Stem Cell Reports, Volume *2*

Supplemental Information

Do Neonatal Mouse Hearts Regenerate following Heart Apex Resection?

Ditte Caroline Andersen, Suganya Ganesalingam, Charlotte Harken Jensen, and Søren Paludan Sheikh

Detailed Experimental Procedures

Animals

C57BL/6 mice were purchased from Taconic Europe and bred according to general guidelines. Pregnant ICR/CD1 mice were obtained from Charles River laboratories, and allowed to acclimatize before labor. Animals were housed in plastic cages with a 12/12 light/dark cycle, and fed ad libitum with a chow appropriate for pregnant mice. All animal experiments were approved by the Danish Council for Supervision with Experimental Animals (#2011/561-1966).

Apex Resection

Apex resection was performed at postnatal day 1 (P1) as previously described (Porrello et al., 2011). We performed surgery on more than 400 animals in total. Briefly, neonates were anaesthetized by hypothermia for 4 min. using an ice bed covered with a plastic sheet to prevent freeze damage of the animals. The mice were closely observed, and turned a couple of times,

quickly reaching a surgical plane of anesthesia. Anaesthetized mice were given 0.05mL 0.9%NaCl subcutaneously to avoid dehydration during surgery. They were then fixed under a stereomicroscope, and a left parasternal and horizontal skin incision was performed at intercostal space 4 (ICR/CD1)/ 5 (C57Bl/6) followed by thoracotomy using blunt dissection. A microsurgical forceps was utilized to gently fix the apex, and a pair of iridectomy scissors was used to resect the apex. Left ventricle chamber exposure was used as a landmark for resection, and immediate blood clotting sealed the heart and prevented the use of suturing. Apex resected animals were then removed from ice beds and the thoractic wall and skin incision were sutured with a 8-0 non-absorbable Prolene suture (Ethicon), and further protected by adhesive glue (Vetbond, 3M). Mice quickly recovered from anesthetics under a heat lamp, and returned to their mother when natural movements and a red/pink complexion were achieved. Sham mice underwent the exact same procedure without resecting the apex of the heart.

At indicated time points, mice were weighed, and depending on the age (P1-P37) sacrificed by decapitation or cervical dislocation. Hearts were then carefully dissected avoiding any further damage, washed in PBS/heparin (SAD, 5000IE/mL), and weighed prior to further processing being 1) cryopreservation, 2) Stereology, 3) fixing/paraffin embedding, and 4) RNA extraction.

***In vivo* 5-ethynyl-2-deoxyuridine (EdU) pulse-chase labeling experiments**

Neonatal mice were injected 1 day after surgery with 50µL EdU (Molecular Probes, Life Technologies; 50 mg/kg) subcutaneously to AR and sham mice (n=5). One week after injection, hearts were dissected and used to quantify the number of proliferating cells in total as well as proliferating cardiomyocytes during 1-7 days after surgery. EdU detection was performed as previously described (Mortensen et al., 2012), and pictures captured/processed as described below. A twenty-field grid was overlayed on each picture and the number of EdU positive cells per area tissue was counted by two independent observers (one blinded). Only fields completely filled with

tissue were included in the analysis, and the EdU+ cell number per mm^2 tissue was calculated for each zone.

Relative quantitative real time PCR (qRT-PCR)

Total RNA was extracted from hearts using the semi-automated 6100 Nucleic Acid Prep Station system, according to manufacturer's instructions (Applied Biosystems). For cDNA synthesis, 0.3-0.4 μg of total RNA was reverse transcribed with High Capacity cDNA RT kit (#4368813, Applied Biosystems), and qRT-PCR reactions were performed in technical triplicates using commercially available Taqman assays or custom designed primers. The PCR was run on a 7900HT Fast Real-time PCR system (Applied Biosystems) and robust and valid qRT-PCR data was obtained by normalizing the raw data against multiple stably expressed endogenous control genes as determined by the qBase Plus platform (Hellemans et al., 2007; Vandesompele et al., 2002).

Immunohistochemistry, stereology, and tissue morphology stains.

Immunohistochemistry was performed as previously described (Andersen et al., 2009). For cryopreservation, hearts were embedded in Tissue-Tek and frozen in isopentane to avoid freezing artifacts. Cryosections were fixed in 4% normal buffered formaldehyde (NBF) (10 min.), blocked in 2%BSA/TBS (10 min.), and then treated as described for paraffin embedded sections. For IHC, hearts were fixed o/n in 4%NBF and embedded in paraffin. Paraffin-embedded sections were de-paraffinized and rehydrated before antigen retrieval (heating in Tris-EGTA; pH 9). Blocking was performed (10 min.) in 2%BSA/TBS, and primary antibodies diluted in 1%BSA/TBS were applied o/n at 4°C. Secondary antibody used was Alexa 555 or 488 or 647 donkey anti-IgG (1:200, Molecular Probes), and mounting medium contained DAPI (Vectashield, Vector Lab.). Collagen deposition was evaluated by Sirius Red staining. Briefly, sections were deparaffinised and rehydrated as in general, incubated with Weigerts Iron Haematoxylin (Sigma-Aldrich; 15 min.), and then in 0.1% Sirius Red (Sigma-Aldrich)/Saturated Picric acid (Sigma-Aldrich) (60 min.) before mounting in pertex.

Microscopic examinations were performed using a Leica DMI4000B Cool Fluo Package instrument equipped with a Leica DFC340 FX Digital Cam and a Leica DFC 300 FX Digital cam. In all experiments, exposure (camera settings) and picture processing (slight adjustment of contrast/brightness and color balance by using Photoshop) were applied equally to sample sections and controls (Isotype or no primary antibody present). Antibodies used included: rat anti-CD45 (1:50, BD Pharmingen); rat anti-CD31 (1:50, BD Pharmingen); rabbit anti-Collagen I (1:100, Abcam); goat anti-Desmin (1:50, Santa Cruz Biotechnology); mouse anti-smooth muscle actin (aSMA, 1:200, Sigma-Aldrich); goat anti-CD31 (1:200, Santa Cruz); rat anti-Ki67 (1:50, DAKO); rabbit anti-NG2 (1:300, Millipore), mouse anti-Cardiac Myosin (1:400, Abcam); and mouse anti-non Muscle Myosin (1:400, Abcam).

Statistical analysis

All analyses comprised at least 4 independent experiments. Two-way ANOVA with a Bonferroni Multiple Comparison posttest, One-Way ANOVA with a Dunnett's Multiple Comparison posttest, or two-tailed *t*-tests were performed as indicated using GraphPad Prism to test significance ($p<0.05$).

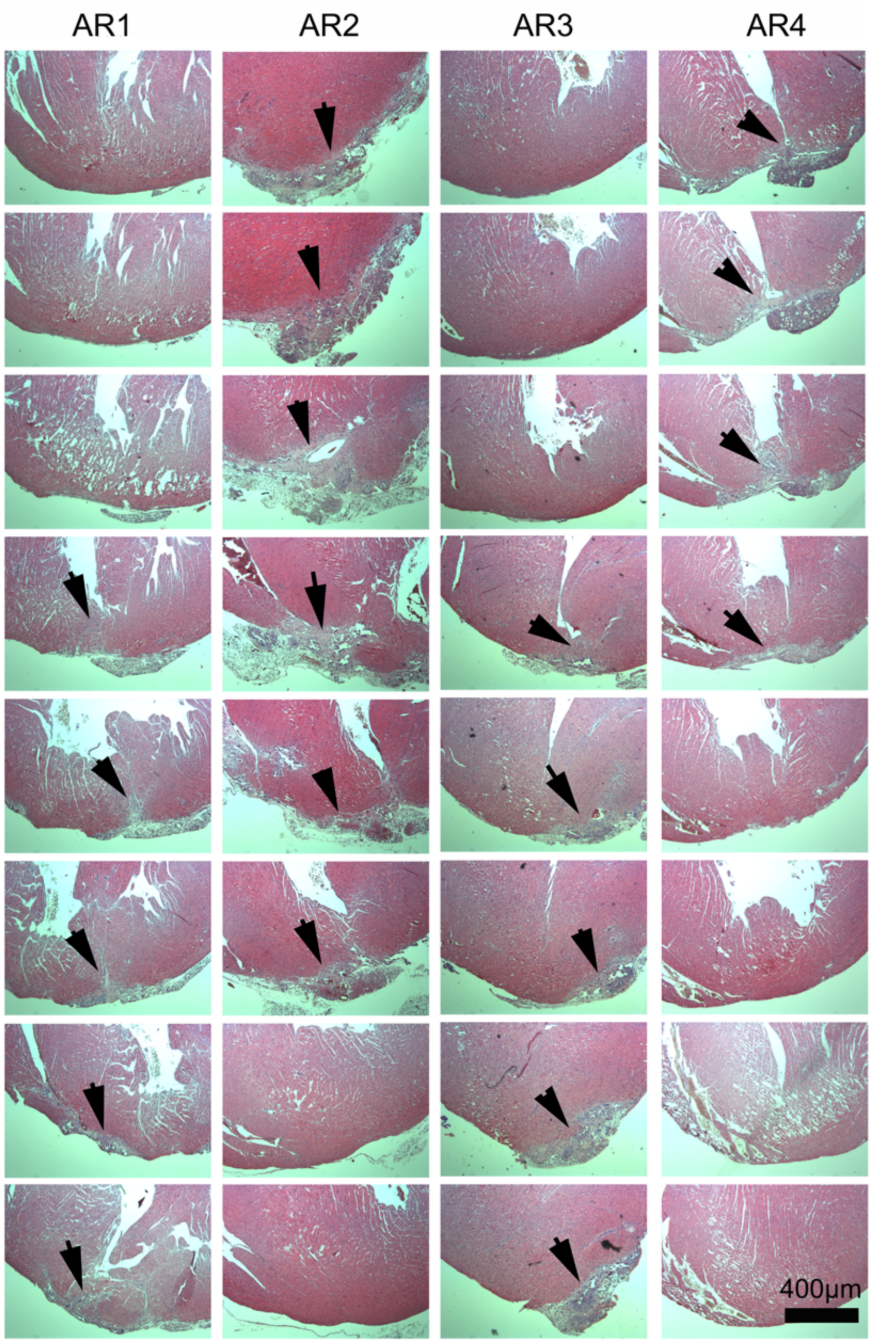

Figure S1. Localization of the lesioned apex zone 21 days following apex surgery (Related to Figure 1).

(A) Sectioning throughout hearts was required to identify the lesioned area in apex resected hearts. Paraffin embedded hearts from C57Bl/6 animals with AR were sectioned completely. Eight hematoxylin stained sections from the 4 representative hearts are shown. Arrowheads indicate sections enclosing AR lesion.

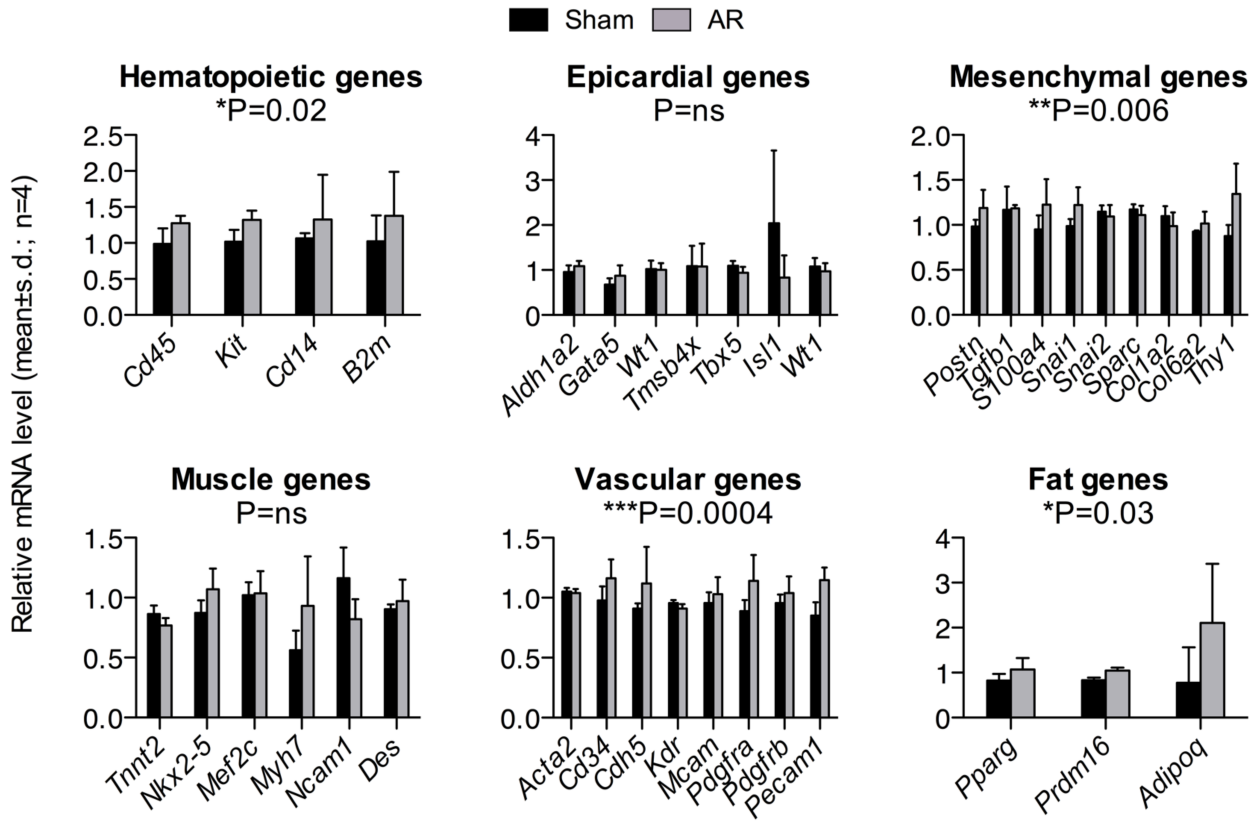

Figure S2. Differential gene expression profiles in sham and AR hearts (Related to figure 2). qRT-PCR was performed on RNA isolated from sham (n=4) and AR (n=4) C57Bl/6 hearts at day 21 following surgery. qPCR raw data were normalized against *Gapdh* and *Tbp,* which were stably expressed as determined by the qBase+ platform (M:0.126, CV: 0.044) (Hellemans et al., 2007; Vandesompele et al., 2002). Genes were assigned to specific gene programs prior to experiment, and statistical significance of a gene program was tested by 2-WAY ANOVA as indicated.

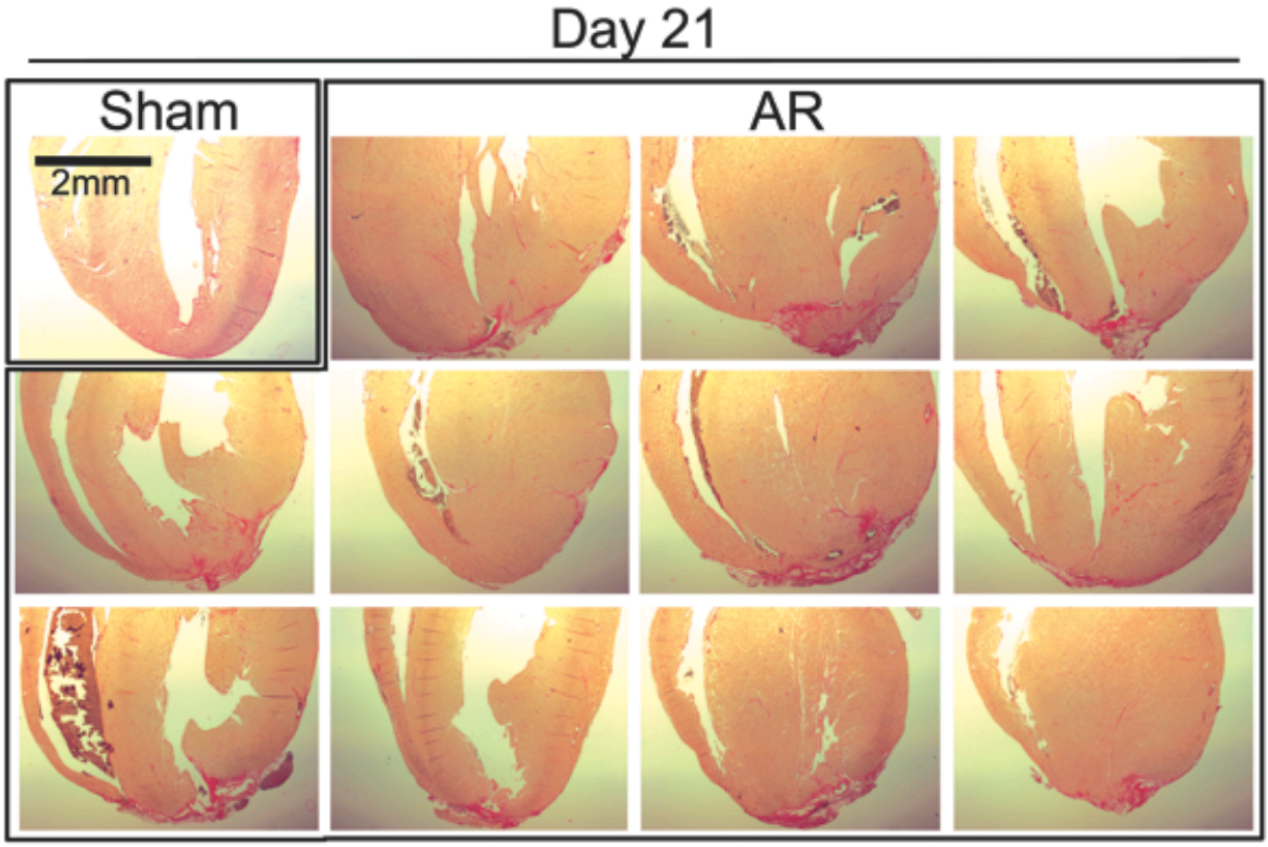

Figure S3. The damaged area of ICR/CD1 apex resected mice contains a fibrotic scar at day 21 (Related to Figure 4). Apex resected ICR/CD-1 hearts heal by fibrotic scarring. Sirius red staining of representative sections from sham (n=3) and AR (n=11) hearts of ICR/CD-1 mice identifies collagen disposition in the apex lesion area.

Supplemental references

Andersen, D.C., Petersson, S.J., Jorgensen, L.H., Bollen, P., Jensen, P.B., Teisner, B., Schroeder, H.D., and Jensen, C.H. (2009). Characterization of DLK1+ cells emerging during skeletal muscle remodeling in response to myositis, myopathies, and acute injury. Stem cells *27*, 898-908.

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 biology *8*, R19.

Mortensen, S.B., Jensen, C.H., Schneider, M., Thomassen, M., Kruse, T.A., Laborda, J., Sheikh, S.P., and Andersen, D.C. (2012). Membrane-tethered delta-like 1 homolog (DLK1) restricts adipose tissue size by inhibiting preadipocyte proliferation. Diabetes *61*, 2814-2822.

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.

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 biology *3*, RESEARCH0034.