Stem Cell Reports, Volume *9*

Supplemental Information

SCA1^{+} Cells from the Heart Possess a Molecular Circadian Clock and Display Circadian Oscillations in Cellular Functions

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SUPPLEMENTAL FIGURES

Figure S1

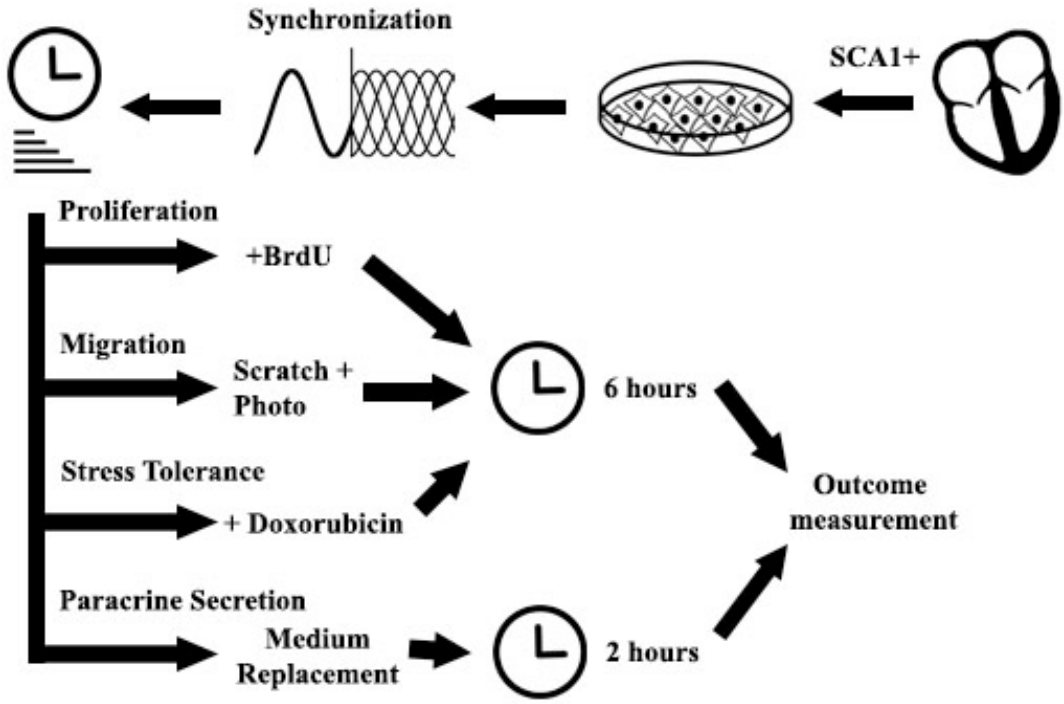


Figure S2

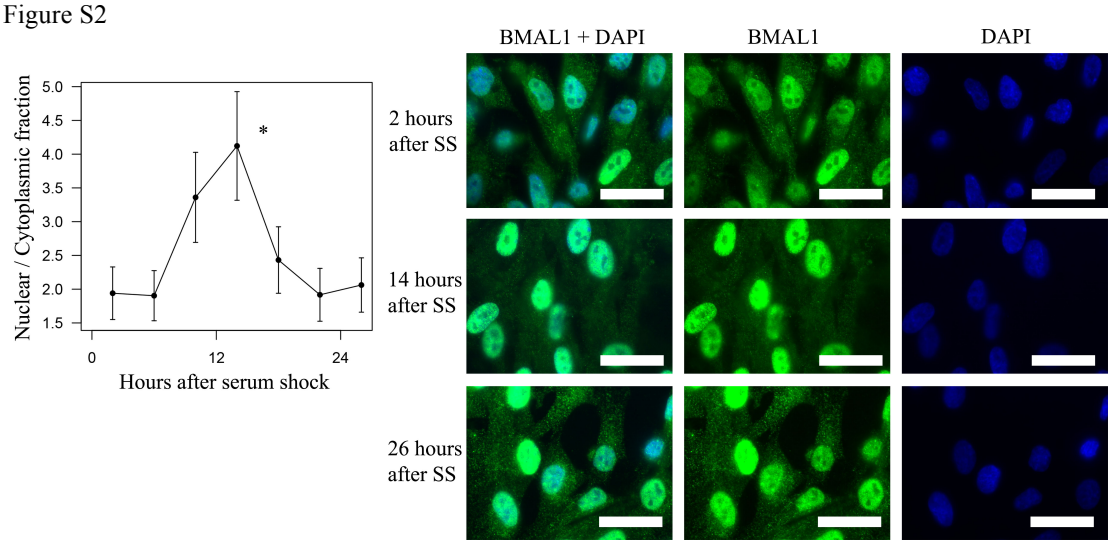


Figure S3

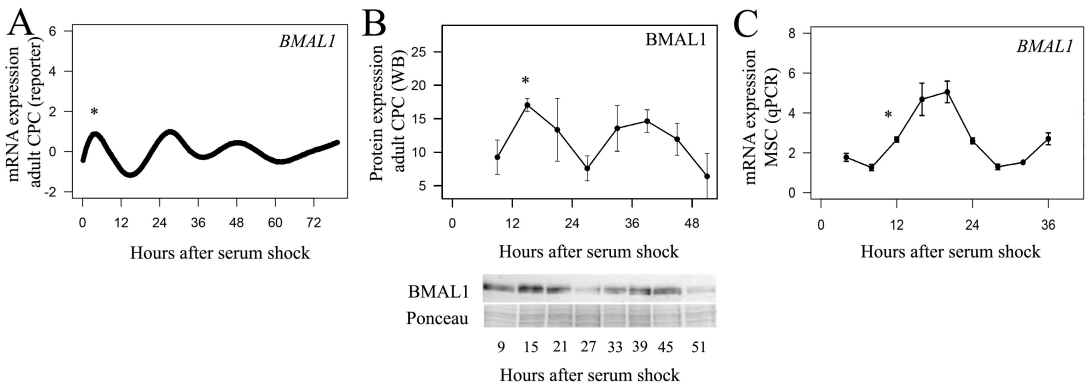
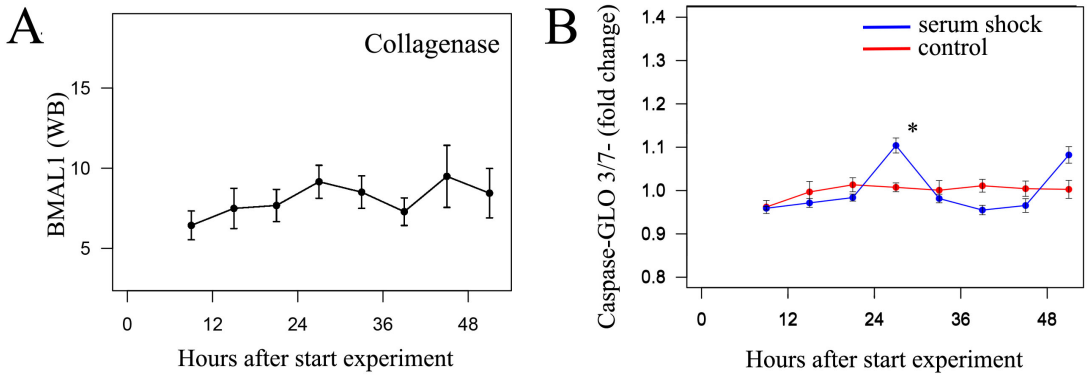


Figure S4



SUPPLEMENTAL LEGENDS

Figure S1. Related to experimental procedures. Sca1-expression was used to isolate cells. Cells were cultured and synchronized before the experiment. After subsequent time-periods, BrdU was added for the proliferation experiment. For migration, a scratch was made in the culture dish and a picture of the well was taken. For stress tolerance, doxorubicin was added and for paracrine secretion, medium was replaced. After a subsequent period (2 or 6 hours), outcome was measured according to procedures described in the methods.

Figure S2. Intracellular shuttling of BMAL1. Related to Figure 1 During 24-hours after serum shock (SS), BMAL1 was measured using immunofluorescence. There was 24-hour rhythmicity in the localization of BMAL1, as quantified by the nuclear / cytoplasmic fraction ($p=0.003$; $n=3$). * Indicates $p<0.05$ for significant 24-hour oscillation. Data are represented as mean ± SEM. Scale bars represent 40uM.

Figure S3. Molecular circadian clockwork within adult SPCs and MSCs. Related to Figure 1 24-hour oscillations in core clock component BMAL1 were present at the transcriptional and protein level. A) 80-hour analysis of mRNA in adult SPCs using bioluminescence reporters ($p<0.001$; $n=3$). B) BMAL1 protein expression in adult SPCs during 51 hours. A 24-hour rhythm was present ($p<0.01$; $n=3$). C) Bmal1 expression in fetal MSCs. (fold change 1.64, peak at 18.2h, $p<0.001$; $n=3$). SPC: human fetal Sca1+ cell; MSC: Mesenchymal stem cell. * Indicates $p<0.05$ for significant 24-hour oscillation Data are represented as mean ± SEM.

Figure S4. Related to Figure 2. A) Collagenase of SPCs disrupts the 24-hour rhythm in BMAL1 protein expression. ($P=0.344$; $n=3$). B) Apoptosis was measured after serum shock, subsequent time-periods (as depicted on x-axis) and 1 hour of hydroxyperoxide exposure using a Caspase-Glo 3/7 assay. Apoptosis was 13% higher 27 and 51 hours compared to 15 and 39 hours after serum shock ($P<0.001$; $n=4$). In non-synchronized cells, no circadian rhythm was present ($P=0.312$; $n=4$). Data are represented as mean ± SEM.

SUPPLEMENTAL TABLES

Cycle threshold values of cardiac transcriptional factors

	PPIA	*NKX2-5*	*GATA4*	*MYH7*
HFH	23,7	29,13	21,34	25,43
SPC	25,43	35,53	32,66	ND
hESC	22,91	ND	ND	ND
H_2O	ND	ND	ND	ND

Table S1. *NKX2-5* and *GATA4* and cardiomyocyte marker *MYH7*. Related to experimental procedures. Human undifferentiated embryonic stem cells and H_2O are used as negative controls, human fetal heart tissue as positive control for transcription factors. HFH: Human fetal heart; SPC: human fetal Sca1+ cell; hESC: human embryonic stem cell;

Release of paracrine factors from SPCs

Paracrine factor	24h Average (pg/ml)	Significance of 24h rhythm (P-value)	24h Amplitude (pg/ml)
BDNF	98.4	0.374	na
bNGF*	244	0.024	56.5
EGF	29.2	0.523	na
FGF-2	67.4	0.149	na
HGF	$10\square210^3$	0.054	$3.17\square10^3$
LIF	830	0.269	na
PIGF-1*	32.5	0.005	8.28
SCF*	19.6	0.020	4.94
VEGF-A*	$5.28\square10^3$	0.017	$1.23\square10^3$
VEGF-D	61,1	0.056	ND

Table S2. Related to Figure 4. bNGF, PIGF-1, SCF, and VEGF-A release has a 24hour rhythm. SPC: Sca1+ cell. * Indicates $P<0.05$ for significant circadian oscillation. n=3 per paracrine factor.

Primer sequences used for PCR

Gene	Forward primer	Reverse primer
PPIA	TTCTGCTGTCTTTGGGACT	CACCGTGTTCTTCGACATTG
BMAL1	GGCTCATAGATGCAAAAACTGG	CTCCAGAACATAATCGAGATGG
CRY1	TTGGAAAGGAACGAGACGCAG	CGGTTGTCCACCATTGAGTT
NKX2-5	TTCTATCCACGTGCCTACAGC	CTGTCTTCTCCAGCTCCACC
GATA4	CGACACCCCAATCTCGATATG	GTTGCACAGATAGTGACCCGT
TNN	GCGGGTCTTGGAGACTTTCT	TTCGACCTGCAGGAGAAGTT
MYH7	TCTTTCCCTGCTGCTCTC	GACTGCCATCTCCGA ATC

Table S3. Related to experimental procedures.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Quantitative RT-PCR

qRT-PCR was used for gene expression analysis. RNA was isolated with a phenol-chloroform (Merck) extraction method, DNase-treated, and quantified using a spectrophotometer. After cDNA synthesis, quantitative measurements of *BMAL1*, *CRY1*, *NKX2-5*, *GATA4*, *TNN*, and *MYH7* were done using SYBR-green (BIORAD) RT-PCR. PPIA was used as housekeeping gene. Primer sequences are given in Supplemental Table 1.

Western Blotting

RIPA-buffer was used to lyse cells for proteins analysis as described previously.[27] Protein concentrations were measured using BCA assay. Lysates were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Reverse Ponceau staining was used to quantify protein loading to prevent any misinterpretation caused by circadian rhythms in control proteins. Membranes

were blocked with 5% Protifar (Nutricia), probed with anti-BMAL1 (1:2000, #93806, Abcam) or anti-CRY1 (1:2000, #13474-1-A, Proteintech) antibodies, followed by a peroxidase-conjugated antibody (1:7000, #170-6515, Biorad), and ECL chemiluminescence (sc-2048, SantaCruz) for detection. Ponceau-corrected protein quantification was performed with Image Lab (Version 5.1, Biorad).

Intracellular shuttling of BMAL1
SPCs were plated on 1% gelatin coated 12mm Ø glass cover slips and synchronized using serum shock. At subsequent periods after synchronization (2-26 hours with 4-hour intervals) SPCs were fixed with 4% paraformaldehyde. BMAL1 was quantified using a standard immunofluorescence protocol. In short, SPCs grown on coverslips were permeabilized using 0.5% Triton-X100, treated with 50mM Glycine, and incubated with an anti-BMAL1 antibody (1:50, #93806, Abcam) overnight. After incubations with a secondary antibody and 4',6-diamidino-2-phenylindole (DAPI) for nuclear labeling, cells were mounted in Vectashield. Pictures were taken with a Nikon Eclipse 80i light microscope at 4 standardized, equally distributed locations on each coverslip. BMAL1 fluorescence intensity was measured in ImageJ software (Version 1.48u4) by a blinded researcher. Nuclear/ cytoplasmic ratio was calculated and all locations on a coverslip were averaged. Subsequent time-periods were compared.

Bioluminescent reporter
Lentiviral plasmids harboring luciferase reporters of BMAL1 and CRY1 promoters were described previously and kindly provided by professor Liu (Liu et al., 2008; Liu et al., 2007; Ramanathan et al., 2012). Viral particles were concentrated via ultracentrifugation after 3 harvests in HEK293T cells. SPCs were then transduced with BMAL1-dLuc or CRY1-dLuc lentivirus and selected with 10ug/ml blasticidin for at least 5 days. Stable polyclonal lines were propagated.

Bioluminescence was monitored via the use of a LumiCycle32 device (Actimetrics). In short, cultures of SPCs were grown confluent and synchronized. Then medium was switched to recording medium (Phenol Red-free DMEM, 10%FCS, 10mM HEPES, 0.035% Bicarbonate, 4.5g/L glucose and Pen/Strep +100μM D-Luciferin (Promega). Culture dishes were sealed with high vacuum grease (Dow Corning) and put in a LumiCycle32, kept in a 37°C incubator for recording. Bioluminescence from each dish was continuously recorded (integrated signal of 70 seconds with intervals of 10 minutes). Raw data (counts/seconds) were base-line subtracted (polynomial order 3).

Cell detachment assay
SPCs were cultured in 0.1% gelatin coated plastic 75cm^2 flasks and synchronized using serum shock. After subsequent time-periods (9-51 hours with 6 hour intervals), the culture medium was removed temporarily and stored under sterile conditions during the time needed for either trypsinization or collagenase treatment. Cells were washed twice with PBS, treated with trypsin (Trypsin-EDTA 0.25%, Lonza) or collagenase A (1mg/ml, Sigma-Aldrich), and re-plated in their own (temporarily removed) medium to prevent medium-induced re-synchronization. After 12 hours, when cells were reattached, cells were lysed and BMAL1 was quantified using western blotting.

Matrix assay
SPCs (2x10^6 cells per ml) were combined with collagen-type1 (1mg/ml, ThermoFisher) and plated in 50μl drops in a 24-well plate. After 30 minutes, culture medium was added. Wells with SPC loaded matrixes were synchronized using serum shock (or served as controls) and were collected after subsequent time-periods (9-51 hours) for western blot analysis.

Proliferation assay
Incorporation of 5-Bromo-2-deoxyuridine (BrdU) was used to quantify proliferation. For each time-point, SPCs were plated in several dilutions on 1% gelatin coated 12mm Ø glass cover slips. At the start of the experiment (t=0), culture medium was synchronized for 30 minutes. After subsequent time periods (2-50 hours with 4 hour intervals), BrdU (final [10μg/mL], BD Pharmingen) was added to the medium for 6 hours. Identical culture confluency (+/- 30%) was used for all time points. After this period, cells were fixed with 4% paraformaldehyde. BrdU quantification is described in the supplemental methods.

Migration assay
SPC migration was measured using a scratch wound cell migration assay (van Mil et al., 2012). Cells were cultured in a 0.1% gelatin coated 24-wells plate and synchronized at 80-90% confluency. After subsequent time periods (2-50 hours with 4 hour intervals) a horizontal and vertical scratch were made

within the wells using a standard 1ml pipet tip. Pictures of 4 standardized locations were taken directly and 6 hours after the scratch. Migration back into the scratch was measured by calculating the surface area right after the scratch (=100%) minus surface area after 6 hours using ImageJ software (Version 1.48u4). Percentages were averaged per well and reduced by the moving average to correct for any non-circadian variations.

Cell death assay
To measure stress tolerance, SPCs were cultured in a 96-well plate and synchronized. After subsequent time periods (9-51 hours with 6-hour intervals) doxorubicin (10uM, 6 hours Sigma) or tert-butyl hydroperoxide (10 μM, 1 hour, Sigma) was added to the culture medium. Subsequently, apoptosis was measured using a luminescent Caspase-Glo 3/7 assay (Promega) according to the manufacturer's instructions.

Cellular secretion assay
To measure growth factor secretion, SPCs were cultured in a 6-well plate and synchronized. After subsequent time periods (12-62 hours with 5-hour intervals), medium was changed and conditioned medium by SPCs was collected for 2 hours. The growth factor levels of SPCs were determined using a 11-multiplex panel (eBioscience, EPX110-12170-901), measured with a Luminex-200 instrument (Bio-Plex 200). The luminex assay was performed according to manufacturer's protocol.

BrdU quantification
To quantify BrdU incorporation, coverslips were incubated with 2N HCl for 30 minutes to allow for DNA denaturation. Samples were blocked with 5% goat serum (Dako) in 0.1% Triton X-100 (VWR chemicals) and incubated with a primary anti-BrdU antibody (1:200, #347580, Pharmigen) overnight. After incubation with secondary antibody (1:100, #715-095-150, Jackson), cells were co-stained with DAPI (1:40.000, Invitrogen) and mounted with Vectashield (Vector laboratories). Pictures of all coverslips were taken with a Nikon Eclipse 80i light microscope at 5 standardized locations equally distributed within the coverslip. A blinded researcher manually counted the amount of DAPI and BrdU positive cells using ImageJ (Version 1.48u4). Percentages were calculated, averaged per coverslip, and reduced by the moving average to correct for any non-circadian variations. Non-synchronized samples served as controls.

Doubling time
SPCs were cultured in 0.1% gelatin coated 35mm plastic culture dishes for at least 2 days. At the start of the experiment (t=0), half of the culture dishes were synchronized. After subsequent time-periods (0-48 hours with 4-hour intervals) cells were loaded in Bürker's chamber and photos taken. Cell numbers were quantified in ImageJ

Scratch assay sub-analyses
In a sub-analysis, a blinded researcher analyzed the effects of cell density and cell orientation on migration. Cell density was derived from the photos taken directly after the scratch. Using ImageJ, % area covered with SPCs was measured semi-automatically. Migration of the 25% lowest and highest density samples was compared. For cell orientation, photos were manually subdivided in 3 groups: parallel, perpendicular, or a mixed orientation as compared to the scratch. Migration of samples with parallel and perpendicular orientation was compared.