ctnna3* deficiency enhances cardiomyocyte proliferation in hearts from P7 mice by upregulating *Yap* expression. Our study demonstrates that *Ctnna3* deficiency is sufficient to promote heart regeneration and cardiomyocyte proliferation in neonatal mice and indicates that functional interference of α -catenins might help to stimulate myocardial regeneration after injury.

Introduction

Heart failure resulting from the loss of cardiomyocytes during injury and disease is a primary cause of human morbidity and mortality [1]. The adult mammalian heart only has limited cardiomyocyte turnover, and this is not sufficient to restore contractile function after heart injury. In contrast, the neonatal mammalian heart possesses a remarkable capacity for cardiac regeneration, but this heart regeneration ability after injury is lost by 7 days of age, a time point that coincides with the onset of cardiomyocyte proliferative arrest [2–4]. Multiple experimental strategies have been explored to restore functional myocardium for repairing the injured heart. These are: (a) cell therapy using embryonic stem cells, induced pluripotent stem cells (iPS) and cardiac progenitor cells; (b) reprogramming of nonmyocytes, e.g. cardiac fibroblasts, to a cardiac cell fate (cardiomyocytes) using cardiogenic genes and small molecules; (c) re-activation of cardiomyocyte mitosis in the adult heart [5–8]. Genetic fate-mapping experiments in the neonatal mice [3] and adult zebrafish [9, 10] indicate that the regenerated cardiomyocytes are mainly derived from reactivation of preexisting cardiomyocytes, rather than activation of undifferentiated stem or progenitor cells. Thus, the identification of genes and a thorough understanding of the mechanisms underlying the regulation of cardiomyocyte proliferation and regeneration may provide critical information for design of new therapeutic strategies to heart failure.

Alpha-catenins (α -catenins) play fundamental roles in cadherin-mediated cell-cell adhesion and actin dynamics [11–13]. They function widely in regulating cell differentiation, proliferation, and regeneration [12–14]. There are three subtypes of α -catenins: namely α E-catenin, α N-catenin, and α T-catenin, encoded by *Ctnna1*, *Ctnna2*, and *Ctnna3* respectively, in mammals [15, 12, 14]. They share substantial similarity of amino-acid sequences but have distinct tissue distribution. α E-catenin is a well-known tumor suppressor with broad expression profile [12]. α N-catenin is restricted to neuronal tissues [16] and α T-catenin is the newest identified member of α -catenin family and primarily expressed in heart tissue and testis [15]. Although cardiac-specific deletion of *Ctnna1* resulted in progressive dilated cardiomyopathy and susceptibility to wall rupture in adult mice [17] and *Ctnna3* deficient mice displayed ventricular arrhythmia and dilated cardiomyopathy after acute ischemia [18], enhanced cardiomyocyte proliferation was observed in hearts from cardiac-specific *Ctnna1* and *Ctnna3* double knockout mice [19]. In addition,

mutations in human *CTNNA3* were identified in patients with arrhythmogenic right ventricular cardiomyopathy [20]. However, whether α E-catenin or α T-catenin deficiency alone promotes heart regeneration and cardiomyocyte proliferation remains elusive.

Here we present the experimental evidence that *Ctnna3* deficiency solely is sufficient to promote heart regeneration following heart apex resection in neonatal mice. Our study revealed that cardiomyocyte proliferation was increased in neonatal *Ctnna3* deficient mice, probably by up-regulating *Yap* expression. These results suggest that α T-catenin does contribute to regulation of heart regeneration and cardiomyocyte proliferation in neonatal mice.

Materials And Methods

Animals

The *Ctnna3*^{-/-} mice (a kind gift from Dr. Radice's lab at Thomas Jefferson University, Philadelphia, PA, USA) have been characterized previously [18]. *Ctnna3*^{-/-} (C57/129) mice were crossed to wildtype (WT) FVB mice to generate *Ctnna3*^{+/-} mice. These mice were maintained and raised on a C57/129/FVB genetic background in a specific pathogen-free facility. Experiments were performed in accordance with "the National Institutes of Health guide for the care and use of laboratory animals" and approved by Use Committee of the Institute of Developmental Biology and Molecular Medicine at Fudan University, Shanghai, China.

Isolation And Culture Of Neonatal Mouse Cardiomyocytes

Cardiomyocytes from neonatal mice were isolated as previously described [19]. Briefly, hearts from neonatal mice at postnatal day 1 (P1) were dissociated with collagenase II. The dissociated cells were plated in a 10cm plate for 2 hours with DMEM/F12 medium, and the supernatant was collected and cells were re-plated on laminin-coated glass coverslips in 12-well plates at 3x10⁵ cells per well. On the following day, the proliferating cells were labeled by EdU in fresh medium for 24 hours and detected using Cell-light EdU Apollo 643 *In Vitro* Kit (#C10310-2, RiboBio, Guangzhou, China) following the manufacturers' instructions.

Neonatal Mouse Apical Resection

Neonatal mouse apical resection of hearts from P1 pups was performed as described previously [21].

Histological And Immunofluorescent Analyses

Tissue processing, frozen sections and immunofluorescent microscopic analysis were performed as previously described [22]. Briefly, for frozen sections, mouse hearts were dissected out, fixed with 4% PFA in PBS overnight, dehydrated with 30% sucrose at 4°C for 3 days and then embedded in OCT (Richard-Allan Scientific). Sections were collected at 10µm.

For histological analysis, mouse hearts were paraffinized and were sectioned at 4 µm followed by hematoxylin/eosin (H&E) staining or Masson's trichrome (MT) staining (G1340, Solarbio, Beijing, China) as previously described[23].For quantification of cardiac fibrosis, images of the MT stained sections were captured with Leica Aperio VERSA microscope (Leica Biosystems, Germany) and the area of fibrosis in heart apex was determined using Visiopharm software (Visiopharm, Horsholm, Denmark).

For *in vivo* EdU (5-ethynyl-2'-deoxyuridine) assay, WT or *Ctnna3*^{-/-} mice at P2 were injected intraperitoneally with 50mg/kg EdU. Twenty four hours after injection, the mouse hearts were dissected out and processed for frozen section. EdU assay was performed using the Kit (#C10310-2, RiboBio, Guangzhou, China) following the manufacturers' instructions.

For immunofluorescent analysis, the sections were stained with antibodies against the following proteins: sarcomeric α-Actinin (#A7732, Sigma, USA); Phospho-histone H3(PH3) (#53348) and PCNA(#2586) from Cell Signaling Technology, USA. Fluorescence micrographs were acquired using a Zeiss LSM710 confocal microscope.

Western Blotting

Standard Western blot protocol was followed. Protein extracts were prepared with RIPA buffer and then subjected to SDS-PAGE. The antibodies against the following proteins were used in our study: αT-catenin (#13974-1-AP, Proteintech, USA), β-catenin(#51067-2-AP, Proteintech, USA), and Yap (14074T, Cell Signaling, USA). The protein bands were detected with an ECL Western Blotting Analysis System. The images were obtained by Tanon-5200, and the density of bands was determined with Image J.

Statistical analysis

Unpaired Student's t-test by GraphPad Prism was conducted for statistical analysis.

Results

Loss of *Ctnna3* accelerates heart regeneration in neonatal mice after heart apex resection.

Although the mammalian adult heart is generally considered nonregenerative, neonatal mouse hearts have a genuine capacity to regenerate following apex resection [1, 3]. To evaluate whether *Ctnna3* affects heart regeneration, we performed surgical apical resection of hearts (5%~10% of the ventricular myocardium) of WT and *Ctnna3*^{-/-} neonatal mice at P1 and harvested hearts at 7 and 14 day(s) post-

resection (dpr) (Fig. 1A) for histological analysis and immunofluorescence staining with antibody against PCNA, separately. The results revealed that, as reported by Porrello [24], the resection plane was characterized by progressive regeneration of the apex with some restoration of the resected myocardium within 14 days (Fig. 1B). The Masson's trichrome staining showed that the accumulation of fibrotic tissue (blue staining in Fig. 1C) in hearts from *Ctnna3*^{-/-} mice at 14dpr dramatically decreased compared with WT mice (Fig. 1C and D). The number of PCNA-positive cells in the border zone of regenerated hearts of *Ctnna3*^{-/-} neonatal mice at 7 dpr was significantly higher than the counterpart in hearts of WT neonatal mice (Fig. 1E and F). As heart regeneration is thought to occur primarily through cardiomyocyte proliferation [25, 10], our results suggest that loss of *Ctnna3* may enhance heart regeneration by promoting cardiomyocyte proliferation in neonatal mice.

Ctnna3 deficiency promotes cell proliferation in neonatal mouse hearts

Since cardiomyocytes in neonatal mouse heart retain active proliferation before postnatal day 7 [26], we tested whether *Ctnna3* deficiency promoted cardiomyocyte proliferation in neonatal mice. The ventricles of WT and *Ctnna3*^{-/-} mice at P1, P3, P7 were sectioned and stained separately with antibodies against phospho-histone H3 (PH3) (a marker of mitosis)[27], and Sarcomeric α -actinin (a specific marker for α -skeletal and α -cardiac muscle actinins) [28]. Quantification of PH3-positive cardiomyocytes revealed that cardiomyocyte proliferation was strikingly increased in the ventricles of *Ctnna3*^{-/-} neonatal mice at P3 and P7 (Fig. 2A, B), but not at P14 compared to WT counterparts.

To further verify the enhanced cell proliferation in *Ctnna3*^{-/-} neonatal mice, the proliferating cells in *Ctnna3*^{-/-} and WT neonatal mice at P7 were *in vivo* EdU-pulse labeled and detected by EdU staining (see Materials and Methods). The results showed that the number of EdU-positive cells was significantly increased in ventricles and atriums of *Ctnna3*^{-/-} neonatal mice compared to those in control littermates (Fig. 2C-F). These results demonstrate that *Ctnna3* deficiency promotes cell proliferation in neonatal mouse hearts.

Ctnna3 deficiency promotes proliferation of primary cardiomyocytes

Since heart is mainly composed of cardiomyocytes, in addition to several other types of cells, such as cardiac fibroblasts, endothelial cells, and smooth muscle cells [29], we hypothesized that *Ctnna3* deficiency promoted the proliferation of cardiomyocytes. To test this hypothesis, primary cardiomyocytes were isolated from P5 WT and *Ctnna3*^{-/-} mouse hearts and pulse-labelled with EdU *in vitro* followed by immunostaining with antibody against sarcomeric α -actinin and EdU staining. Our study revealed that a significant increase of the proportion of the EdU-positive cardiomyocytes (proliferating cardiomyocytes) from *Ctnna3*^{-/-} neonatal mice compared to that from WT mice at P5 (Fig. 3A and B). These results demonstrate that the loss of *Ctnna3* promotes proliferation of cardiomyocytes in neonatal mice.

Loss of Ctnna3 enhances Yap expression

Alpha-catenins directly bind to both β -catenin and actin filaments, thereby coupling stable actin filaments to the cadherin adhesion molecules [11, 16, 13]. β -catenin is also a well-documented positive regulator for cell proliferation. To study the mechanism(s) by which α T-catenin deficiency promotes cell proliferation, the expression level of β -catenin in hearts from *Ctnna3*^{-/-} and WT mice at P7 was evaluated by Western blotting. The result showed that the protein level of β -catenin was not significantly changed between WT and *Ctnna3*^{-/-} hearts (Fig. 3C and D).

The Hippo pathway is a key regulatory signaling pathway for heart development and organ size [30]. Thus, we inspected whether loss of *Ctnna3* only had any effect on *Yap* expression in the neonatal heart. Our study revealed that the protein level of Yap significantly increased in hearts from P7 *Ctnna3*^{-/-} mice compared to that in control mouse hearts (Fig. 3C and D). This result suggests that *Ctnna3* deficiency may promote neonatal cardiomyocyte proliferation by enhancing *Yap* expression.

Discussion

Previous study showed that cardiac-specific *Ctnna1* (α E-catenin) deficient adult mice displayed progressive dilated cardiomyopathy [17] and *Ctnna3* (α T-catenin) deficient adult mice exhibited dilated cardiomyopathy only after acute ischemia [18], but without affecting the number of cardiomyocytes. Li, et al., also demonstrated that simultaneously cardiac-specific deleting *Ctnna1* and *Ctnna3* in mice at perinatal stage resulted in an increased cardiomyocyte number and cardiomyocyte proliferation in the postnatal heart [19]. Although these results strongly suggest that *Ctnna1* and *Ctnna3* jointly play an important but redundant role in the inhibition of cardiomyocyte proliferation, whether *Ctnna3* contributes to heart regeneration or *Ctnna3* is adequate to suppress cardiomyocyte proliferation at neonatal stage needs to be addressed. Here we present *in vivo* evidences that *Ctnna3* deficient only is sufficient to enhance heart regeneration and cardiomyocyte proliferation in neonatal mice. Hence, functional interference of α -catenins may provide a potential strategy to promote myocardial regeneration after injury.

Declarations

Declaration of competing interest

The authors declare that they have no conflicts of interest with the contents of this article.

Authors' contribution: Zou and Dai work at data analysis.

Sha Zou and Wuhou Dai were responsible for the data acquisition, data analysis and writing. Jifen Li and Hongyan Wang provided the *Ctnna3*^{-/-} mice, Yuan Lin, Juan Liu and Wufan Tao provided ideas of research, paper modification and critical reading of the manuscript. All authors have approved the final manuscript.

Ethics approval:

Experiments were performed in accordance with “the National Institutes of Health guide for the care and use of laboratory animals” and approved by Use Committee of the Institute of Developmental Biology and Molecular Medicine at Fudan University, Shanghai, China.

Acknowledgements

This work was supported by the National Key Research and Development Program of China (Grant No. 2016YFC1000500), National Natural Science Foundation of China (Grant No. 31671330) and Science and Technology Commission of Shanghai Municipality (Grant No. 201409001500).

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Figures

Figure 1

Figure 1

Ctnna3 deficiency promotes heart regeneration in neonatal mice after heart apical resection. A. The scheme of the open thoracotomy experiment. P1: postnatal day 1; dpr: day(s) post-resection. B-C. Representative images of paraffin heart sections from WT and *Ctnn3*^{-/-} mice at 14 dpr stained with HE (B) or Masson's trichrome (C). Low panels in (C) are enlarged views of the cropped regions in up panels in (C). Bars=50µm. D-E. Statistics analysis of the area of fibrotic tissue per field in C (WT, n =3; *Ctnn3*^{-/-},

n=3) and PCNA-positive cells per field at apex in (F) (WT, n =5; *Ctnna3*^{-/-}, n=5). F. Representative fluorescent images of frozen sections of mouse hearts at 7 dpr stained with anti-PCNA. White dash lines mark the resection boundaries. Bars=50µm. Values in D and E represent the means ± SD. *, p<0.05.

Figure 2

Figure 2

Enhanced cell proliferation in hearts of *Ctnna3*^{-/-} neonatal mice. A. Representative florescent images of mouse heart sections from P7 WT and *Ctnna3*^{-/-} mice co-stained with anti-PH3 (for prophase mitotic

cells) and anti-Sarcomeric- α -actinin (for Cardiomyocytes). Bar=200 μ m. B. Statistics analysis of PH3-positive cells per field (WT, n=5; *Ctnna3*^{-/-}, n=5). C and E. Representative fluorescent images of the sections of atrium (C) and ventricle (E) from P7 WT and *Ctnna3*^{-/-} neonatal mice pulse-labelled with EdU. Bars=50 μ m. D and F. Statistics analysis of EdU-positive cells per field (WT, n=5; *Ctnna3*^{-/-}, n=5) in C and E. Values in B, D and F represent the means $\pm$ SD. **, p<0.01, *, p<0.05 ns, not significant.

Figure 3

Figure 3

Ctnna3 deficiency promotes proliferation of primary cardiomyocytes and up regulates YAP expression. A. Representative fluorescent images of cultured primary mouse cardiomyocytes from neonatal WT and *Ctnna3*^{-/-} mice. The cultured cells were pulse-labeled with EdU for 24 hrs followed by fluorescent staining. Bar=100 μ m. B. Statistics analysis of EdU-positive cardiomyocytes per field in A. (WT, n=9; *Ctnna3*^{-/-}, n=12). C-D, Western blot analysis (C) and quantification (D) of α -catenin and Yap protein expression in

hearts from WT and Ctnna3 deficient mice. (n=3 for WT and Ctnna3^{-/-}, respectively). Values in B and D represent the means $\pm$ SD. *, p<0.05.