Do Neonatal Mouse Hearts Regenerate following Heart Apex Resection?

Ditte Caroline Andersen,1,2,* Suganya Ganesalingam,1,2 Charlotte Harken Jensen,1 and Søren Paludan Sheikh1,3,*

1Laboratory of Molecular and Cellular Cardiology, Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Winsloewparken 21th, 5000 Odense C, Denmark
2Clinical Institute, University of Southern Denmark, 5000 Odense C, Denmark
3Institute of Molecular Medicine, University of Southern Denmark, 5000 Odense C, Denmark

*Correspondence: danandersen@health.sdu.dk (D.C.A.), soeren.sheikh@rsyd.dk (S.P.S.)
http://dx.doi.org/10.1016/j.stemcr.2014.02.008
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

SUMMARY

The mammalian heart has generally been considered nonregenerative, but recent progress suggests that neonatal mouse hearts have a genuine capacity to regenerate following apex resection (AR). However, in this study, we performed AR or sham surgery on 400 neonatal mice from inbred and outbred strains and found no evidence of complete regeneration. Ideally, new functional cardiomyocytes, endothelial cells, and vascular smooth muscle cells should be formed in the necrotic area of the damaged heart. Here, damaged hearts were 9.8% shorter and weighed 14% less than sham controls. In addition, the resection border contained a massive fibrotic scar mainly composed of nonmyocytes and collagen disposition. Furthermore, there was a substantial reduction in the number of proliferating cardiomyocytes in AR hearts. Our results thus question the usefulness of the AR model for identifying molecular mechanisms underlying regeneration of the adult heart after damage.

INTRODUCTION

A key question in cardiovascular biology is to what degree the heart is able to regenerate after tissue damage from either cardiac stem cells or cardiomyocyte division. Cardiovascular disease including myocardial infarct is currently one of the leading causes of death worldwide, and the general view is that this is mainly caused by a genuine inability of the mammalian heart to regenerate upon damage (Vieira and Riley, 2011). Yet, this dogma was recently challenged by exciting data suggesting that the mouse heart retains regenerative ability up to 1 week after birth (Porrello et al., 2011), and without being reproduced by others, it has now been accepted as an established principle that neonatal mammalian hearts do enclose a true cardiac-regenerative potential following apex resection (AR) (Aguire et al., 2013; Garbern and Lee, 2013). As a minimal requirement, complete cardiac regeneration should include the restoration of the functional continuity of cardiomyocytes, as well as blood supply in the necrotic area of the damaged heart with no sign of scar formation. Indeed, urodele amphibians and zebrafish have been shown to possess a high capacity to repair the heart following damage such as AR that meets these minimal criteria (Garbern et al., 2013). Accordingly, the zebrafish heart is regenerated in 60 days following AR, with full recovery of the myocardium (Poss et al., 2002). The mammal and zebrafish heart anatomy/physiology diverge substantially (Garbern et al., 2013). It was therefore a breakthrough in regenerative medicine of the mammalian heart when Porrello et al. in 2011 showed that the neonatal mouse heart (1 day old) holds an intrinsic capability to regenerate completely following resection of 10% of the heart apex (Porrello et al., 2011). As in the zebrafish heart (Jopling et al., 2010), the regenerative response in mice was primarily accomplished through reentry of cardiomyocytes into the cell cycle (Porrello et al., 2011). Interestingly, this ability was only transient and lost by postnatal day 7 (P7) (Porrello et al., 2011), a scenario the authors most recently suggested is caused by the homeobox transcription factor Meis1 inhibiting cardiomyocyte proliferation (Mahmoud et al., 2013). Remarkably, the repairing response seems to be faster in mice (21 days) (Porrello et al., 2011) than in teleost fish (60–180 days) (Lafontant et al., 2012; Poss et al., 2002). Furthermore, the regenerated neonatal mouse heart reportedly showed no signs of major scarring after 21 days (Porrello et al., 2011), which is in contrast to the mammalian adult heart that lacks substantial regenerative capacity (Garbern et al., 2013; Vieira and Riley, 2011). In addition, urodele amphibians and teleost fish show substantial scarring up until 60–180 days postinjury (Lafontant et al., 2012; Oberpriller and Oberpriller, 1974; Poss et al., 2002).

The reported availability of the neonatal mouse heart regeneration model is thus extremely valuable to researchers in order to identify factors that may be used for improving regeneration of the adult heart in the large group of patients suffering from cardiac infarcts. We thus originally set out to identify factors enabling regeneration of the heart. However, our data do not yield evidence of a complete regenerative response in the neonatal mouse heart following AR.
RESULTS

Establishing the AR Model in Inbred C57Bl/6 Mice

The study by Porrello et al. was performed in the outbred ICR/CD-1 mouse strain (Porrello et al., 2011). However, many transgenic mouse models including ours use inbred mouse strains. We therefore set out to clarify and evaluate the regenerative potential of AR hearts in C57Bl/6 mice. We used the exact same surgery protocol (Figure 1A) as described previously by Porrello et al. (2011) for AR. The heart-to-body weight was reduced by 8.4% in AR hearts as compared with sham hearts immediately after resection (Figures 1B and 1C). The average resection size is thus similar to the approximate 10% accomplished by Porrello et al. (2011). Supportive of that, we achieved a 90% survival of AR animals following surgery, whereas maternal cannibalization further lowered this to 80%–85% (Figure 1D), which is moderately higher than the 70% survival reported for ICR/CD-1 mice (Porrello et al., 2011). Thus, our surgery setup for AR in C57BL/6 mice seems comparable to that reported for the ICR/CD1 mice.

Neoatal C57Bl/6 Mice Do Not Regenerate Their Hearts following AR

Unexpectedly, however, we found that the AR heart weights were compromised throughout the study, with a persistent 14% reduction in AR hearts at day 21 (Figure 1E). Additionally, the resected hearts were 9.8% shorter at day 21 than sham controls (Figures 1F and 1G). These results thus indicated that the apex was healed but not regenerated with healthy tissue replenishing the removed apex. In accordance, stereomicroscopic (Figure 1G) and histological (Figure 1H; Figure S1 available online) examinations of AR and sham hearts showed an extensive remodeling of the damaged area in 100% of AR hearts at day 21, which was absent in sham specimens. Of note, scars were observed in 15.7% ± 4.1% of hematoxylin and eosin (HE)-stained sections (778 ± 54 per heart; n = 7). These data thus surprisingly suggested that C57BL/6 hearts were unable to regenerate.

Apex-Resected C57Bl/6 Hearts Are Healed with Accumulation of Many Noncardiomyocytes

An extensive quantitative real-time PCR study showed that mesenchymal, fat, hematopoietic, and vascular gene
programs were significantly induced in AR hearts at day 21 as compared to sham hearts (Figure S2), whereas epicardial and cardiomyocyte gene programs overall remained unchanged at this endpoint (Figure S2). Hematopoietic cells such as macrophages are responsible for cleaning up necrotic constituents in areas of heart damage but are also main drivers of cardiac fibrosis (Santini and Rosenthal, 2012). We found increasing mRNA levels of *Cd45*, a hematopoietic marker (Figure 2A), already 3 hr after AR, reflecting an immediate influx of inflammatory cells as confirmed by immunohistochemistry (Figure 2B). The majority of these inflammatory cells disappeared between days 7 and 14 (Figures 2A and 2B), but some were still evident at day 21 in the lesioned area of AR hearts (Figure 2B). Heart fibrosis is characterized by deposition of collagen I and a transition of cardiac fibroblasts to myofibroblasts secreting large amounts of fibronectin. We found the inflammatory response in AR hearts to be clearly accompanied by an increase in both *Pro-collagen I* and *Fibronectin* (Figure 2A). This was confirmed by substantial amounts of collagen in the lesioned area as visualized by Sirius Red staining (Figure 2C) and immunohistochemistry (Figure 2D). In contrast to sham hearts, 100% of AR hearts contained numerous noncardiomyocytes (cardiac myosin*/β*-actin) in the apex (Figure 2D). Moreover, mRNAs for *Atrial Natriuretic Peptide* (ANP) and *Brain Natriuretic Peptide* (BNP), two markers of cardiac damage and hypertrophy, were increased in the AR hearts (Figure 2E) with a concomitant 7.5% increase in cardiac width (Figure 2F), the latter possibly indicating hypertrophic growth. Together, these data demonstrated that resection of heart muscle in neonates induced a massive fibrotic response resulting in substantial scarring at day 21.
The Damaged Apex Comprises Modest Vascularization, but Few Proliferating Cardiomyocytes

Ideally, cardiac regeneration should be able to restore the functional continuity of cardiomyocytes, as well as blood supply (endothelial and smooth muscle cells) in the necrotic area of the damaged heart. We did encounter blood vessels with CD31+ endothelia, and surrounding smooth muscle cells (aSMA+ and NG2+) in the lesioned area (Figures 2D and 3A). These vessels may either be preexisting vessels developed in the border zone prior to lesion, but they may also represent newly formed vascularity in the damaged area because both Cd31 and numerous other vascular genes (Figure S2) were increased in AR hearts as compared with sham controls. Yet, the vascularization was still incomplete as compared with
normal vascularized myocardium (Figures 2D and 3A). Porrello et al. suggest that the regenerative heart response is due to an increased number of cardiomyocytes undergoing division 1–7 days following AR (Porrello et al., 2011). We therefore quantified the total number of proliferating cells (5-ethynyl-2-deoxyuridine positive [EdU+]) as well as the number of proliferating cardiomyocytes (EdU+/cardiac myosin+/collagen I–) 1–7 days postsurgery by pulse-chase labeling experiments (Figures 3C–3F). As such, there was a clear tendency (p = 0.06) for lower numbers of proliferating cells in the AR hearts compared to sham hearts, and a profound reduction in the overall number of dividing cardiomyocytes throughout the heart, with remarkably few being present in the apex area (Figures 3G and 3H). Instead, the proliferating cells in the apex region of AR hearts (Figures 3F and 3H) were fibroblastic in phenotype (EdU+/cardiac myosin−/collagen 1+) and accounted for the majority of proliferating cells in this zone (Figure 3F). In addition, we did not observe any difference in AR and sham hearts of Meis1b transcripts (Figure 3G), and levels were decreasing with normal cardiac development (Figure 3H). This contradicts the suggested ability of increasing Meis1b levels to inhibit cardiomyocyte proliferation beyond P7 (Mahmoud et al., 2013). Therefore, our data do not support enhanced cardiomyocyte proliferation 1–7 days following AR as previously reported by Porrello et al. (2011). To exclude that the regeneration process merely was delayed in C57Bl/6 mice, we prolonged the examination period with 16 days, thus analyzing the hearts histologically at day 37. Yet, the hearts were still substantially compromised at day 37 with large visual fibrotic scars (Figures 1H, 1I, and 2C), numerous noncardiomyocytes in the lesion area (data not shown), and insufficient vascularization (data not shown), further suggesting that C57Bl/6 hearts heal by remodeling rather than by true regeneration.

ICR/CD1 Mice Also Fail to Accomplish Complete Regeneration of Their Hearts

Porrello et al. showed a high regenerative capacity of apex-resected hearts in outbred ICR/CD1 mice (Porrello et al., 2011). Although no difference in regenerative capacity has been reported for C57Bl/6 and ICR/CD1 mouse strains, other regeneration schemes such as wound healing in the ear are highly dependent on the strain (Heber-Katz, 1999). We therefore next performed a new series of AR in 24 ICR/CD1 mice to reevaluate the cardiac-regenerative response recently described by Porrello et al. (2011). However, we did not find any evidence suggesting a complete cardiac-repairing response in the neonatal heart of ICR/CD1 mice either (Figure 4). As with the C57Bl/6 hearts, we found substantial fibrotic scars in 21.5% ± 4.7% of HE-stained sections (846 ± 60 per heart; n = 11), many noncardiomyocytes (cardiac myosin−/desmin−/nonmuscle myosin+/collagen I+), and an incomplete vascular network in 100% of resected ICR/CD1 hearts at day 21 (Figures 4 and S3). These data thus suggest that the absence of complete heart regeneration following heart muscle resection is mouse strain independent.

DISCUSSION

Mortality after myocardial infarction is relatively high, and surviving patients are often severely compromised due to insufficient heart-pump function. At the cellular level, damage to the heart’s contractile constituents, the cardiomyocytes, is irreversible, and treatments merely serve to reduce symptoms, and only a minority of patients receives a heart transplant. Novel therapeutics to reestablish the cardiac tissue following infarcts are therefore needed, and factors improving heart regeneration will be of enormous value. The groundbreaking discovery of a mammalian regenerative heart model in mice (Porrello et al., 2011) has thus gained immense attention and hope for identifying factors that may improve cardiac regeneration. However, none of our results here suggests that the neonatal mammalian heart is able to accomplish complete regeneration following heart muscle resection as recently suggested by Porrello et al. (2011). Instead, we demonstrate that the resected heart apex remains lost and that the resection border is healed by a fibrotic scar composed of myofibroblasts, adipocytes, and sparse vessels. These nonmyogenic cells likely originate from the pericardium/epicardium (Smart et al., 2007; Zhou et al., 2008), the only structure in the apex that seemed completely regenerated. In contrast, we observed a lack of cardiac regeneration likely caused by a reduced number of proliferating cardiomyocytes within AR hearts as compared with noninjured hearts. Although the absolute number of proliferating cardiomyocytes would have been optimally determined by quantifying a proliferation marker in combination with a nuclear cardiomyocyte genetic label (Ang et al., 2010), our approach using EdU with cardiac myosin/collagen I seems valid for the relative measurements we performed, and importantly, Porrello et al. used a similar approach (Porrello et al., 2011). Our results are thus contradictory to those reported in the original study performed by the Sadek research group (Porrello et al., 2011).

We can only speculate on the reasons for the contradictions between our study and the Porrello study (Porrello et al., 2011). Importantly, we resected equal amounts of apex tissue, an obvious key parameter that otherwise could have influenced the outcome. Likewise, we used the same time schedule during surgery as previously described by Porrello et al. (2011), where the extent of hypothermia may be critical for the heart tissue survival and therefore...
also the regenerative ability. The use of different mouse strains may impose another problem to the results; yet, this seems less likely because we performed AR in both C57Bl/6 and ICR/CD1 mice and found no difference in scar formation at day 21. Supportive of that, the Sadek research group as well as others have reported a regenerative capacity of the heart in B6C3F1 (hybrid of C57Bl/6 × C3H/He) (Xin et al., 2013) and C57Bl/6 (Haubner et al., 2012) mice. Even so, ICR/CD1 mice from different breeding facilities have been shown to diverge (Aldinger et al., 2009), and unknown factors associated with the mice thus cannot be absolutely excluded for having an impact on the heart’s ability to regenerate. Finally, one may consider the analysis procedures used in our study and the ones performed by Porrello et al. Both studies base a substantial amount of the results on histological examinations. Porrello et al. report in their supplemental information that they observe a small fibrotic scar in 2 out of 140 (1.4%) sections from damaged hearts (Porrello et al., 2011). Yet, they do not describe if this accounts for each heart examined or just a single outlier. It is also unclear how many hearts were examined and if the 140 sections were randomly chosen throughout the heart or in one series. For comparison, we observed substantial scarring in 100% (31 out of 31) of AR hearts using histology, and the lesioned area was present in 19.5% ± 5.2% of sections, regardless of mouse strain. Notably, Porrello et al. sliced 140 sections (each 5 μm) from one, day 21 heart, whereas we obtained 820 ± 66 sections (each 5 μm). Thus, it may be that Porrello et al. overlooked the lesioned area, which often is located in the beginning or the end of sectioning (Figure S1).

Our results therefore contradict that the AR model may be used for identifying factors that enable cardiomyocyte replenishment and improve heart regeneration following damage. We cannot, however, eliminate the possibility that a few cardiomyocytes are being regenerated following AR, but the amplitude of this response, if present, may therefore be insufficient for a complete cardiac repair. One obvious limitation of the model seems to be the ongoing hyperplastic and hypertrophic growth present in the neonatal mouse hearts, which makes it difficult to distinguish an eventual small true regenerative response from simple developmental features. In that regard, it is important to note that the well-recognized zebrafish heart resection model is performed in adult animals (Jopling et al., 2010; Lafontant et al., 2012; Poss et al., 2002) free of immediate hyperplastic growth.
Yet, from our results, we cannot exclude that other cardiac regeneration models such as the left anterior-descending (LAD) artery ligation in the neonatal myocardium (Haubner et al., 2012; Mahmoud et al., 2013; Xin et al., 2013) do unravel a true and complete regenerative potential of the heart. Still, we find it questionable that a full cardiac regenerative response is accomplished in only 21 days (Mahmoud et al., 2013; Porrello et al., 2011), and in one study (Haubner et al., 2012), after only 7 days following LAD damage of the mouse heart. For comparison, Jesty et al. recently showed that cryo-injury to the neonatal heart is associated with scar formation even after 94 days (Jesty et al., 2012), a scenario that is also seen up until 60–180 days after cardiac injury in simpler organisms like teleost fish (Jopling et al., 2010; Lafontant et al., 2012). However, in a previous study, a genetic model of cardiomyocyte ablation showed that the cardiomyocyte pool is restored in the fetal mouse heart by enhanced proliferation of remaining cardiomyocytes (Drenckhahn et al., 2008). One may speculate if a similar scenario could take place after neonatal mouse heart LAD damage, whereas the AR model lacks an intact 3D heart architecture, a feature that likely is important to some extent for mammalian regeneration to be accomplished. In summary, we thus believe that additional clarifying data are required from the scientific community on this controversial matter to firmly establish whether the mammalian heart is regenerative, otherwise our data substantiate the view that it is not.

**EXPERIMENTAL PROCEDURES**

A detailed version of our Experimental Procedures can be found in the Supplemental Experimental Procedures. All animal experiments were approved by the Danish Council for Supervision with Experimental Animals (#2011/561-1966). AR was performed at P1 as previously described by Porrello et al. (2011). Briefly, neonates were anesthetized by hypothermia, and the apex was resected until left ventricle chamber exposure, after which the thoracic wall and skin were sutured. Sham mice underwent the exact same procedure without resecting the apex of the heart. For pulse-chase labeling experiments, mice were injected 1 day after surgery with EdU, and the number of proliferating cardiomyocytes and cells in total was counted after 7 days. Relative quantitative PCR (qPCR) and histology were performed as previously described (Andersen et al., 2009). All analyses comprised at least four independent experiments, and statistical significance (p < 0.05) was tested as indicated.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and three figures can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.02.008.
Haubner, B.J., Adamowicz-Brice, M., Khadayate, S., Tiefenthaler, V., Metzler, B., Atman, T., and Penninger, J.M. (2012). Complete cardiac regeneration in a mouse model of myocardial infarction. Aging (Albany, N.Y. Online) 4, 966–977.

Heber-Katz, E. (1999). The regenerating mouse ear. Semin. Cell Dev. Biol. 10, 415–419.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19.

Jesty, S.A., Steffey, M.A., Lee, F.K., Breitbach, M., Hesse, M., Reining, S., Lee, J.C., Doran, R.M., Nikitin, A.Y., Fleischmann, B.K., and Kotlikoff, M.I. (2012). c-kit+ precursors support postinfarction myogenesis in the neonatal, but not adult, heart. Proc. Natl. Acad. Sci. USA 109, 13380–13385.

Jopling, C., Sleep, E., Raya, M., Martí, M., Raya, A., and Izpisúa Belmonte, J.C. (2010). Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. Nature 464, 606–609.

Lafontant, P.J., Burns, A.R., Grivas, J.A., Lesch, M.A., Lala, T.D., Reuter, S.P., Field, L.J., and Frounfelter, T.D. (2012). The giant danio (D. aequipinnatus) as a model of cardiac remodeling and regeneration. Anat. Rec. (Hoboken) 295, 234–248.

Mahmoud, A.I., Kocabas, F., Muralidhar, S.A., Kimura, W., Koura, A.S., Thet, S., Porrello, E.R., and Sadek, H.A. (2013). Meis1 regulates postnatal cardiomyocyte cell cycle arrest. Nature 497, 249–253.

Oberpriller, J.O., and Oberpriller, J.C. (1974). Response of the adult newt ventricle to injury. J. Exp. Zool. 187, 249–253.

Porrello, E.R., Mahmoud, A.I., Simpson, E., Hill, J.A., Richardson, J.A., Olson, E.N., and Sadek, H.A. (2011). Transient regenerative potential of the neonatal mouse heart. Science 331, 1078–1080.

Poss, K.D., Wilson, L.G., and Keating, M.T. (2002). Heart regeneration in zebrafish. Science 298, 2188–2190.

Santini, M.P., and Rosenthal, N. (2012). Myocardial regenerative properties of macrophage populations and stem cells. J. Cardiovasc. Transl. Res. 5, 700–712.

Smart, N., Risebro, C.A., Melville, A.A., Moses, K., Schwartz, R.J., Chien, K.R., and Riley, P.R. (2007). Thymosin beta-4 is essential for coronary vessel development and promotes neovascularization via adult epicardium. Ann. N Y Acad. Sci. 1112, 171–188.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, H0034.

Vieira, J.M., and Riley, P.R. (2011). Epicardium-derived cells: a new source of regenerative capacity. Heart 97, 15–19.

Xin, M., Kim, Y., Sutherland, L.B., Murakami, M., Qi, X., McNally, J., Porrello, E.R., Mahmoud, A.I., Tan, W., Shelton, J.M., et al. (2013). Hippo pathway effector Yap promotes cardiac regeneration. Proc. Natl. Acad. Sci. USA 110, 13839–13844.

Zhou, B., Ma, Q., Rajagopal, S., Wu, S.M., Domian, I., Riverafeliciano, J., Jiang, D., von Gise, A., Ikeda, S., Chien, K.R., and Pu, W.T. (2008). Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. Nature 454, 109–113.