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Recapitulation of developmental cardiogenesis governs the morphological and functional regeneration of adult newt hearts following injury

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ABSTRACT

Urodele amphibians, like the newt, are the "champions of regeneration" as they are able to regenerate many body parts and tissues. Previous experiments, however, have suggested that the newt heart has only a limited regeneration capacity, similar to the human heart. Using a novel, reproducible ventricular resection model, we show for the first time that adult newt hearts can fully regenerate without any evidence of scarring. This process is governed by increased proliferation and the up-regulation of cardiac transcription factors normally expressed during developmental cardiogenesis. Furthermore, we are able to identify cells within the newly regenerated regions of the myocardium that express the LIM-homeodomain protein Islet1 and GATA4, transcription factors found in cardiac progenitors. Information acquired from using the newt as a model organism may help to shed light on the regeneration deficits demonstrated in damaged human hearts.

Introduction

Adult human hearts have a very limited ability to generate new cardiac myocytes. Although this mechanism might be important for turnover or homeostasis of the cardiac cells, it has insufficient capacity for regenerating significant regions of the myocardium following injury (Bergmann et al., 2009; Torella et al., 2007). Instead of functional replacement of cardiomyocytes with electro-mechanical stabilization (reverse remodeling), the damaged myocardium is infiltrated by fibrotic tissue (Chachques, 2009). This type of wound healing response where the functional myocardium is replaced by the scar tissue may lead to progressive heart failure. Experimental and clinical trials involving the use of cell-based therapies, delivery of growth factors and cardiac tissue engineering are producing promising but conflicting results (Bartunek et al., 2009; Genovese et al., 2007; Reffelmann et al., 2009). Along with identifying the optimal cell type to transplant, with respect to stage of commitment or diversification within a myocardial sub-lineage, there still remain the issues of cell delivery, homing of the cells to the damaged areas of the heart and electrical coupling into the host myocardium that have to be addressed before cell-based therapies can be considered as a suitable substitute to established heart intervention therapies (Mummery et al., 2010).

In order to address these problems it is necessary to obtain information from organisms that can regenerate the myocardium following damage. Some elegant work has recently shown that the two-chambered heart of the zebrafish can undergo extensive regeneration following injury (Poss et al., 2002). Another species that has comprehensive regeneration abilities is the red-spotted newt or *Notophthalmus viridescens* (Brockes and Kumar, 2002). This urodele amphibian has the remarkable ability to regenerate multiple organs and tissues, with the most impressive example being the regeneration of a fully functioning limb following amputation (Brockes, 1997). However, earlier studies concluded there was a regeneration deficit when it came to cardiac repair in these animals (Becker et al., 1974; Neff et al., 1996; Oberpriller and Oberpriller, 1974).

There have been some intriguing results indicating that the newt heart does have an ability to repair. For example, if minced newt cardiac tissue is transplanted to the site of a resected ventricle, not only is there increased proliferative events at the transplantation site but there is also functional integration and restoration of the resected newt ventricle (Bader and Oberpriller, 1978). Furthermore, it has been shown that following crush injury to the newt ventricle there is a severe reduction in sarcomeric proteins, with levels of these proteins restored several weeks following the initial injury (Laube et al., 2006). However, many of the heart regeneration models in the newt remain poorly characterized in terms of cellular contribution and length of time necessary to restore a fully functioning myocardium following injury.

To address the extent of regeneration in the newt heart, we have developed a standardized ventricular resection model in the red-spotted newt (*N. viridescens*) where we can follow functional and structural restoration for extended periods of time after the initial injury. We show via histological and functional echocardiogram

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analysis that the newt heart morphologically and functionally regenerates, without the presence of scarring. We further show the identification of separate populations of proliferating Islet1 and GATA4 positive cells within the regenerating regions of the heart, implying that cardiac progenitors could be present and participate in the regeneration of newt myocardium.

Materials and methods

Antibodies

The following primary antibodies were used: mouse monoclonal anti-myosin heavy chain IgG (MF20; Developmental Studies Hybridoma Bank), mouse monoclonal anti-Islet1 IgG (40.2D6; Developmental Studies Hybridoma Bank), goat polyclonal anti-cardiac troponin T (Abcam plc, UK), rat monoclonal anti-BrdU IgG (Trichem ApS), rabbit polyclonal anti-GATA4 (H-112), mouse monoclonal anti-GATA4 (G4), rabbit polyclonal anti-NKX2.5 (H-114) and rabbit polyclonal anti-p-Histone H3 (Ser 10) (GATA4, NKX2.5 and Histone H3 antibodies are from Santa Cruz Biotechnology). For immunofluorescence studies, primary antibodies were detected with appropriate species-specific Alexa Fluor-conjugated secondary antibodies (Invitrogen).

Animals and procedures

All experiments were performed according to European Community and local ethics committee guidelines. Adult red-spotted newts, *N. viridescens*, were supplied by Charles D. Sullivan Co., Inc. and maintained in a humidified room at 15–20 °C. Newts were anesthetized by placing them in an aqueous solution of 0.1% ethyl 3-aminobenzoate methanesulfonate salt (Sigma-Aldrich) for 15 min. Newts were positioned supine and an incision was made into the ventral body wall exposing the pericardium. The thoracic cavity was exposed fully with a single anchoring stitch, using a reverse cutting (3/8 circle 8 mm) needle with monofilament 8.0 non-absorbable suture, placed into both sides of the incised pericardium. Blunted 5.0 forceps were placed under the exposed aorta, and lifted, in order to expose the heart exteriorly. Starting just below where the atria enter the ventricle, a 27 G needle (0.41 mm) was then used to make a diagonal left to right puncture of the ventricle wall. Using iridectomy scissors, the portion of the tissue pierced by the needle was removed from the ventricle. Hemorrhaging was stemmed via rapid clot formation, brought about by the application of local pressure with cotton swabs. The damaged hearts were maneuvred back into the thoracic cavity and the pericardium and body wall were closed with interrupted stitches using sutures previously mentioned. Newts were left to recover for 24 h in an aqueous solution of 0.5% sulfamerazine (Sigma-Aldrich) on ice, before being placed back into a 15–20 °C water environment. Heart masses were measured using a Sartorius electron microscope (BP210D), which has a readability of 10 μg. For BrdU labeling experiments animals were injected intraperitoneally with 25 μl of 2 mg/ml BrdU 1 day, 3 days, 7 days, 10 days, 14 days and then weekly up until indicated time-points.

Echocardiogram analysis

The ultrasonic imaging was performed on anesthetized newts using a Philips HDI 5000 system with a CL 15–7 probe. Each recording was performed within 15 min after onset of anesthesia. The diastolic and systolic diameters were measured blindly, with at least three recordings made on each animal at each session. The average value was used as a representative recording for the animal at each specified time-point. Fractional shortening measurements were made using the following equation:

$$((\text{Diastole}-\text{Systole})/\text{Diastole})\times 100 = \text{Fractional shortening}(\%).$$

To compare mean fractional shortening percentages between uninjured and resected hearts, a Mann–Whitney *U* test was performed. Statistical significance was accepted at $P<0.05$.

RNA extraction and cDNA synthesis

RNA was extracted from newt A1 myoblasts and ventricular myocardium using the RNAqueous®-4PCR kit (Ambion) according to the instructions of the manufacturer. Ventricular myocardium was obtained by dissecting newt hearts free from the thoracic cavity and removing the connecting aorta and atria with micro-surgical scissors. One microgram of total RNA was used for cDNA synthesis, performed in a 25 μl reaction using 0.8 mM dNTPs, 2 μg 3′RACE Adaptor (Ambion), 1× first strand buffer, 10 U RNase Inhibitor (Invitrogen) and 50 U M-MuLV reverse transcriptase (Fermentas). RNA, primers and dNTPs were first incubated at 37 °C for 90 min followed by 70 °C for 10 min cycle to heat-inactivate the transcriptase. The reaction mixture was diluted with 25 μl of 1× first strand buffer. For every RNA sample, a negative control, lacking reverse transcriptase, was also performed.

PCR protocols

Partial cDNAs for cardiac transcription factors from *N. viridescens* were cloned using degenerate PCR (Table 1). All PCRs were carried out using High Fidelity PCR Enzyme Mix (Fermentas) according to manufacturer's instructions. Primers against the partial cDNA sequences for NKX2.5 (HM367109), GATA4 (HM367110), GATA5 (HM367111), HAND2 (HM367112) and Islet1 (HM367113) were designed using Primer-BLAST (NIH) and Oligo Property Scan (Eurofins MWG Operon—Table 2). For the semi-quantitative PCR, 1 μl of cDNA was mixed with 0.2 μM of each primer, 0.4 mM of each dNTP, 1.5 mM $MgCl_2$, 1× PCR buffer with $(NH_4)_2SO_4$ and 1 U Taq DNA Polymerase (recombinant—Fermentas) in a 25 μl reaction. After an initial denaturation step at 94 °C for 2 min, the samples were put through 10 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 10 s, followed by 19–25 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 10 s, followed by a final elongation step at 72 °C for 10 min. Comparative samples were run on 2% agarose gels containing ethidium bromide and visualized on a UV table. Real-time PCR was carried out using Fast qPCR 2× buffer (KAPA Biosystems, USA) on a Qiagen Rotor-Gene Q real-time PCR machine, according to manufacturer's instructions. Real-time PCR expression analysis of each gene was normalized to GAPDH, a ubiquitously expressed gene used previously as a normalization factor (Vascotto et al., 2005). Eurofins MWG Operon supplied all primers used. To compare mean relative gene expression between uninjured and resected hearts, a two-tailed Student's *t*-test was performed. Statistical significance was accepted at $P<0.05$.

Histology

Tissue samples were mounted as previously described (Morrison et al., 2006). Six micrometer frozen serial sections were cut through the entire ventricle using a Leica Jung CM1800 cryostat. A hematoxylin and eosin (H&E) staining was performed to observe overall cardiomyocyte and nuclei structure. Acid fuchsin-orange G staining (AFOG), which

Table 1
Degenerate primers used to identify cardiac transcription factors.

Gene	Forward	Reverse
Islet1	5′-TCNCCGGAYYTGGARTGGCA-3′	5′-CANCKCTTGTTYTGAAACCA-3′
NKX2.5	5′-CAGGTNAARATHTGGTT-3′	5′-ACCADGCYCKDATBCCATG-3′
GATA4	5′-GGMYTNTAYCAYAAGATGAA-3′	5′-GCYARGACCAGGYTGTTCCA-3′
GATA5	5′-CAAAGCTTTAYCAYAARATGAA-3′	5′-TCGAATTCCCRTGNARCTTCAT-3′
HAND2	5′-GAGTGCATCCCCAAYGTKCC-3′	5′-CCAKACYTGCTGVGGCCA-3′