Supplemental Materials and Methods

Isolation of cardiac and bone marrow SP cells.

Minced cardiac tissue was digested with 0.1% collagenase B (Roche Molecular Biochemicals), 2.4 U/ml dispase II (Roche Molecular Biochemicals), and 2.5 mmol/L $CaCl_2$ at 37°C for 30 minutes, filtered through 70 μm and 40 μm filters, and washed with HBSS buffer supplemented with 2% fetal calf serum and 10 mmol/L HEPES. Cardiomyocyte depleted mononuclear cell suspensions were incubated with Hoechst 33342 (5 μg/ml) (Sigma) at 37°C for 90 minutes in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) (2% fetal calf serum, 10 mmol/L HEPES) at a concentration of 10^6 nucleated cells/mL. Verapamil (50 mmol/L), fumitremorgin C (FTC, 1 μmol/L) and 2-deoxyglucose (50 mmol/L) were used as inhibitors of the Hoechst 33342 dye efflux ability. Bone marrow SP cells were isolated form the tibia and femur as previously described [1]. Cell Surface antigen staining was performed at 4° C for 30 minutes using fluorochrome-conjugated monoclonal rat anti-mouse antibodies reactive to Sca-1, CD31, and CD45 (Pharmingen). Respective isotype controls (Pharmingen) were used as negative controls. Propidium iodide (PI) (2 μg/mL) was added prior to FACS to exclude dead cells.

FACS analysis.

FACS was performed using MoFlo (Cytomation Inc.) and FACSAria (BD) both equipped with triple lasers. Acquired data were analyzed using Summit software (Cytomation, Inc.) and BD FACSDIVA software (BD Biosciences). SP cells were identified as Hoechst-low cells as previously described [1]. Samples co-incubated with Hoechst and verapamil were used as negative controls to set the threshold of the respective SP gate as previously described [1].

Experiments defining the proliferative capacity of cSP cells.

Total cell number. Twenty thousand WT and *Abcg2 -/-* or ten thousand WT and *Abcg2* overexpressing cSP cells from passage 4-6 were plated onto culture dishes. Cardiac SP cells were trypsinized and the cell number was calculated using a hematocytometer at day 9 or 6 for *Abcg2 -/-* or *Abcg2* overexpressing, respectively. Culture medium was replaced every 72 hours.

Immunocytochemistry for phospho-histone H_3. cSP cells from passage 4-6 were plated and cultured in proliferation media for 5 days. Cells were washed twice with PBS, fixed with 4% paraformaldehyde solution and permeabilized with methanol. cSP cells were subsequently incubated in 1% BSA solution for one hour and then stained with a primary antibody against phospho-histone H_3 (Abcam) for 2 hours followed by 2 hours staining with secondary antibody (Alexa-555, Molecular probes) at room temperature. Coverslips were mounted on the slides using a DAPI-containing mounting medium (Vector Vectashield). Cells were visualized using Zeiss epi-fluorescent microscopy (Zeiss, Axiovert 200M).

Expression of Ki67 via FACS analysis. cSP cells were fixed (4% paraformaldehyde), permeabilized (Perm/Wash solution, BD Biosciences) and stained with FITC-conjugated polyclonal Ki67 antibody (Santa Cruz). Respective isotype control (BD Biosciences) was used as negative control.

***In cell western for total protein and DNA*.** Equal numbers of WT and Abcg2 -/- cSP cells from passages 4-6 were cultured in the proliferation media. Two days following culture, cSP cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. Total protein was measured by the staining of Alexa-680 conjugated antibody against succinimidyl ester (Molecular probes, 1:50000 for 15min). The total DNA content was determined by nuclear staining with the TOPRO-3 DNA dye (Molecular probes, 1:2500 for 1hr). The in-cell Western analysis was performed in the Licor Odyssey infrared imager.

Co-culture experiments.

Adult rat cardiomyocytes were isolated from male Wistar rats as described previously [2]. The isolated cardiomyocytes were seeded at low density onto

laminin-coated culture dishes or coverslips. Cardiomyocytes were maintained in DMEM culture medium supplemented with creatine (5 mmol/L), L-carnitine (2 mmol/L), taurine (5 mmol/L), penicillin/streptomycin (1 %), FBS (7%) and bromodeoxyuridine (BrdU, 100 µmol/L) and the culture medium was replaced every 72 hours. For co-culture experiments cardiac SP cells were transfected with either GFP-lentivirus (control) or GFP-Abcg2-lentivirus (sample). GFP-positive cardiac SP cells were added onto the cardiomyocyte feeder layer at a ratio of 1:10 at day 10 and maintained for additional 10-12 days. The cardiomyogenic differentiation of cSP cells was evaluated by Immunocytochemical staining for a-sarcomeric actinin, as previously described (Otmar's paper). Nuclear staining was performed by supplementation of 4, 6-diamidino-2-phenylindole (DAPI).

Quantitative RT-PCR.

Total RNA was extracted from freshly isolated CSP cells and CDNA was synthesized using the iScript cDNA synthesis kit from BIO-RAD. Real-time PCR (BioRad-MyIQ) was performed using the following primers:

GADPH: 5'-TCACCACCATGGAGAAGGC-3'
and 3'-GCTAAGCAGTTGGTGGTGCA-5',
Abcg2: 5'-GAACTCCAGAGCCGTTAGGAC-3'
and 3'-CAGAATAGCATTAAGGCCAGG-5',
Mdr1a: 5'-TGGGTGCAGCTTTTCTCCTTA-3'
And 3'-CAGTGAGCACTTGTCCAATAGAG-5'
Mdr1b: 5'-CTG TTGGCGTATTTGGGATGT-3,'
and 3'-CAGCATCAAGAGGGGAAGTAATG-5'.

Data analysis was performed based on the standard curve method. Starting quantity (SQ) of Abcg2, Mdr1a and Mdr1b genes were compared to SQ of GAPDH.

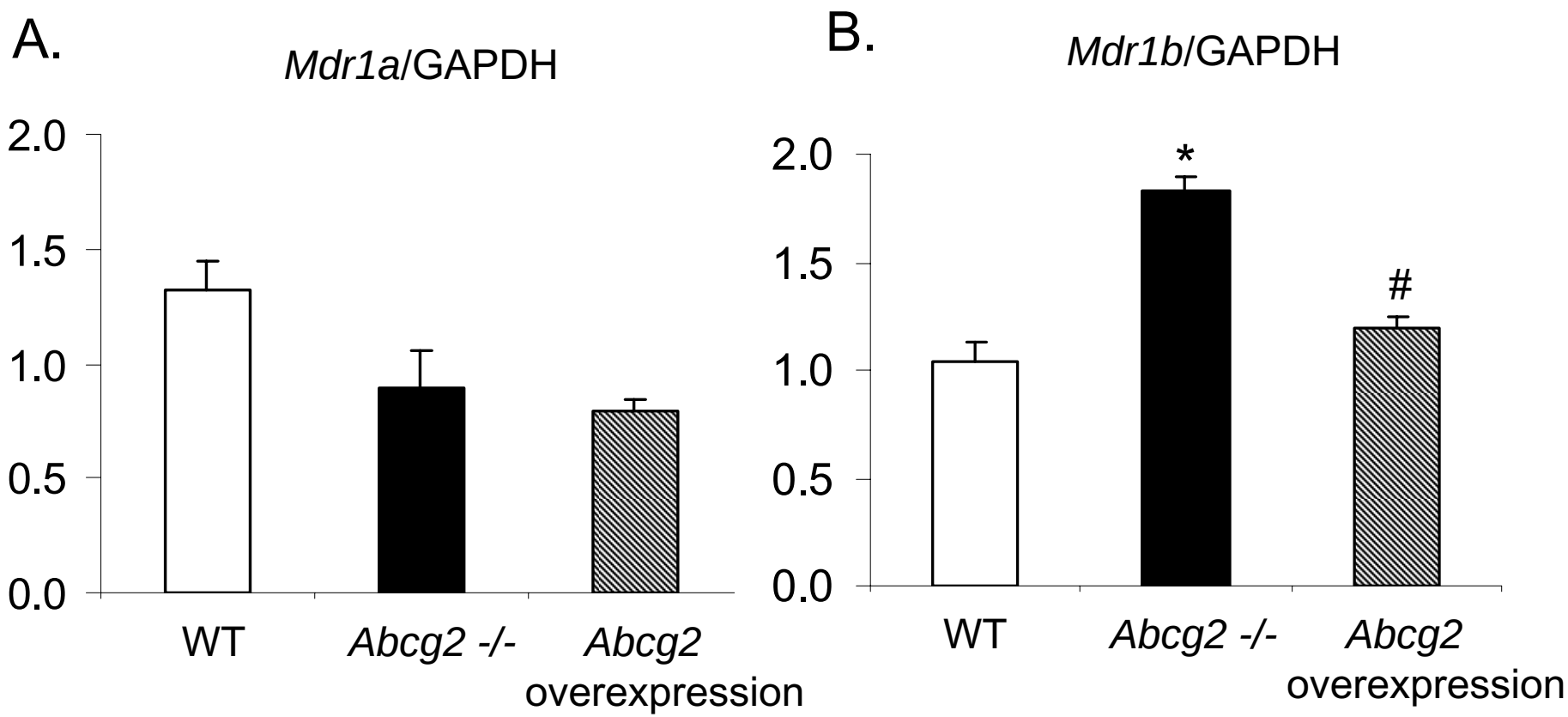
Immunocytochemistry.

cSP cells were cyto-spinned (500rpm for 5min) onto slides at a concentration of 5000 cells/slide (Shandon Cytopsin 3). Cells were fixed immediately in 4% paraformaldehyde and permeabilized with methanol. Subsequently cSP cells were incubated for 1 hr with 1% BSA solution and were stained for Mdr1 (Santa Cruz, clone C-19, FITC-conjugated) for 2hr at room temperature. Coverslips were mounted on the slides as described above.

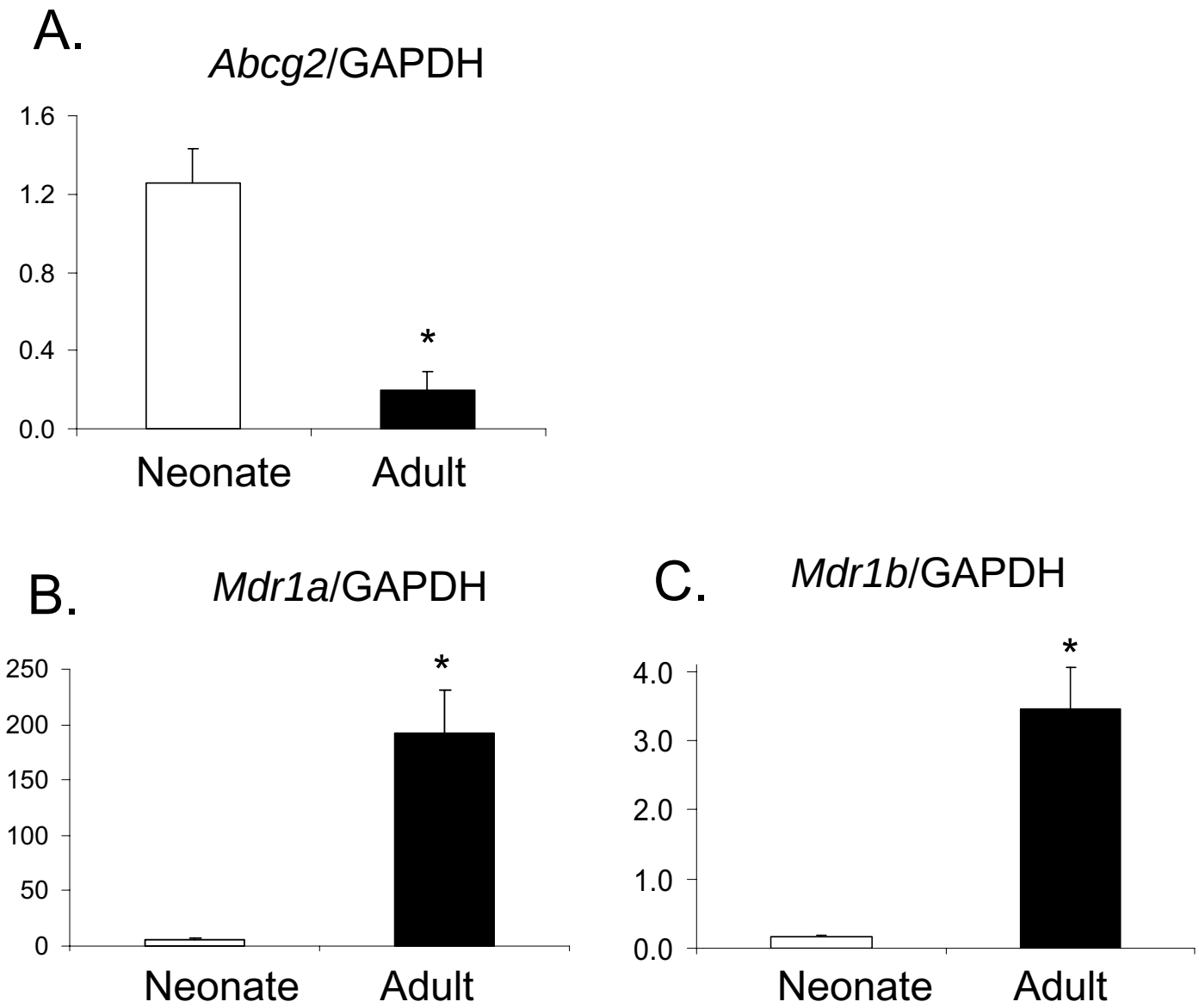
References.

1. Pfister O, Mouquet F, Jain M, Summer R, Helmes M, Fine A, Colucci WS, Liao R. CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. *Circ Res*. 2005;97:52-61.
2. Lim CC, Zuppinger C, Guo X, Kuster GM, Helmes M, Eppenberger HM, Suter TM, Liao R, Sawyer DB. Anthracyclines induce calpain-dependent titin proteolysis and necrosis in cardiomyocytes. *J Biol Chem*. 2004;279:8290-9.

Online Figure I



Online Figure II



Online Figure I: Mdr1a/b gene expression in expanded WT, *Abcg2 -/-* and *Abcg2* over-expressing cSP cells. (A) *Mdr1a* and (B) *Mdr1b* mRNA levels normalized with GAPDH determined by quantitative RT-PCR (*, $p<0.05$ vs. WT, #, $p<0.05$ vs. *Abcg2 -/-*). Data are presented as relative fold changes to the WT cSP cells.

Online Figure II: *Abcg2 and Mdr1a/b gene expression is regulated in an age-dependent fashion.* **(A)** *Abcg2*, **(B)** *Mdr1a*, and **(C)** *Mdr1b* mRNA normalized to GAPDH using quantitative RT-PCR ($p < 0.05$).