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Differentiation induction of mouse embryonic stem cells into sinus node-like cells by suramin

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Abstract

Background—Embryonic stem (ES) cells differentiate into cardiac phenotypes representing early pacemaker-, atrial-, ventricular-, and sinus node-like cells, however, ES-derived specification into sinus nodal cells is not yet known. By using the naphthylamine derivative of urea, suramin, we were able to follow the process of cardiac specialization into sinus node-like cells.

Methods—Differentiating mouse ES cells were treated with suramin (500 μ M) from day 5 to 7 of embryoid body formation, and cells were analysed for their differentiation potential via morphological analysis, flow cytometry, RT-PCR, immunohistochemistry and patch clamp analysis.

Results—Application of suramin resulted in an increased number of cardiac cells, but inhibition of neuronal, skeletal muscle and definitive endoderm differentiation. Immediately after suramin treatment, a decreased mesendoderm differentiation was found. Brachyury, FGF10, Wnt8 and Wnt3a transcript levels were significantly down-regulated, followed by a decrease in mesoderm- and cardiac progenitor-specific markers BMP2, GATA4/5, Wnt11, Isl1, Nkx2.5 and Tbx5 immediately after removal of the substance. With continued differentiation, a significant up-regulation of Brachyury, FGF10 and GATA5 transcript levels was observed, whereas Nkx2.5, Isl1, Tbx5, BMP2 and Wnt11 levels were normalized to control levels. At advanced differentiation stages, sinus node-specific HCN4, Tbx2 and Tbx3 transcript levels were

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significantly up-regulated. Immunofluorescence and patch-clamp analysis confirmed the increased number of sinus node-like cells, and electrophysiological analysis revealed a lower number of atrial- and ventricular-like cardiomyocytes following suramin treatment.

Conclusion—We conclude that the interference of suramin with the cardiac differentiation process modified mesoderm- and cardiac-specific gene expression resulting in enhanced formation of sinus node-like cells.

Keywords

Embryonic stem cell; Mouse; Cardiac differentiation; Suramin; Brachyury; Sinus node-like cells

1. Introduction

Mouse embryonic stem (ES) cells have been used as *in vitro* model to study cardiac development, to analyze toxic effects of drugs on cellular differentiation, and for human ES cells, as a potential source for cardiac repair [1–4]. Cardiomyocytes with electrophysiological properties characteristic of pacemaker-, ventricular-, atrial-, His-purkinje- or sinus node-like cells have been generated from mouse [5–7] and human [8–11] ES cells. A comparison of gene expression patterns and electrophysiological properties revealed that both mouse and human ES-derived cardiomyocytes are representative of early developmental stages [8,12].

To enrich the number of ES cell-derived cardiomyocytes, strategies for directed cardiac differentiation have been established based on cardiac-specific promoters (e.g. α -MHC, MLC2v, cardiac α -actin) driving antibiotic resistance or reporter gene constructs [13–18]. To enhance the efficiency of cardiac differentiation, growth factors and signaling molecules involved in heart development have been applied. Such cardiac-inducing factors are, for instance, signaling molecules of the bone morphogenetic protein (BMP), fibroblast growth factor (FGF) [19–21] and WNT [22,23] families. These factors act in a spatiotemporal-dependent manner during mesoderm and cardiac development and initiate a cardiac-specific gene expression program, e.g. via the activation of Nkx2.5, GATA4/5/6 transcription factors and T-box (Brachyury) factors. Cytokines like cardiotrophin-1 [24], endothelin [25] and neuregulin-1 [26] also promote development of specific cell types of the cardiac conduction system. The vitamin A-derivative retinoic acid (RA) accelerates the differentiation of ES cells into the cardiac lineage and specifically induces ventricular cardiomyocytes [15].

Suramin, a symmetrical polysulphonated naphthylamine derivative of urea, is known for its anti-parasitic, anti-viral (HIV) and anticancer activities and exerts diverse biological effects on various cell functions including proliferation, migration and differentiation [27]. These activities have been attributed to its interaction with specific growth factors or cytokine receptors. Specifically, suramin prevents receptor binding of bFGF [28], hFGF-1 [29], EGF [30], IGF [31], PDGF [32], TGF- β [33], Wnt family members [34], IL1 [35], IL2 [36], IL4 [37] and TNF α [38]. In the case of hFGF-1, suramin directly binds to residues of the heparin and FGF receptor binding site and causes oligomerization of hFGF-1 [29], which results in the inhibition of FGF signaling. Besides the disturbance of growth factor-receptor binding, suramin has also been shown to affect growth factor signaling related to protein

kinase C [39,40], phosphatidylinositol kinase, diacylglycerol kinase [41], protein tyrosine phosphatases [42], or uncoupling of G-proteins [43]. In *Xenopus* embryos, suramin in a concentration-dependent manner alters the fate of the Spemann's organizer resulting in a shift of the dorso-ventral differentiation pattern and at high concentrations increased cardiac differentiation. These *in vivo* data led to our hypothesis that suramin would directly modulate cardiac differentiation of mouse embryonic stem (ES) cells.

Indeed, we found that high concentrations of suramin in a time-dependent manner induced formation of sinus node-like cells from differentiating ES cells. The process is concomitant with inhibition of neuronal, skeletal muscle and definitive endoderm (DE), but not mesendoderm cell differentiation. More specifically, suramin induced pleiotropic effects on transcripts of early mesoderm- and cardiac-specific genes, whereas at advanced stages, sinus node-specific genes, such as HCN4, Tbx3 and Tbx2 were significantly up-regulated. These data thus provide new insights into the process of ES-derived cardiac differentiation and specialization into sinus node-like cells. Because ES cell-derived cardiomyocytes can function as biological pacemakers [44,45], these findings may foster the generation of patient-specific biopacemakers with therapeutic value for sick sinus syndrome and other bradycardiac disorders.

2. Material and methods

2.1. Culture of undifferentiated mouse ES cells

Embryonic stem (ES) cells of line R1 were cultivated on feeder layer of primary mouse embryonic fibroblasts on gelatin (0.1%)-coated petri dishes (Nunc, Wiesbaden, Germany) in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 15% heat-inactivated fetal calf serum (FCS), L-glutamine (2 mM), non-essential amino acids (NEAA, stock solution diluted 1:100), penicillin–streptomycin (stock solution diluted 1:100, all from Invitrogen), β -mercaptoethanol (Serva, Heidelberg, Germany, final concentration 0.1 mM), and 10 ng/ml LIF (see [46]).

Feeder-free CGR8 ES cells were cultured as described [47] and used for comparative flow cytometrical (FACS) determination of cardiac cells.

2.2. Differentiation of mouse ES cells and suramin treatment

For spontaneous differentiation, R1 and CGR8 ES cells ($n = 400$) were used to form embryoid bodies (EBs) that were cultivated in hanging drops with Iscove's modified Dulbecco's medium (IMDM, Invitrogen) supplemented with 10% FCS, 450 μ M α -monothioglycerol (MTG, Sigma, St. Louis, CA, USA), L-glutamine, NEAA and penicillin-streptomycin (see above). At day 2, EBs were transferred into bacteriological petri dishes and cultured in suspension. Suramin (500 μ M, Sigma, dissolved in PBS) was added to the culture medium at day 5 for 48 h. At day 7, suramin-treated and control EBs ($n = 20$ –30) were plated onto gelatin-coated 6 cm tissue culture dishes (Nunc) and cultivated in IMDM supplemented with 10% FCS, MTG, L-glutamine, NEAA and penicillin-streptomycin (see above). The R1 EB-derived cells were analysed morphologically, by RT-PCR, immunofluorescence and FACS analysis and cardiac cells were investigated by the patch

clamp method at different stages of *in vitro* differentiation. In parallel, CGR8-derived cells were used for FACS analysis.

2.3. Morphological differentiation analysis

For morphological analysis, EBs were separately plated onto single wells of gelatin-coated 24-well culture plates (Nunc) and cultured in IMDM with supplements (see above). The differentiation into cardiac, neuronal and skeletal muscle cells was monitored by microscopical analysis of 10 independent experiments. To determine the size of the EBs at days 5 (control) and 7 (=2 d of suramin treatment versus control), EBs were measured by a digital camera connected to the inverse microscope (Eclipse TE 300, Nikon, Düsseldorf, Germany) with the LUCIA Imaging System (Nikon), and images were processed by Powerpoint (Microsoft).

2.4. RNA extraction and semi-quantitative RT-PCR analysis

ES-derived cells were collected and suspended in lysis buffer composed of 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% (w/v) sarcosyl and 0.1 M β -mercaptoethanol.

Total RNA was isolated by the single step extraction method [48]. mRNA was reverse transcribed using Oligo d(T)₁₈ and RevertAisTM M-MuLV reverse transcriptase (both from MBI Fermentas, St. Leon-Rot, Germany). cDNA was amplified using oligonucleotide primers (summarized in Table 1) and recombinant Taq DNA polymerase (MBI Fermentas). For analysis of HCN4, Cx30.2, Cx40, Cx43, Cx45, Tbx2 and Tbx3, additionally beating cardiac clusters were isolated at days 7 + 4, 7 + 6, 7 + 9, 7 + 12 and 7 + 15. Polyadenylated mRNA was isolated from the clusters using the mRNA DIRECT Microkit (DynaL Biotech ASA, Oslo, Norway). Reverse transcription was performed with MuLV reverse transcriptase according to the protocol supplied by the manufacturer. cDNA amplification was carried out with HotStart Taq Plus DNA polymerase (Qiagen, Hilden, Germany).

The PCR products were electrophoretically separated on 2% (w/v) agarose gels containing 1% (w/v) ethidium bromide and gels were illuminated with UV light, stored by the E.A.S.Y. system (Herolab GmbH, Wiesloch, Germany) and analysed by the TINA2.08e software (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany).

The data of target genes were plotted as percentage changes in relation to the expression of the housekeeping gene β -tubulin. The transcript level of β -tubulin was used to normalize the amount of RT products of all samples. For each transcript 3 to 5 independent experiments were analysed.

2.5. Immunocytochemistry and confocal microscopy analysis

For immunocytochemistry, EB outgrowths on gelatine-coated cover slips (18 × 18 mm, *n* = 4 in 60 mm tissue culture plate) were either fixed with 4% paraformaldehyde (PFA) in PBS at room temperature (RT) for 20 min or in methanol: acetone (=7:3) at -20 °C for 10 min, depending on the antibody used (see Table 2). After rinsing (3×) in PBS, bovine serum albumin (BSA, 1% in PBS) or 10% goat serum plus 1% BSA in PBS were used to inhibit

unspecific labelling (30 min) at RT. Cells were incubated with the primary antibodies in specific dilutions at 37 °C for 60 min or over night at 4 °C (Table 2). Samples were washed (3×) in PBS and incubated with fluorescence-labelled secondary antibodies (diluted in 0.5% BSA in PBS, from Jackson ImmunoResearch Lbs., West Grove, USA and Molecular Probes, Invitrogen, respectively) at 37 °C for 45 min (Table 2). To label the nuclei, cells were incubated with Hoechst 33342 (5 µg/ml in PBS) at 37 °C for 10 min. After washing (3×) in PBS and (1×) in Aqua dest, the specimens were embedded in Vectashield mounting medium (Linaris, Wertheim, Germany).

To analyse the distribution of HCN4-positive cells in EB outgrowths, beating cardiac clusters were isolated as described [46] and re-plated onto cover slips (Ø 10 mm) in wells of 4-well plates (Nunc). The clusters were fixed by methanol: acetone (7:3) and blocked as described above. Immunofluorescence analysis was performed with primary antibodies against either, titin and HCN4 (Alomone Labs Ltd., Jerusalem, Israel) or MF20 and HCN4 or with triple staining for Cx30.2 (treatment overnight at 4 °C), HCN4 (Abcam Ltd., Cambridge, UK) and titin or α -sarcomeric actin (90 min, 37 °C) followed by incubation with appropriate secondary antibodies. Labelled cells of 3 to 5 independent experiments were analysed by the fluorescence microscope ECLIPSE E600 (Nikon) and the confocal laser scanning microscope LSM 510 META (Carl Zeiss, Jena, Germany).

2.6. Flow cytometry

R1- and CGR8-differentiated cells were washed twice in PBS and dissociated with 1 mg/ml collagenase B (Roche) at 37 °C for 20 min. The dissociation process was blocked by serum-containing medium. Cells were washed with 50 ml PBS and fixed at RT with 2% PFA for 20 min. After washing with 50 ml PBS, cells were stored at 4 °C until FACS analysis. For FACS analysis of cardiac Troponin I-positive cardiomyocytes, cells were centrifuged at 2000 rpm for 5 min. After permeabilization with 0.1% Triton-X in PBS on ice for 3 min, 1 ml PBS was added and the cells centrifuged at 2000 rpm for 5 min. Cells were incubated at RT in 50 mM glycine (30 min) followed by 1% BSA in PBS (30 min), and with the primary antibody against cardiac Troponin I (Thermo Scientific/Neomarker, Fremont, CA, USA; ready to use) for 1 h. Cells were washed with glycine (50 mM) and incubated with the secondary antibody chicken anti-mouse Alexa 488 (1:500 in 0.5% BSA, Molecular Probes) for 30 min. After washing in PBS, cell suspension was stored in 400 µl PBS at 4 °C until analysis. FACS analysis of 3 independent experiments was performed with the Aria Cell Sorter (BD Biosciences) and the data analysed with the Diva software.

2.7. Electrophysiology

Action potentials of ES cell-derived cardiomyocytes were studied during continuous superfusion with solution containing 150 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 2 mM MgCl₂ and 10 mM glucose (pH adjusted to 7.4 with NaOH) [49]. A system for rapid solution exchange was used for drug application (Cell Micro Controls, Virginia, VA; ALA Scientific Instruments, Long Island, NY, USA). Action potentials were measured at physiological temperature (37 °C) with standard single-electrode current-clamp technique (Axopatch 200, Axon Instruments, Foster City, CA, USA), ISO 2 software was used for data acquisition and analysis (MFK, Niedernhausen, Germany). Electrode solution contained 40

mM KCl, 8 mM NaCl, 100 mM D,L-K-aspartate, 5 mM Mg-ATP, 5 mM EGTA, 2 mM CaCl_2 , 10 mM HEPES, 0.1 mM GTP-Tris, (pH adjusted to 7.4 with NaOH), free Ca^{2+} -concentration was calculated to be ~50 nM (computer program EQCAL, Biosoft, Cambridge, UK). Membrane capacitance is closely correlated with cell size. The cell capacitance was measured by means of fast depolarising ramp pulses (from -40 to -35 mV, duration 5 ms) at the beginning of each experiment. Since the membrane conductance is constant in this range, a change in current level is due to the capacitive properties of the cell membrane.

The ratio of different types of cardiomyocytes with variable action potentials was determined based on the following parameters: membrane potential before activation of action potential, duration of action potentials measured as the time period from depolarisation to 90% repolarisation (shown as $\text{APD}_{90\%}$), presence of a long action potential plateau and shape of action potential (dome- or triangle-like, typical for ventricular- and atrial-like cardiomyocytes, respectively), upstroke velocity and presence or absence of carbachol-sensitivity (reduction of action potential duration and/or frequency and hyperpolarisation of the cell membrane). The number of cells measured is indicated in Table 3.

2.8. Statistical analysis

Statistical significance was tested by the unpaired *t*-test with significance levels: +++*P* < 0.001, ++*P* < 0.01, +*P* < 0.05.

3. Results

3.1. Suramin treatment of EBs increases the number of spontaneously beating cardiac clusters and represses neuronal and skeletal muscle cell differentiation

Application of 500 μM of suramin to EBs between days 5 and 7 resulted in a significant increase in the number of beating cardiac clusters (Fig. 1A) and a suppression of neuronal and skeletal muscle cell differentiation (see Fig. 1I, O). Suramin treatment resulted in the formation of irregular and fragile EBs, however, the size of the EBs increased from day 5 to day 7 and was not significantly different from non-treated EBs (controls). Shortly after plating of suramin-treated EBs, cellular outgrowths were reduced. Surprisingly, beating cardiac cluster formation in EB outgrowths showed a unique bi-phasic distribution. During the first days after EB plating (up to day 7 + 5) the number of beating clusters was not significantly different in control and suramin-treated cultures. Between days 7 + 6 and 7 + 9, the number of beating clusters in suramin-treated EB outgrowths was slightly reduced, whereas at advanced and late stages (days 7 + 12 to 7 + 28) a significant increase in the number of beating clusters was observed (Fig. 1A). These findings were confirmed by FACS analysis of anti-cardiac Troponin I (cTnI)-labeled cells at early (7 + 2 days) and advanced (7 + 15 days) differentiation stages (Fig. 1F–H). At day 7 + 2, no significant increase in the amount of cTnI-positive cells in suramin-treated cultures (1.2 fold change) was observed, whereas at day 7 + 15 the number of cTnI-positive cardiomyocytes increased by 5.6 fold (*n* = 3 experiments). A comparable positive influence of suramin on cardiomyocyte differentiation was also demonstrated for feeder-free CGR8 ES cells

(however, at earlier differentiation stage) with a 4 fold increase in cTnI-positive cardiomyocytes ($n = 3$ experiments, data not shown). The increase in the percentage number of beating clusters was not observed by morphological analysis, when EBs were either cultured at lower concentration (100 μ M suramin) or treated at earlier time points (0 to 2 days). Instead, neuronal differentiation was increased (data not shown). These pivotal experiments indicated a concentration- and time-dependent influence of suramin on ES cell differentiation.

Immunofluorescence analysis performed at intermediate (7 + 6 days) and late (7 + 19 days) stages of differentiation showed clear differences in the number and morphology of cardiac, skeletal muscle and neuronal cells in suramin-treated compared to control variants. Cardiac cells labeled by titin (Z-band epitope)-specific antibodies showed the typical sarcomeric structural organization. Although control and suramin-treated cardiomyocytes revealed no differences at the intermediate stage (day 7 + 6, Fig. 1B, C), prominent titin-positive clusters were observed in suramin variants at the advanced stage (7 + 19 days, Fig. 1D, E, see Fig. 1A). Immunolabelling by sarcomeric α -actin and MF20 revealed similar results (not shown).

Neuronal differentiation in control cultures was demonstrated by the presence of β III-tubulin-positive clusters with typical neuronal extensions (Fig. 1K), whereas in suramin-treated cultures, only a few punctate-like β III-tubulin-positive structures were found at day 7 + 6 (Fig. 1L). Similarly, nestin labelling was abundant in controls, but reduced in suramin-treated EB outgrowths (data not shown). At an advanced stage of differentiation (day 7 + 19), a significant number of (β III-tubulin-positive clusters with neuronal extensions was observed in control cultures (Fig. 1M), whereas suramin-treated variants showed a lower number and less organized neuronal cells (Fig. 1N, see Fig. 1I). These observations suggest an inhibitory effect of suramin (at high concentration) on neuronal differentiation.

Skeletal muscle cell-specific labelling by the anti-sarcomeric myosin (MF20)-specific antibody was almost lacking in both variants at the early time point (7+ 6 days, not shown). At day 7 + 19, myotube-like structures demonstrated the presence of skeletal muscle cells in controls (Fig. 1P), whereas in suramin-treated cultures a reduced number of MF20-immunostained myocytes was observed (Fig. 1Q, see Fig. 10).

Because suramin is known as an anti-angiogenic agent, expression of the pan-endothelial cell marker, PECAM, was analysed. However, no significant differences between control and suramin-treated cell cultures were found by immunocytochemical analysis (data not shown).

In summary, differentiation into beating cardiac clusters was specifically supported by treatment with high concentration of suramin at a late differentiation stage.

3.2. Suramin alters transcript levels of cardiac-, skeletal muscle-, neuron-, mesendoderm- and definitive endoderm-specific genes

To confirm the effects of suramin on cardiac versus neuronal and skeletal muscle cell differentiation observed by morphological and immunofluorescence analysis, we analysed transcript levels of the cardiomyocyte structural proteins α -myosin heavy chain (MHC) and

myosin-light chain (MLC)-2v. Whereas the α -MHC transcript levels were similar in control and suramin-treated variants (not shown), the relative MLC-2v transcript levels were significantly decreased at intermediate, but were up-regulated at late stages of differentiation (7 + 22 days; Fig. 2A).

Furthermore, we analysed the transcription of the ryanodine receptor (RyR)-2 gene, which regulates Ca^{2+} release in the sarcoplasmic reticulum membrane, crucial to progressive increase of beating rate during cardiac differentiation of ES cells [50]. RyR-2 mRNA levels of suramin-treated cells were in the range of the control variant throughout early and intermediate stages, but increased at late stages (7 + 15 to 7 + 28 days) in comparison to the control (Fig. 2A) suggesting an altered Ca^{2+} release mechanism in suramin-treated cardiomyocytes.

Cardiac-specific connexin (Cx) transcript levels showed a significant down-regulation in the case of Cx40 at early and intermediate stages and an up-regulation to the control level at the late stage, whereas Cx45 levels were slightly down-regulated at the early and intermediate stage following suramin treatment (Fig. 2A). In contrast, transcripts for Cx43 were not significantly different in suramin and control cells, perhaps because Cx43 is not only expressed in cardiac cell types, but also in cells of other lineages [51].

For the characterization of neural cell differentiation, nestin, NFM, GFAP, En-1 and synaptophysin mRNA levels were determined. Reduced nestin, NFM, En-1 and GFAP transcript levels at intermediate and/or late stages confirmed the data of decreased neural differentiation by suramin (Fig. 2B, see Fig. 1I–N). Surprisingly, a higher synaptophysin transcript level compared to the control was found under the influence of suramin in 7 day EBs. Following differentiation, the synaptophysin mRNA levels were down regulated at the intermediate stage, but at day 19, did not differ from controls (Fig. 2B).

Transcripts of the skeletal muscle-specific myogenic regulatory genes myf5, myogenin, and myoD were significantly suppressed throughout all stages of differentiation and the transcript levels never reached control values thus supporting our morphological observations (Fig. 2C, see Fig. 1O–Q).

Endoderm-specific HNF4 α and specifically HNF6 α transcripts were down-regulated immediately after suramin treatment and during the entire differentiation course, whereas mesendoderm-specific Sox17 levels were significantly reduced only shortly after suramin treatment. At later stages a slight, but insignificant upregulation of Sox17 transcript levels was found in comparison to control (Fig. 2D) suggesting that only definitive endoderm differentiation is inhibited, whereas at the intermediate developmental stage Sox17 expression would be required for the activation of a cardiac developmental program.

Thus, the gene expression analysis also reflected our results obtained at the cellular level. Skeletal muscle, neuronal and additionally endoderm-specific transcripts were reduced due to suramin treatment whereas cardiac specific gene expression was maintained and/or activated.

3.3. Suramin influences the transcript levels of mesoderm- and cardiac specific developmental control genes

To further investigate the mechanisms of suramin-induced changes in cardiac differentiation, we focused our analysis on genes encoding signalling molecules that regulate early mesoderm and cardiogenic differentiation, e.g. Wnt8, Wnt3a and FGF10 and transcription factors like the T-box factor Brachyury (Fig. 3A).

RT-PCR analysis demonstrated a slight reduction of Wnt8 mRNA level 12 h after suramin treatment, but a significant reduction of Brachyury and FGF10 transcript levels during the 48 h of suramin treatment (Fig. 3A). Reduced Wnt3a transcript levels were detected in the cells 36 and 48 h after the onset of suramin-treatment compared to control cells (Fig. 3A).

Next, we analysed genes that are involved in the development of early mesoderm and cardiac progenitors, including BMP2/4, GATA4/5, Isl1, Nkx2.5, Wnt11, and Tbx5. Transcript levels of these genes were not different in cells of both variants during suramin treatment between days 5 and 7 (data not shown). However, after removal of suramin, BMP2, GATA4/5, Isl1, Nkx2.5, Wnt11 and Tbx5 levels were decreased (Fig. 3B). The transcription of Wnt11 (known to activate the cardiac transcription factors Nkx2.5 and GATA4), was down-regulated at this stage, whereas Wnt3a and Wnt8 mRNA levels in both variants were detected only at very low levels in the EB outgrowths (not shown).

We went on to study the process of cardiac differentiation during the subsequent differentiation process. Most significantly, in suramin-treated cells, Brachyury transcripts were strongly (but only transiently) up-regulated 5 to 7 days after suramin removal and EB plating (7 + 5 and 7 + 7 days, Fig. 3B), this increase was followed by an immediate decrease to control levels at stage 7 + 9 days. This was paralleled by the up-regulation of FGF10 (7 + 7 and 7 + 12 days), GATA5 (7 + 9 to 7 + 15 days) and GATA4 (7 + 12 days) mRNA levels during differentiation. The other cardiac developmental control genes (BMP2, Wnt11, Isl1, Nkx2.5 and Tbx5) showed no remarkable differences in transcript abundance between suramin and control variants (Fig. 3B).

RT-PCR analyses of cells from late stages (7 + 22 to 7 + 28 days) revealed no differences in BMP4/2, GATA4, FGF10, Isl1 and Nkx2.5 transcript levels between suramin-treated and control variants. It would suggest that with respect to transcriptional regulation of those genes, both populations followed a normal differentiation program. However, some other markers showed an up-regulation of transcription at late stages. GATA5 levels were up-regulated at 7 + 28 days, and Tbx5 transcript levels were significantly up-regulated at 7 + 22 days and 7 + 28 days (see Fig. 3B), whereas the pattern of GATA6 expression was not different from controls (not shown).

In summary, transcript analysis demonstrated that mesoderm- and early cardiac progenitor-specific gene expression was indeed increased after suramin treatment. Transcript levels of genes known to be mainly involved in the differentiation of working myocardium, however, were transiently reduced.

3.4. Electrophysiological analysis of ES-derived cardiac phenotypes of suramin-induced and control cells

Electrophysiological measurements of ES-derived cardiomyocytes permit a phenotypic analysis that distinguishes among cardiac cells with early pacemaker, atrial-, ventricular- and sinus node-like action potential (see [5–7,52]). Initially, action potentials of control cardiac cells were measured by the whole cell patch-clamp technique. From day 1 after plating of the EBs (7 + 1 day), the differentiating cardiomyocytes displayed a variety of action potentials, which were activated spontaneously or following stimulation with injected current. As predicted, early and late cardiomyocytes with pacemaker activity differed in their action potential amplitude and upstroke velocity, and by their sensitivity to 20 μ M carbachol (Fig. 4A). Only sinus node-like cells treated with carbachol showed a reduced frequency of action potentials with a hyperpolarised membrane potential. The action potential duration of sinus node-like cells was reduced, and atrial- and ventricular-like types of action potentials differed in their plateau shape and action potential duration at 90% of repolarisation (Table 3).

Thus and based on the unique action potential traits (Fig. 4A), we identified three main stages of cardiac differentiation of (control) ES cells: early (7 + 2 to 7 + 4 days, Fig. 4B), intermediate (7 + 6 to 7 + 9 days, Fig. 4C) and advanced (7 + 11 to 7 + 20 days, Fig. 4D).

Next, we analysed, whether suramin treatment modulated cardiac cell differentiation and formation of specific cardiac subtypes. During all stages of differentiation, in the suramin-treated cell population an increased number of sinus node-like cells was measured (Fig. 4B–D), whereas the number of atrial- and ventricle-like cells was decreased at the intermediate stage (Fig. 4C) and the amount of early pacemaker-like cells was reduced at the advanced stage (Fig. 4D).

The patch-clamp data furthermore suggested that suramin application specifically affected cardiac differentiation by increasing the number of sinus node-like cells.

3.5. The sinus node-specific marker HCN4 is up-regulated at the transcript and protein level by suramin treatment

To test the hypothesis that suramin has the capacity to induce the formation of sinus node-like cells, we analysed the expression of HCN4, a gene encoding the isoform 4 of the hyperpolarization-activated channel that is highly expressed in the sinus node and is required for mature cardiac pacemaker activity [53].

HCN4 mRNA analysed from whole EB outgrowths revealed no significant differences between suramin-treated and control variants at early and intermediate stages, and showed elevated HCN4 transcript levels in suramin-treated cells only at the late stage (5+ 28 days, Fig. 5A). However, significant differences during intermediate and advanced stages were seen, when RT-PCR analysis was performed with cells from isolated cardiac clusters. HCN4 transcript levels in suramin-treated cells were reduced immediately following treatment, up-regulated at intermediate and advanced stages (Fig. 5B).

Immunofluorescence analysis confirmed the transcript data. The number of HCN4-positive (HCN4+) cells in titin-labelled cardiac clusters was increased (Fig. 5C). Confocal microscopy showed a higher number of HCN4+ cells in MF20 (Fig. 5G, D)- and titin (Fig. 5H, E)-labelled clusters in the suramin-treated (Fig. 5G–I) versus the control (Fig. 5D–F) variant.

The data furthermore supported our observation that the application of suramin facilitates cardiac differentiation into sinus node-like cells.

3.6. Transcriptional regulation involved in the formation of ES-derived sinus node-like cells

To further investigate the development of suramin-induced sinus node-like cells, cardiac conduction system-specific T-box genes, Tbx2 and Tbx3, and connexins (Cx30.2) were analysed in whole EBs (Fig 6A) and in isolated cardiac clusters (Fig. 6B).

Tbx2 and Tbx3 are known to play a role in the diversification of the specialized conduction system during vertebrate embryogenesis [54–56]. Transcript analysis of isolated beating clusters showed a down-regulation of Tbx2 and Tbx3 levels at intermediate stages followed by a significant up-regulation at the advanced stage of differentiation (Fig. 6B). This up-regulation of Tbx2 and 3 levels at advanced stages correlated with increased HCN4 transcript levels in the cardiac clusters (see Fig. 5C). In contrast to isolated cardiac clusters, in whole EBs Tbx2 and Tbx3 transcripts were up-regulated only at later differentiation stages (Fig. 6A).

Connexins were also analysed, including Cx30.2, which is a marker of the conduction system and is normally found in the sinus node and the atrioventricular node (AVN) of the adult mouse heart [57]. Cx30.2 mRNA levels isolated from suramin-treated beating clusters were reduced in comparison to control cells at intermediate stages, but reached control levels at advanced stages (Fig. 6). Transcript levels of Cx40 and Cx43, which are highly expressed in the atrial and ventricular working myocardium, respectively [58], were significantly lower in clusters of suramin-treated cells at early and intermediate stages, but not different from controls at advanced stages. Transcript levels of Cx45 showed no differences in suramin-treated and control cells in isolated clusters. In cells isolated from whole EB outgrowths (see Fig. 2A), the connexins showed reduced transcript levels in suramin-treated cells at early (Cx40, Cx45, Cx43) and intermediate (Cx40, Cx45) stages, but no significant differences to the control at the late differentiation period. An explanation may be that in whole EB outgrowths besides cardiac cells, also cells of other lineages are present.

Immunofluorescence (triple staining) analysis demonstrated that the level of Cx40-labeling in titin-positive cardiac cells isolated from beating clusters was similar in suramin-treated and control cells (Fig. 7A, B). A reduced Cx43 immunolabeling of titin-positive cells was, however, evident in the suramin-treated cell population (Fig. 7B), suggesting that these differences are attributed to a modification of connexin structural organization, rather than protein abundance.

In α -sarcomeric actin (α SA)-positive control cells, both markers of the sinus node (Cx30.2 and HCN4) showed a similar labeling intensity (Fig. 7C); however, cardiac cells from isolated beating clusters of the suramin variant showed a lower Cx30.2 protein abundance (Fig. 7D). One interpretation may be that – although HCN4 is strongly expressed – the regular tissue-specific connexin coupling of sinus node-like cells is irregular in the suramin-induced cells.

In summary, the transcript level and protein analysis of sinus node-specific factors additionally supported our data of the increased number of sinus-node like cells measured after suramin treatment. This may be directly correlated with the increased number of beating cardiomyocytes observed by morphological analysis.

4. Discussion

ES cell-derived cardiomyocytes represent a potential source for biologically relevant cells capable of functionally integrating into host cardiac tissues [59,60]. Here, we demonstrate that treatment of mouse ES cells with suramin in a time- and concentration-dependent manner, during definitive stages of EB formation promotes the selective differentiation of ES cells to sinus node-like cardiac cells. These findings are of translational interest because they provide a unique pharmacology-based approach that promotes cardiac sub-lineage formation and thus enhance the production of potential biopacemakers that could overcome some of the current limitations associated with electronic pacemakers [61]. Our findings provide a basis for future studies to elucidate the mechanisms for pacemaker cell induction *in vitro*.

Differentiation of suramin-treated cells was characterized by a specific transcriptional pattern of genes that regulate early mesoderm development, cardiac progenitor formation, cardiac differentiation and specification of sinus node-like cells, and the suppression of skeletal muscle, neuronal and definitive endodermal cells. The dramatic increase in the appearance of beating cardiomyocytes (paralleled by a decrease in skeletal muscle and neuronal cell formation) was only detected, when ES-derived EBs were cultured in the presence of high concentrations of suramin between days 5 and 7 (at late stages of EB formation). A similar shift in the differentiation pattern induced by suramin has been described in *Xenopus*. Culture of the *Xenopus* dorsal blastopore lip in the presence of suramin resulted in the complete prevention of neural induction [62]. The differentiation pattern was shifted from dorsal mesoderm (notochord and somites) to ventral mesoderm (heart) structures [62], whereas endoderm tissue was still present [63]. The ventralizing effect of suramin in *Xenopus* was explained by the inhibition of activin A-induced dorsalization [64] and premature expression of BMP4, an antagonist of several dorsal-specific genes [65]. Transcription of Sox17, a mesendoderm-specific marker playing a substantial role in early mesoderm and cardiac differentiation [66] was reduced immediately after suramin treatment, but showed a slight upregulation at later stages. This profile suggested the formation of Sox17-expressing mesendoderm (necessary for cardiac development) however, transcript levels of the definitive endoderm-specific markers HNF4 α and HNF6 α were suppressed, suggesting that definitive endoderm differentiation was inhibited.

The reduction of FGF10, Wnt8 and Wnt3a transcript levels in the presence of suramin suggested a direct involvement of these signaling factors in the suramin-induced differentiation process. Canonical Wnt3a and Wnt8 signalling suppress cardiogenesis in avians *in vivo* [67] and in differentiating ES cells *in vitro* [68]. Expression of Wnt8 during mouse heart development between E8.5 d p.c. to E11.5 d p.c. suggests a regulatory function of Wnt8 in early cardiogenesis [69]. In P19 embryonal carcinoma cells, Wnt3a-conditioned medium induced an up-regulation of early cardiac markers, but no spontaneous beating capacity [23] suggesting that additional signals are required for terminal differentiation. However, Wnt3a and Wnt8 may not only trigger ES-derived cardiogenesis, but also affect the differentiation pattern, because Wnt3a and Wnt8 play an important role in neurogenesis, as demonstrated for mouse (Wnt3a, [70]), *Xenopus* (Wnt8, [71]) and ES cells [68]. The significant decrease of neuronal differentiation in suramin-treated cells could be also correlated with the immediate down-regulation of Wnt8 and Wnt3a. The inhibition of skeletal muscle cell differentiation in ES-derived cells by suramin may be also the result of down-regulation of Brachyury (a direct Wnt3a target gene) and FGF10, because Brachyury controls paraxial mesoderm development [72], while FGF10 is required for the development of the prospective limb mesenchyme [73]. In addition, suramin has been shown to interrupt Wnt and FGF signalling pathways by inducing the release of Wnts [34] and FGFs [28] from their receptors. We therefore speculate that Wnt3a/Wnt8 and FGF10 receptor binding and specific signalling pathways interrupted by suramin during this time frame of EB differentiation, result in a down-regulation of these genes thereby affecting downstream target genes. In summary, our findings suggest that down-regulation of Wnt3a/Wnt8 during late EB formation stages in suramin-induced cells has a minor impact on cardiac differentiation in comparison to suppressed neuronal and skeletal muscle cell differentiation.

The most significant response of ES cells during EB differentiation in the presence of suramin is the down regulation of Brachyury transcripts that are significantly, but only transiently up-regulated after removal of suramin. This finding is consistent with the idea that suramin exerts its function in an extra-cellular manner. Because Brachyury was described as a direct Wnt3a target gene [72], the reduced Brachyury level (in suramin-treated cells at days 7 to 7 + 1) could be a consequence of Wnt3a down-regulation at days 6.5 to 7 in the EBs (see Fig. 3). Alternatively, Brachyury has been shown to play a significant role in early cardiac development. In this context, the study of Kouskoff et al. is important. They showed that cardiac differentiation of Brachyury-positive cells is restricted to a narrow window during mesoderm development in ES-derived EBs, and only a subpopulation of Brachyury-positive cardiac progenitor cells differentiate into cardiomyocytes [74].

The transcript analysis of other cardiac-specific genes revealed characteristic profiles in cells after suramin treatment. For example, BMP2 [rev [75]], Wnt11 [68], and the cardiac-specific transcription factors GATA4/5, Isl-1, Nkx2.5 and Tbx5 showed a temporary down-regulation of transcript levels in suramin-treated cells immediately after suramin removal, but normalized to the control level at advanced (BMP2, Wnt 11, Isl1, Nkx2.5, Tbx5) and late (BMP2/4, GATA4, FGF10, Isl1, Nkx2.5) stages, respectively. With respect to BMP factors, our findings support previous data showing that the inhibition of BMP signaling in

ES cells or during early phases of ES cell differentiation is crucial for cardiomyocyte differentiation [76]. The transient decrease of cardiac-specific transcripts as consequence of suramin treatment followed by normalization to control levels thus may reflect the bi-phasic pattern in the formation of cardiac beating clusters (see Fig. 1A). We speculate that this pattern of differentiation is the result of a direct suramin inhibition of (i.e. Wnt8, Wnt3a, FGF10) signaling pathways that affect the activity of transcription factors, such as Brachyury. After removal of the substance Brachyury is induced and ES-derived cells show stable expression of cardiac-specific genes (like Nkx2.5, GATA5, Wnt11, BMP2/4). Moreover, our analyses revealed that the “second phase” characterized by the significant enhancement in the amount of cardiac beating clusters (representing pacemaker cell activity) is paralleled by a temporary suppression of genes involved in the formation of the working myocardium (i.e. MLC-2v, Cx40, Cx43).

Consistent with this bi-phasic response, the patch-damp technique (see [5–7,52]) permitted us to distinguish among early pacemaker-, atrial-, ventricle- and sinus node-like cells. The most important finding was that the number of sinus node-like cells was enhanced in suramin-treated variants at all stages, whereas the number of atrial- and ventricle-like cells was decreased especially at the intermediate stage (see Fig. 4). Consistently, the up-regulation of HCN4 at the transcript and protein level confirmed the increase in the number of sinus node-like cells following suramin-treatment.

Our data showed that atrial and ventricle-like cardiomyocytes, as components of the working myocardium, are reduced in the suramin-induced cardiac population. Specifically, the reduced transcript levels of Cx40 and Cx43 in suramin-treated cells are consistent with a decreased differentiation towards working myocardium (versus increased development of sinus node-like cells). In development, Cx40 is the dominant connexin isoform in the atria and the fast conducting components of the conduction system of adult hearts, and Cx43 is expressed both in the working myocardium of the ventricles and the atria [77,78]. Hence, our *in vitro* results confirm increased HCN4, and lower levels of Cx43 and Cx40 levels in pacemaker-like cells analogous to those of the sinus node [79], Cx30.2, another marker of nodal cells in the adult murine heart [57], however, showed decreased abundance at transcript and protein level in ES-derived cells at early and intermediate stages.

In vivo, atrial cells do not differentiate into sinus nodal cells [55]. We, therefore, speculate that the reduced number of atrial and the increase in sinus node-like cells is not a result of re- or transdifferentiation of atrial cells into sinus node-like cells.

The preferential induction of sinus node-like cells by suramin in these studies was also supported by the demonstration of enhanced Tbx2 and Tbx3 transcription levels in suramin-treated cells at late stage. Both genes are key regulators in the formation of the sinus node *in vivo* [54,55,80]. Tbx2 and Tbx3 show an overlap in expression suggesting a possible redundant function in early heart development, while at later stage, Tbx2 expression gradually fades [56]. Tbx3 is expressed in the embryonic and adult heart and becomes restricted to the proximal components of the conduction system [54], and both Tbx2 and 3 are expressed in the primary myocardium and suppress the transformation of primary myocardium into working myocardium [54,80]. The finding that Tbx3 repressed the activity

of atrial myocardial marker genes, including Cx40 [54] would be consistent with our data that show a significant down-regulation of Cx40 transcript levels at the intermediate stage.

In addition, the activation of Tbx2 and Tbx3 expression in the developing heart (atrioventricular canal and sinus venosus) is directly linked to the BMP/Smad-mediated signalling pathway [81]. Because BMP expression in our culture system is not directly affected by suramin treatment at late stages, BMP-/Smad-signalling mechanisms may be involved in Tbx2 and Tbx3 transcript up-regulation in suramin-treated cells.

Moreover, the absence of the cardiac transcription factor Nkx2.5 early in development is a prerequisite for the development of sinus nodal cells [82]. Indeed, our data showing the temporary down-regulation of Nkx2.5 and BMP transcript levels immediately following suramin treatment (see Fig. 3) are consistent with the finding that Nkx2.5 expression is regulated by BMP-dependent mechanisms [83]. Whether the specific expression pattern of BMPs is important for late processes of cardiac differentiation and specialization in suramin-treated cells, however, needs to be clarified in the future.

In conclusion, we postulate that the suramin-induced activation of cardiac differentiation paralleled by suppression of neural, skeletal muscle and definitive endoderm cell differentiation is the result of pleiotropic effects on genes regulating ES-derived mesoderm and early cardiac differentiation and specification into sinus node-like cells (see Fig. 8). The down-regulation of Wnt3a, Wnt8, Brachyury and FGF10 transcription in cells differentiating in the presence of suramin, followed by a reduction of Nkx2.5 and other cardiac developmental control genes and the transient up-regulation of Brachyury, FGF10 and GATA5 seemed to be necessary for the continuation of cardiac differentiation that results in the activation of sinus node-specific genes HCN4, Tbx2 and Tbx3.

Thus a specific and temporal gene expression pattern is required for the direct differentiation of ES cells into sinus node-like cells. Because no methods are currently available to generate sinus node-like cells *in vitro*, this knowledge of transcriptional regulation by suramin during cardiogenesis and sinus node development may be crucial for the establishment of strategies for the efficient differentiation of ES-derived sinus node-like cells.

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Fig. 1.

Effects of suramin on the differentiation of R1 ES-derived embryoid bodies (EBs) into cardiac, neuronal and skeletal muscle cells. (A) Number of EBs containing spontaneously beating cardiac clusters (in % of EBs) after plating of individual 7 day EBs from day 7+1 following differentiation up to day 7+28. Error bars indicate standard deviation ($n=10$ experiments). (B–E) Immunocytochemical analysis of titin-labeled cardiomyocytes of control (B) and suramin-treated (C) cells in EB outgrowths at day 7+6, and of control (D) and suramin-treated (E) cells at day 7 + 19 of differentiation. (F) FACS analysis of R1 ES-derived cardiac Troponin I (cTnI)-positive cardiomyocytes at days 7+2 and 7+15. Shown is the fold increase in the number of CTnI-positive cells in suramin-treated versus untreated cells (control, $n=3$ experiments). (G–H) Representative FACS analysis of cTnI-positive R1 ES-derived control (G) and of suramin-treated (H) cells. (I) Number of EBs containing neuronal cells (in % of EBs) of control and suramin-treated variants during differentiation up to day 7+28 (for details see A). (K–N) Immunocytochemical analysis of βIII-tubulin-positive cells of control (K) and suramin-treated (L) EB outgrowths at day 7+6 and of

control (M) and suramin-treated (N) cells at 7+19 days of differentiation. (O) Number of EBs containing skeletal muscle cells (in % of EBs) of control and suramin-treated variants during differentiation up to 7+28 days (for details see A). (P, Q) Immunocytochemical analysis of MF20-positive skeletal muscle cells of control (P) and suramin-treated (Q) EB outgrowths at day 7+19 of differentiation. The earlier stage did not show representative skeletal muscle differentiation. Hoechst 33342 was used to label the nuclei, Bar=50 μ m.

Fig. 2.

RT-PCR analysis of mRNA levels of genes expressed in cardiac (A)-, neural (B)-, skeletal muscle (C)-, mesendoderm- and endoderm (D)-specific cells. Samples were collected prior to treatment (d5), and throughout the differentiation period up to stage 7+28 days at the indicated time-points from untreated (control) and suramin-treated (suramin) cultures. Shown are (A) cardiac-specific myosin light chain (MLC)-2v, ryanodine receptor 2 (RyR2) and connexin (Cx) 40, 43 and 45 transcript levels, (B) neural-specific nestin, neurofilament protein (160 kDa, NFM), engrailed (En-1), glial fibrillary acidic protein (GFAP) and synaptophysin transcript levels, (C) skeletal muscle cell-specific myogenin, MyoD and Myf5 transcript levels and (D) mesendoderm-specific Sox17 and definitive endoderm cell-specific HNF4 α and HNF6 α transcript levels. The data were plotted (in %) in relation to the transcript levels of the housekeeping gene β -tubulin ($n=5$ experiments). Error bars represent standard error of the mean. Open circle: non-treated control, closed circle: suramin-treated samples.

Fig. 3.

RT-PCR analysis of mesoderm- and early cardiac-specific genes. (A) Samples were collected at day 5 (prior to suramin-treatment) and during the time range of suramin-treatment (days 5.5 to 7). Shown are transcript levels of Wnt8, FGF10, Brachyury and Wnt3a in control and suramin-treated cells. (B) Samples were collected during the process of cardiac differentiation of EBs at the indicated time-points. Shown are transcript levels of Brachyury, BMP2, BMP4, GATA5, GATA4, FGF10, WNT11, Isl-1, Nkx2.5 and Tbx5. The data were plotted (in %) in relation to β -tubulin mRNA levels [$n = 5$ (A) and $n = 3$ (B) experiments]. Error bars represent standard error of the mean. Open circle: non-treated control, closed circle: suramin-treated samples.

Fig. 4.

Patch clamp analysis of R1 ES-derived control and suramin-treated (suramin, 500 μ M) cells at three different stages of cardiac differentiation. (A) Specialization of ES cell-derived cardiomyocytes into early pacemaker, atrial-, ventricle- and sinus node-like cells shown by expression of different types of action potentials. (B–D) The number of specialised cardiomyocytes of early pacemaker-, atrial-, ventricle-like and sinus node-like types determined at early (B, 7+2 to 7+4 days), intermediate (C, 7+6 to 7+9 days) and advanced (D, 7+11 to 7+20 days) stages of differentiation. Open columns: non-treated control; closed columns: suramin-treated cells.

Fig. 5.

RT-PCR and immunocytochemical analysis of sinus node-specific HCN4 (hyperpolarization-activated and cyclic nucleotide-gated channel) at the transcript and protein level. (A) HCN4 transcript level of cells isolated from whole EB outgrowths and (B) from beating cardiac clusters of control and suramin-treated cells, respectively, through the differentiation period at the indicated time-points. The data were plotted (in %) in relation to β -tubulin transcript level ($n = 5$ experiments). Error bars represent standard error of the mean. Open circle: non-treated control, closed circle: suramin-treated samples. (C) Immunocytochemical analysis of the number of HCN4-positive cells in titin-labeled cardiac clusters isolated from control and suramin-treated cells at day 7+12 in %. (D–I) Immunocytochemical analysis of control (D–F) and suramin-treated (G–I) cells isolated from beating clusters at day 7+12. Cells were immunostained by anti-HCN4/anti-MF20 (D, G)-, and anti-HCN4/anti-titin (E, F and H, I)-specific antibodies, respectively. Hoechst 33324 was used to label the nuclei. Bar = 50 μ m (D, G) and = 10 μ m (E, F, H, I).

Fig. 6. RT-PCR analysis of cardiac-specific connexins (Cx) and conduction system specific T-box transcription factors Tbx2 and Tbx3. (A) Tbx2, Tbx3 and Cx30.2 transcript levels in cells of whole EB outgrowth of control and suramin-treated cultures and (B) Cx40, 43, 45, 30.2, Tbx2 and Tbx3 mRNA levels in beating cardiac clusters of control and suramin-treated cells. The data were plotted (in %) in relation to β -tubulin transcript levels. Error bars represent standard error of mean. Open circle: non-treated control, closed circle: suramin-treated samples.

Fig. 7. Immunocytochemical (triple) analysis of isolated beating clusters by cardiac (Cx40, Cx43, titin, alpha-sarcomeric actin, (αSA)- and sinus node (Cx30.2, HCN4)-specific markers. (A, B) Representative confocal images of control (A) and suramin-treated (B) cells immunolabeled by Cx40, Cx43 and titin at stage 7 + 12 days. (C, D) Representative confocal images of control (C) and suramin-treated (D) cells immunostained for Cx30.2, HCN4 and αSA at stage 7 + 12 days. Bar = 10 μm.

Fig. 8.

Summary data describing the effects of suramin treatment on ES-derived cardiac differentiation. (A) The experimental system demonstrating stages of embryoid body (EB) differentiation, (B) Sequential inactivation/activation of mesoderm- and cardiac-specific genes during suramin-induced cardiac differentiation and specialization into sinus node-like cells, and (C) Hypothetic model of cardiac differentiation of ES cells into sinus-node-like cells.

Table 1

Primers for RT-PCR analysis.

Gene	Primer sequence (forward/reverse)	$T_a(^{\circ}\text{C})$	No. of cycles	Product size (bp)
Wnt3a	ATTGAATTTGGAGGAATGGT CTTGAAGTACGTGTAACGTG	55	40	318
Wnt8a	AAGTCAGCCAGCTGCAGCCAA GGTGAATTGTCCTGAGCAT	65	40	418
Wnt11	GCCATGAAGGCCTGCCGTAG GATGGTGTGACTGATGGTGG	65	35	153
BMP2	GAGATGAGTGGGAAAACG GCAGTAAAGGCATGATAGC	55	40	606
BMP4	AAGTTTCCACCGTGTACATT CCGAGCCAACACTGTGAGGAGT	59	40	307
FGF10	ACATTGTGCCTCAGCCTTTC TTCCATTCAATGCCACATACAT	55	45	529
Nkx2.5	CGACGGAAGCCACGCGTGCT CCGCTGTGCTTGCACTTG	63	35	181
Gata4	AAACGGAAGCCCAAGAACCTGAAT GAGCTGGCCTGCGATGTCTGAGTG	60	35	311
Gata5	CGACGTAGCCCTTCGTGG GCCACAGTGGTGTAGACAG	55	40	179
Tbx2	TTCCACAACTGAAGCTGAC GCTGTGTAATCTTGTCATTCTG	60	40	204
Tbx3	CAGCCGCGGTTCCACATCGTCA GGGCCGTGCTCCTCTTGCTCTC	60	40	410
Tbx5	CACAGCCCCTTCAGCAGCGAGAC AGGGGCCCCGAGGTGAAATGAG	60	40	495
Brachyury	GAGAGAGAGCGAGCCTCCAAAC GCTGTGACTGCCTACCAGAATG	59	45	230
Islet-1	GGACTCCCAGATCTGCGAGG ATTTGATTGCCGAACCAAC	60	40	243
α MHC	CTGCTGGAGAGGTTATTCCTCG GGAAGAGTGAGCGGCGCATCAAGG	64	36	301
Mlc2v	TGTGGGTCACCTGAGGCTGTGGTTCAG GAAGGCTGACTATGTCCGGGAGATGC	60	45	189
HCN4	CGGAGGGCCTTCGAGACGGTT GAGGGGCTGCTGGCGGGTGAA	60	40	443
HCN1	GGGCGCCACCTGCTACG CCGGGCTGGAACACCTCAA	65	40	354
RyR2	GACGGCAGAAGCCACTCACCTGCG CCTGCAGAGAACTGACAACTGGA	60	40	332
Connexin 30.2	GCGCCGCCGGTGTGTTCGT GCCGCCTCGCCCTCGCTGTC	55	40	525
Connexin 40	CCACGGAGAAGAATGTCTTCA TGCTGCTGGCCTTACTAAGG	62	40	447
Connexin 43	TGGGGGAAAGGCGTGAG CTGCTGGCTCTGCTGGAAGGT	61	40	1274
Connexin 45	ATCATCCTGGTTGCAACTCC CTCTTCATGGTCTCTTCCG	62	40	169
NFM	TATTGTGACTGAGGGCTGTCCG GAGGCACTAAGGAGTCCCTG	60	45	297
GFAP	TCGGAGTTGAAAGTTACAGG AGGATGGTTGTGGATTCTTC	50	35	234

Gene	Primer sequence (forward/reverse)	$T_a(^{\circ}\text{C})$	No. of cycles	Product size (bp)
Nestin	CTACCAGGAGCGCGTGGC TCCACAGCCAGCTGGAACCTT	60	35	220
Synaptophysin	TGCAGAACAAGTACCGAGAG CTGTCTCCTTGAACACGAACC	60	39	296
En-1	TGGTCAAGACTGACTCACAGCA TCTCGTCTTTGTCCTGAACCGT	61	39	390
myogenin	GACTCCCCACTCCCATTACATA GGCGGCAGCTTTACAAACAACACA	58	40	441
myf5	AGCCCACCAGCCCCACCTC TTCTGCCCAGCTTGCTTTTCCTTC	60	40	395
myoD	GCTCTGATGGCATGATGGATTACAGCG ATGCTGGACAGGCAGTCGAGGC	55	40	144
Sox17	CCATAGCAGAGCTCGGGGTC GTGCGGAGACATCAGCGGAG	62	40	627
HNF4 α	ACACGTCCCCATCTGAAG CTTCCTTCTTCATGCCAG	55	40	253
HNF6 α	CGCGCAGGTCAGCAATGGAAGTAA GCTGCTGGGAGATGGTGATTTGTA	65	35	434

Table 2

Antibodies used for immunofluorescence analysis.

Primary antibody	Dilution	Fixation	Company	Secondary antibody ^a (dilution)
Sarcomeric alpha actin (5C5)	1:100	Methanol:acetone (7:3, v/v)	Sigma, St. Louis, CA, USA	FITC-conjugated rabbit (1:100) or AMCA-conjugated donkey anti-mouse IgG (1:100)
Titin (T12)	1:100	Methanol:acetone (7:3, v/v)	A gift from Dr. D. Fürst	Cy3- (1:800) or Alexa488- (1:100) - conjugated goat or AMCA-conjugated donkey anti-mouse IgG (1:100)
MF20	Undiluted	Methanol:acetone (7:3, v/v)		Alexa488-conjugated goat anti-mouse IgG2a (1:100) or Cy3-conjugated goat anti-mouse IgG (1:800)
β-III-tubulin (TU-20)	1:100	4% paraformaldehyde in PBS	Chemicon International Inc., Temecula, CA, USA	FITC-conjugated rabbit anti-mouse IgG (1:100)
Nestin (Rat 401)	1:3	4% paraformaldehyde in PBS	Developmental Studies Hybridoma Bank, IA	FITC-conjugated rabbit anti-mouse IgG (1:100)
PECAM (CD31, MEC 7.46)	1:20	4% paraformaldehyde in PBS	Abcam, Cambridge, UK	Cy3-conjugated goat anti-rat IgG (1:800)
HCN4	1:20	Methanol:acetone (7:3, v/v)	Alomone Labs, Jerusalem, Israel	Cy3-conjugated goat anti-rabbit IgG (1:600) Alexa488-conjugated chicken anti-rabbit IgG (1:100)
HCN4	1:20	Methanol:acetone (7:3, v/v)	Abcam, Cambridge, UK	Alexa488-conjugated donkey anti-rat IgG (1:100)
Cx40	1:50	Methanol:acetone (7:3, v/v)	Chemicon International Inc., Temecula, CA	Cy3-conjugated goat anti-rabbit IgG (1:600)
Cx43	1:50	Methanol:acetone (7:3, v/v)	Sigma, St. Louis, CA	FITC-conjugated donkey anti-mouse IgM (1:100)
Cx30.2	1:50	Methanol:acetone (7:3, v/v)	A gift from Dr. M.Kreuzberg	Cy3-conjugated donkey anti-rabbit IgG (1:600)
Cardiac Troponin I	Ready to use	2% paraformaldehyde in PBS	Thermo Scientific/ Neomarker, Fremont, CA, USA	Alexa 488-conjugated chicken anti-mouse IgG (1:500)

^a AMCA-, Cy3- and FITC-conjugated secondary antibodies were from Jackson ImmunoResearch Lbs., West Grove, PA. Alexa488- conjugated secondary antibodies (Molecular Probes) were from Invitrogen, Karlsruhe, Germany.

Table 3

Electrophysiological properties of control (A) and suramin-treated (B) ES-cell derived cardiomyocytes at the early (7 + 2–4 days), intermediate (7 + 6–9 days) and advanced (7 +11–20 days) stages.

Cell type	V_m (mV)	APD90% (ms)	Amplitude (mV)	Number of cells
<i>A) Control cells</i>				
Control (7 + 2–4 days)				$n = 12$
Pacemaker-like	-50.0 ± 4.0	229.0 ± 17.0	66.0 ± 14.0	2 (17%)
Atrial-like	-52.0 ± 3.9	77.5 ± 21.7	59.5 ± 8.2	4 (33%)
Ventricular-like	-76.6 ± 9.7	279.2 ± 98.2	101.4 ± 14.0	5 (42%)
Sinus node-like	-55.0	354.0	85.0	1 (8%)
Control (7+6–9 days)				$n = 31$
Pacemaker-like	-40.3 ± 3.5	252.1 ± 48.4	60.2 ± 4.9	9 (29%)
Atrial-like	-72.3 ± 7.2	52.0 ± 7.4	85.5 ± 10.2	4 (13%)
Ventricular-like	-57.7 ± 4.5	266.7 ± 33.1	81.9 ± 6.3	10 (32%)
Sinus node-like	-71.1 ± 5.0	312.6 ± 64.1	95.1 ± 3.2	8 (26%)
Control (7 + 11–20 days)				$n = 30$
Pacemaker-like	-39.1 ± 3.6	230.4 ± 28.2	62.5 ± 5.0	14 (47%)
Atrial-like	-52.8 ± 6.5	90.0 ± 4.2	71.3 ± 8.3	4 (13%)
Ventricular-like	-59.0 ± 1.8	232.0 ± 38.6	85.6 ± 7.0	7 (23%)
Sinus node-like	-62.4 ± 2.3	105.2 ± 34.6	98.0 ± 2.1	5 (17%)
<i>B) Suramin-treated cells</i>				
Suramin (7 + 2–4 days)				$n = 11$
Pacemaker-like	-41.5 ± 4.5	183.0 ± 100.0	59.5 ± 8.5	2 (18%)
Atrial-like	-49.0 ± 7.0	63.5 ± 10.5	66.5 ± 7.5	2 (18%)
Ventricular-like	-47.8 ± 3.3	121.0 ± 6.6	67.5 ± 8.3	4 (37%)
Sinus node-like	-57.3 ± 1.9	267.3 ± 68.2	101.3 ± 3.7	3 (27%)
Suramin (7 + 6–9 days)				$n = 34$
Pacemaker-like	-37.1 ± 2.1	236.4 ± 38.8	72.9 ± 5.2	7 (27%)
Atrial-like	-51.0	67.0	56.0	1 (3%)
Ventricular-like	-58.0	100.0	82.0	1 (3%)
Sinus node-like	-61.2 ± 2.0	157.0 ± 12.4	96.6 ± 1.8	25 (73%)
Suramin (7 +11–20 days)				$n = 29$
Pacemaker-like	-38.7 ± 4.0	237.0 ± 38.9	62.0 ± 5.0	6 (21%)
Atrial-like	-73.0 ± 4.9	68.0 ± 16.8	116.3 ± 14.4	3 (10%)
Ventricular-like	-59.0 ± 2.8	265.8 ± 84.2	84.2 ± 5.1	6 (21%)
Sinus node-like	-62.5 ± 2.9	209.0 ± 71.1	93.8 ± 2.5	14 (48%)

Specialisation of the cells was based on the action potential parameters: resting membrane potential (V_m), action potential duration at 90% of repolarisation (APD90%) and amplitude of the action potential. Shown are data of pacemaker-, atrial-, ventricular- and sinus node-like cells.