Original Paper

Ascorbic Acid-Induced Cardiac Differentiation of Murine Pluripotent Stem Cells: Transcriptional Profiling and Effect of a Small Molecule Synergist of Wnt/ β -Catenin Signaling Pathway

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Key Words

Pluripotent stem cells • cardiomyocytes • Differentiation • Transcriptome • Small molecules • iPSC • ESC • Sscorbic acid • Wnt pathway

Abstract

Background: Reproducible and efficient differentiation of pluripotent stem cells (PSCs) to cardiomyocytes (CMs) is essential for their use in regenerative medicine, drug testing and disease modeling. The aim of this study was to evaluate the effect of some previously reported cardiogenic substances on cardiac differentiation of mouse PSCs. **Methods:** Differentiation was performed by embryoid body (EB)-based method using three different murine PSC lines. The differentiation efficiency was monitored by RT-qPCR, immunocytochemistry and flow cytometry, and the effect mechanistically evaluated by transcriptome analysis of treated EBs. **Results:** Among the five tested compounds (ascorbic acid, dorsomorphin, cyclic adenosine 3',5'-monophosphate, cardiogenol C, cyclosporin A) only ascorbic acid (AA) exerted a strong and reproducible cardiogenic effect in CGR8 cells which was less consistent in other two PSC lines. AA induced only minor changes in transcriptome of CGR8 cells after administration during the initial two days of differentiation. Cardiospecific genes and transcripts involved in angiogenesis, erythropoiesis and hematopoiesis were up-regulated on day 5 but not on days 2 or 3 of differentiation. The cardiac differentiation efficiency was improved when QS11, a small-molecule synergist of Wnt/ β -catenin signaling pathway, was added to cultures after AA-treatment. **Conclusion:** This study demonstrates that only minor transcriptional changes are sufficient for enhancement of cardiogenesis of murine PSCs by AA and that AA and QS11 exhibit synergistic effects and enhance the efficiency of CM differentiation of murine PSCs.

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Introduction

Cardiovascular diseases are the most frequent cause of death in adults and main non-infectious cause of death in children in the United States and Western Europe [1]. Due to the limited capacity of adult cardiomyocytes (CMs) to proliferate, cell losses of failing heart are irreversible and strategies for replacement of damaged cells by various types of exogenous cells are being developed [2, 3]. Pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), represent an attractive source of clinically useful CMs because they are easily accessible and expandable in culture, have broad developmental potential and high capacity to reproducibly differentiate into spontaneously beating cardiac cells *in vitro* [4, 5]. Patient-specific origin of iPSCs allows to bypass immunologic complications and ethical concerns that complicate the clinical application of ESCs [5]. However, more data is required to establish safety, efficacy and long-term behavior of iPSC-derived grafts. Major effort of many investigators in recent years has been to develop methods for enrichment of stem cell-derived CMs *in vitro* in order to improve their yield, purity and safety [6]. To improve the efficiency of CM differentiation, the directed differentiation of PSCs into CMs was induced by the supplementation with signaling molecules, such as Activin-A and BMP4, Wnt3a, TGF- β as well as small molecule compounds such as the Gsk3 inhibitors CHIR99021 and 6-bromoindirubin-3'-oxime (BIO), inhibitor of Wnt response-1 (IWR1), inhibitor of Wnt production-4 (IWP4) inhibitor of BMP signaling dorsomorphin and ascorbic acid [7-15]. Regulation of pathways which are involved in cardiac development such as Wnt, TGF- β , BMP, and Notch pathways could increase efficiency and purity of desired cell population. Interline variability of existing protocols and low yield of CMs is the major obstacle for use of PSCs in developmental studies, *in vitro* disease modeling and tissue repair. Thus, it is required to compare and validate cardiogenic potential of reported chemical substances as well as search for new factors or their combinations to improve the CM yield, homogeneity, maturation and functionality.

In this study we evaluated cardiac enhancing properties of five reported small molecules (ascorbic acid, dorsomorphin, cyclic adenosine 3',5'-monophosphate, cardiogenol C and cyclosporin A) using two murine ESC lines and one iPSC line. Among tested molecules ascorbic acid (AA) was the only one capable of enhancing cardiac differentiation when applied on day 0-2 of differentiation but most reproducible cardiogenic effect was observed only in CGR8 ESC line while in other two PSC lines the effect was rather inconsistent. We next compared global gene expression profiles of AA treated and untreated CGR8 ESCs during day 0-5 of differentiation to better understand the mechanism of AA-induced cardiac differentiation. Surprisingly, we observed only minor differences in gene expression between treated and non-treated cells despite pronounced enhancement of CM yield in AA-treated group in later stages of differentiation. Most up-regulated transcripts on days 2 and 5 of differentiation belonged to processes related to regulation of transcription and cardiac markers were induced only on day 5 of differentiation along with transcripts involved in angiogenesis, erythropoiesis and hematopoiesis. To further improve our protocol we tested whether addition of QS11, a small molecule synergist of Wnt/ β -catenin signaling pathway [16], during day 4-9 of differentiation alone or in combination with AA would yield more robust and reproducible results. We found that in contrast to treatment with single compounds the combined application of AA and QS11 resulted in a more consistent induction of CM differentiation from all three tested murine PSC lines than each substance alone, although batch-to-batch variability in CM yield could not be completely eliminated. These data indicate that cardiogenic effect of some previously reported compounds is difficult to reproduce and that variable cardiogenic potential of different PSCs and response to effective small molecules can be diminished but not completely abolished by a combination of different inducers of cardiogenesis that target different cellular processes at different stages of differentiation.

Materials and Methods

Cell culture

Murine ESC line CGR8 was cultured without feeder cells on 0.1% gelatin-coated tissue culture plates in Glasgow minimum essential medium (GMEM) supplemented with 10% fetal bovine serum (FBS), 0.1% non-essential amino acids (NEAA), 100 U/mL leukemia inhibitory factor (LIF, ESGRO, Chemicon/Merck Millipore, Billerica, MA, USA), and 50 μ M β -mercaptoethanol (β -ME). Unless otherwise specified, all cell culture reagents were obtained from Life Technologies (Carlsbad, CA, USA). Transgenic murine D3 ESC line α PIG (clone 44) was described earlier [17]. These cells express the puromycin resistance gene N-acetylaminotransferase (PAC) and the IRES-flanked enhanced green fluorescent protein (eGFP) under the control of cardiac alpha myosin heavy chain promoter (α MHC). α PIG44 ESCs were maintained on irradiated mouse embryonic fibroblasts (MEF) in Dulbecco's minimal essential medium (DMEM) containing 15% FBS, 1x NEAA, 2 mM L-glutamine, 50 μ M β -ME and 1000 U/ml LIF. Transgenic murine iPSC line AT25 was engineered from murine iPSC line TiB7.4 to express the PAC and eGFP genes under the control of α MHC promoter (Fatima et al, manuscript in preparation). iPSC line TiB7.4 was kindly provided by Rudolf Jaenisch and Alexander Meissner [18]. AT25 iPSCs were cultured in the same way as described for α PIG44 ESCs.

Cardiac differentiation

To induce cardiac differentiation, CGR8 ESCs were dissociated into single cells with 0.05% trypsin/EDTA and cell suspension containing 20,000 cells/mL was prepared in the differentiation medium (Iscove's modified Dulbecco's minimal essential medium (IMDM) supplemented with 20% FBS, 1% NEAA and 100 μ M β -ME). The differentiation was performed in hanging drops with 500 cells per 25 μ L drop as described previously [19]. After two days, embryoid bodies (EBs) that were formed in hanging drops were transferred into fresh differentiation medium but without small molecules and cultured for additional 3 days in a suspension culture on a shaker under continuous horizontal agitation. On day 5 of differentiation, EBs were plated on 0.1% gelatin-coated dishes and cardiac differentiation efficiency was monitored microscopically by determining the percentage of spontaneously contracting EBs and further quantified by flow cytometry as percentage of cardiac troponin T (TnnT2) positive cells.

Differentiation of α PIG44 ESCs and AT25 iPSCs was performed by using mass culture method as described previously [20, 21]. EBs were formed in non-adherent 10 cm plates under continuous agitation on a horizontal shaker by culturing 7×10^4 cells per ml of differentiation medium. At day 2 of differentiation, EBs were transferred into a fresh medium at the density of 2000 EBs per 10 cm non-adherent plate. Differentiation was continued without medium change until day 9 and afterwards the medium was changed every 2-3 days. Efficiency of cardiac differentiation was analyzed by determining the fraction of eGFP- or TnnT2-positive CMs on day 9, 11, and 13 of differentiation by flow cytometry. Experiments with selected substances were performed using 100 μ M L-ascorbic acid phosphate (AA, Wako Pure Chemicals Industry, Japan), 1 μ M cyclic 3'-5' adenosine monophosphate (cAMP), 2 μ M dorsomorphin, 0.25 μ M cardiogenol C and 2 μ g/ml cyclosporin A (CsA, all from Sigma-Aldrich, St. Louis, MO, USA). PSCs were treated with dorsomorphin, a selective small molecule inhibitor of BMP signaling, in ESC medium for 2 days prior to induction of differentiation and for further 2 days during the EB-formation step [9]. AA, cAMP, cardiogenol C and CsA were applied during the initial two days of differentiation as described previously [8, 9, 22-25]. Ascorbic acid and CsA were also applied during day 4-9 and 0-9 of differentiation. In some experiments AA was present during the whole differentiation period of 12 days. QS11 (Sigma-Aldrich) was added during day 4-9 or 0-9 of differentiation at the final concentration of 2.5 μ M [16]. Scheme summarizing treatment protocols with various compounds is shown in Figure 1.

Purification of CMs

On day 9 of differentiation when first spontaneously beating EBs containing GFP-positive CMs appeared in cultures of AT25 cells puromycin was added to a final concentration of 8 μ g/ml to remove non-CMs. On day 11, the selected cardiac clusters were transferred into new non-adherent culture dishes for further selection with medium change every second day. Analyses were performed with purified cardiac clusters on day 16-18 of differentiation after 7 days of puromycin treatment.

RT-PCR

To validate the enhancement of cardiogenesis by small molecules the expression of cardiac specific transcripts in EBs was determined by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis using ABI 7500 FAST Detection System (Applied Biosystems). Total RNA was isolated from undifferentiated ESCs or iPSCs, and day 8 and day 12 EBs using TRIzol (Invitrogen, Life Technologies). 1 µg of DNase I-pretreated RNA was reverse-transcribed by Superscript II RTase (Invitrogen) using random hexamers for priming. Diluted (1:20) cDNA was amplified using SYBR-Green PCR Master Mix (Syber Green Advantage qPCR premix, Clontech) and qRT-PCR was performed in triplicates for each sample. All reactions began with Taq activation at 95°C for 10 min and were followed by 40 cycles of 15 sec denaturation at 95°C, 30 sec annealing at 58°C, extension for 45 sec at 60°C, and finally ending with a melting curve acquisition. The relative quantities of the target genes were normalized against relative quantities of GAPDH housekeeping gene taking untreated sample as a reference.

Fold-expression changes were calculated by raising to the power of the negative value of delta-delta Ct value ($2^{-\Delta\Delta Ct}$) and the resulting values were plotted as raw quantity values setting the expression value of the reference sample to 1. Primers used for qPCR analyses are listed in the Table 1.

Immunocytochemistry

EBs were plated on day 5 of differentiation on gelatin-coated 6 cm dishes at 50 EBs/plate in differentiation medium. At day 15 EBs were fixed in 4% buffered paraformaldehyde (pH 7.5), permeabilized by Triton X-100 and stained overnight at 4°C with monoclonal α -actinin antibodies (clone EA53, Sigma). Secondary detection was performed with anti-mouse-IgG1a-AlexaFluor-555 (1:1000, Invitrogen) at RT for 60 min. Nuclei were stained with Hoechst 33342 (2 mg/ml, Invitrogen). After washing, samples were kept until analysis in DABCO. Samples were examined using Zeiss Axiovert 200M fluorescence microscope and analyzed with Zeiss Axiovision 4.5 software (Carl Zeiss, Jena, Germany).

Flow cytometry

Day 12 EBs were dissociated with trypsin and single cells were fixed in 4% PFA. Cells were permeabilized in 0.5% saponin solution in 5% BSA for 1 hr at room temperature and stained with TnnT2 primary antibody (1:50, sc20025, Santa Cruz, Dallas, USA) or isotype control murine IgG2a (sc3878, Santa Cruz) in 50 µl 0.8% BSA and 0.5% saponin for 30 min at 4°C. As negative control undifferentiated murine ESCs were used and stained according to the same procedure with TnnT2 antibody. After staining with primary antibody cells were washed in PBS and secondary antibody goat anti-mouse IgG-AlexaFluor-555 (1:100, Life Technologies) were added and incubated for 1 hr at 4°C. After washing, cells were analyzed by flow cytometry (FACScan, BD Pharmingen). Cell debris was gated out and 10000 events were acquired for analysis.

Microarray procedure

Murine ESCs CGR8 were differentiated in the presence and absence of 100 µM AA during day 0-2 of differentiation according to the protocol described above. Total RNA was isolated from three biological

Table 1. Primer sequences used for quantitative RT-PCR

Gene name	Sequence (5'→3')	Product size bp
<i>NKX2.5</i> -Forward	CAGCCAAAGACCCTCGGGCG	142
<i>NKX2.5</i> -Reverse	TGCGCCTGCGAGAAGAG	
<i>Acta2</i> -Forward	TGGGATGGAGTCAGCGGGCA	241
<i>Acta2</i> -Reverse	AGCCAGGATGGAGCCACCGA	
<i>cTnnT2</i> -Forward	GGTGCCACCCAAGATCCCCG	199
<i>cTnnT2</i> -Reverse	AATACGCTGCTGCTCGGCC	
<i>Myh6</i> -Forward	TGTCCCGGAAGGGGGCAA	144
<i>Myh6</i> -Reverse	CCGGCTCGTGCAGGAAGGTC	
<i>Gapdh</i> -Forward	GGCTCATGACCACAGTCCAT	108
<i>Gapdh</i> -Reverse	ACCTTGCCCACAGCCTTG	
<i>Col5a</i> -Forward	AGGAGGACCCACATGTCTCA	264
<i>Col 5a</i> Reverse	GCACCTCTGGGCACTCTATC	
<i>Mef2c</i> -Forward	ACGAGGATAATGGATGAGCGT	113
<i>Mef2c</i> -Reverse	ATCAGTGCAATCTCACAGTCG	
<i>Ets2</i> -Forward	CCCTGTCGCCAACAGTTTTC	84
<i>Ets2</i> -Reverse	GGGAGCACAGCAAACAGAGA	
<i>Klf1</i> -Forward	TTTGGCACCTAAGAGGCAGG	215
<i>Klf1</i> -Reverse	CACAGCAGAAGGGACGATGT	
<i>Gata2</i> -Forward	CACCCCGCGTATTGAATG	130
<i>Gata2</i> -Reverse	CCTGCGAGTCGAGATGGTTG	
<i>Elk3</i> -Forward	GGGCTCTACTCGTCCTTCA	159
<i>Elk3</i> -Reverse	TGCTTGTCGGTTTATTGGTCA	

Fig. 1. Treatment intervals with various small molecules. Green arrows stand for ascorbic acid (AA) and red arrows for other small molecules that were tested for their cardiac differentiation potency with murine ESC and iPSC lines. QS11 is a broad specificity small molecule inhibitor of ARFGAP that activates Wnt/ β -catenin signaling pathway in the presence of Wnt3a [16]. For detailed protocols see the Methods section "Cardiac differentiation".

replicates of undifferentiated ESCs (day 0) and intact AA-treated or untreated EBs on days 2, 3 and 5 of differentiation using TRIzol reagent. Only those samples were processed for further analysis in which we were able to confirm that AA significantly enhanced cardiac differentiation efficiency at later stages (day 8 and 12) of differentiation by assessing the number of beating EBs in control and AA-treated groups. Biotinylated cRNAs were prepared according to the standard Affymetrix protocol using the Mouse Genome 430 version 2.0 Array [26] and taken for global transcriptional analysis. Differentially expressed genes were selected using a fold change/p-value filter with the following criteria: only p-values smaller than 0.05 and an expression change higher than 1.5-fold was considered statistically significant for further analysis. The fold-change was calculated by dividing the mean intensity of the genes in one group by that in the other group. If this number was less than one, the negative reciprocal was used. For comparison of gene expression profiles between AA-treated and untreated EBs we have removed redundant probe sets and genes without annotation or of unknown origin. Among redundant probe sets, we selected the probe set with the highest average expression signal. Principal component analysis (PCA) was performed using Partek Genomics Suite (Partek, Inc.) to visualize data variability in a multidimensional picture. Distance between the samples reflects the degree of dissimilarity between them. To compare our dataset with reference time kinetic data of Gaspar and coworkers [27], a Robust Multi-array Analysis (RMA) algorithm was used to perform background correction, summarization and normalization. Quantile normalization method [28] was employed to normalize the raw dataset at probe feature level executable with the help of Partek Genomic Suite tool (Partek® software, version 6.6, Partek Inc., St. Louis, MO, USA) and PCA plot was generated. There was a clear batch effect noted between our data and the mESC Time kinetic Data set. To remove the batch effect for data presentation the batch removal algorithm ComBat.R was used [29]. The enrichment of specific biological processes, molecular functions, cellular components and pathways among differentially expressed genes was analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, <http://david.abcc.ncifcrf.gov/>) bioinformatics resource [30, 31] and functional categories and biological pathways annotated by the Gene ontology tool [32] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database [33]. Annotations were considered significantly overrepresented when the p-value of the Fisher's exact test as used by DAVID was <0.05.

Electrophysiology

In order to analyze the functional properties of CCs obtained from AA-treated and untreated cultures, puromycin-purified CCs of AT25 iPSC line were dissociated into single CMs by using 0.05% trypsin/EDTA. CMs were plated on gelatin-coated glass cover slips and cultured for 24-48 hours before measurements were performed. The cover slips were placed into a recording chamber (37°C) and cells superfused continuously with extracellular solution. Cell membrane capacitance was determined on-line using the PULSE software (HEKA Elektronik). Action potentials (AP) of spontaneously beating CMs were recorded by the whole-cell

current-clamp technique using an EPC-9 amplifier and the PULSE program. Response of CMs to hormonal regulation was assessed by administering isoproterenol (Iso) and carbachol (CCh) (Sigma-Aldrich). Data are presented as the mean $\pm$ standard error of the mean (S.E.M).

Results

AA robustly enhances cardiac differentiation of CGR8 ESCs

To evaluate cardiac enhancing properties of selected small molecules we first compared the effects of AA, dorsomorphin and cAMP that were reported to promote cardiac differentiation of murine ESCs [8, 9, 22]. First, we evaluated the cardiogenic activity of these molecules in murine ESC line CGR8 utilizing the hanging drop method. Dorsomorphin was used during 2 days before start of differentiation and for further 2 days after initiation of differentiation (Fig. 1). AA and cAMP were applied only during the first 2 days of differentiation and afterwards the medium did not contain any cardiogenic inducers, according to the previously published protocols [8, 9, 22]. Beating EBs were counted among all attached EBs on gelatin-coated dishes to initially estimate the cardiac enhancing potential of tested small molecules. As shown in Figure 2A, spontaneous beating activity appeared as early as on day 8 of differentiation only in AA treated group in 35% of EBs. In control, DMSO, dorsomorphin and cAMP groups the first contracting EBs were observed on day 9 at frequency of 7%, 6%, 8% and 7%, respectively. From day 8 on, the fraction of contracting EBs in AA-treated group was steadily increasing reaching 90% of EBs by day 12 of differentiation (Fig. 2A), whereas the percentage of contracting EBs in control, DMSO, dorsomorphin and cAMP groups was 3.9-fold lower at this time point ($23 \pm 8\%$, $24 \pm 15\%$, $34 \pm 14\%$ and $22 \pm 8\%$, respectively; $p < 0.05$ from three independent experiments), (Fig. 2A).

In these cultures we observed not only that the number of beating EBs in AA treated group increased but also that beating areas in these EBs were larger and contained more CMs as illustrated by the immunostaining for cardiac α -actinin (Fig. 2B). Moreover, AA treatment also strongly enhanced the CM yield as determined at the single cell level by flow cytometric quantification of cTnT-positive CMs. This analysis showed that on day 12 of differentiation the fraction of cTnT-positive CMs was 3.5-times higher in AA-treated EBs ($3.6 \pm 0.9\%$) than in control EBs ($1.2 \pm 0.6\%$), ($n=4$, $p < 0.05$, Fig. 2C). In order to further quantify the enhancement of cardiac differentiation of CGR8 ESCs by AA we used qRT-PCR to determine the expression of transcripts encoding for cardiac specific structural proteins α -actinin (ACTN2), cardiac troponin T (TNNT2) and α -myosin heavy chain (MYH6) in AA-treated and untreated EBs on day 12 of differentiation. As shown in Fig. 2D, the expression of *Actn2*, *Tnnt2* and *Myh6* mRNAs was increased by 7-, 110- and 180-fold in AA group compared to control group, respectively. These data demonstrate that the observed increase in the number of beating EBs and TNNT2-positive cells after AA treatment of CGR8 ESCs during the first two days of differentiation correlates with higher expression of cardiac genes in differentiating EBs.

Cardiogenic effect of AA on two other murine PSC lines

Next we sought to determine whether the selected small molecules are capable of enhancing the CM yield in other murine PSC lines. For this purpose we initiated the differentiation processes in a suspension mass culture using the transgenic ESC line α PIG44 and the transgenic iPSC line AT25, in which the expression of eGFP is controlled by the α MHC promoter enabling simple visualization and quantification of cardiac differentiation efficiency at different time points of differentiation. In these experiments we tested the cardiogenic effects of compounds that were used with CGR8 cells above as well as the effects of two additional substances, cardiogenol C and cyclosporin A (CsA), that were previously reported to robustly induce cardiomyogenesis in mouse ESCs if applied during the first two days of differentiation [24, 25, 34-36]. The efficiency of cardiac differentiation was determined by flow cytometric analysis of eGFP-expressing cells in enzymatically dissociated EBs on day 9, 11 and 13 of differentiation.

Fig. 2. Screening for robust inducers of cardiac differentiation of murine CGR8 ESC line. **A.** Compared to untreated and DMSO treated controls only ascorbic acid (AA, 100 μ M) but not dorsomorphin (2 μ M) or cAMP (1 μ M) strongly and reproducibly enhanced the cardiac differentiation of murine ESC line CGR8. The time windows during which the substances were administered are described in the *Methods* section and in Fig. 1. Data are given as mean $\pm$ S.E.M. of three independent experiments. * $p < 0.05$. **B.** Fluorescence images of adherent EBs in control or AA-treated cultures stained for cardiac α -actinin [49] on day 15 of differentiation. Nuclei are stained with Hoechst 33342 in blue. Scale bars: 100 μ m. **C.** Flow cytometric assessment of

cardiac differentiation efficiency of control or AA-treated CGR8 ESCs by quantification of cardiac troponin T (TnnT2)-positive cells in EBs at day 12 of differentiation ($n=4$, * $p < 0.05$). Isotype control was performed along with each experiment to exclude unspecific staining. FL1 is fluorescence detection channel (515-545 nm wave length). **D.** Compared to untreated control cells, AA increases the expression of cardiac transcripts encoding for α -actinin (Actn2), cTnT (TnnT2) and α -myosin heavy chain (Myh6) as determined by quantitative RT-PCR analysis. Error bars represent S.D. from one biological experiment run in triplicate. * $p < 0.05$.

Similarly to observations with CGR8 ESCs, AA applied from day 0 to day 2 of differentiation was the only small molecule capable of increasing the cardiogenesis of α PIG44 and AT25 cells by 2-4-fold compared to untreated controls (Fig. 3A, B). However, we observed highly variable CM yields in independent experiments with these two cell lines which is reflected in higher standard deviations in Figure 3. Dorsomorphin showed a tendency towards increased yields of CMs in AT25 iPSCs but this effect was inconsistent and non-significant compared to DMSO vehicle control (Fig. 3A), and was not observed with α PIG44 ESCs (Fig. 3B). In control untreated, DMSO vehicle-treated, and cAMP-, cardiogenol C- and CsA-treated groups the fraction of eGFP-positive CMs rarely exceeded 1% of cells in EBs and the differences were not statistically significant independently of PSC line tested. When CsA was administered

Fig. 3. Effect of selected small molecules on cardiac differentiation of murine PSCs. Among five tested compounds only AA enhanced cardiac differentiation of iPSC line AT25 (A) and ESC line α PIG44 (B). The percentage of eGFP positive CMs was determined on differentiation days 9, 11 and 13 by flow cytometry. Dead cells were excluded by PI staining. Data are given as mean $\pm$ S.E.M from six independent experiments (* $p < 0.05$). C. Effect of cyclosporin A (CsA) on CM-differentiation of α PIG44 ESCs and AT25 iPSCs. D. Effect of AA on CM-differentiation of α PIG44 ESCs and

AT25 iPSCs after application in different time intervals during differentiation. In panels C and D the fraction of eGFP-positive CMs was determined on indicated days of differentiation by flow cytometry. Data are shown as means $\pm$ S.E.M. of at least two independent experiments run in triplicates (* $p < 0.05$; ** $p < 0.005$).

between days 4-9 of differentiation there was a tendency towards increased cardiogenesis in α PIG44 ESC and AT25 iPSC lines, however this effect did not reach statistical significance and was not consistent in different experiments (Fig. 3C). Upon application through day 0-9 the CSA did not increase the yield of CMs (data not shown).

To determine the time window in which AA exerts its strongest effect on cardiac enhancement AA was applied during day 0-2, 0-9 and 4-9 of differentiation. In agreement with data shown above, the addition of AA during days 0-2 was sufficient for enhancing cardiac differentiation in this experiment (Fig. 3D). Similar effect was also achieved when cells were treated with AA continuously until day 9 of differentiation. In contrast, addition of AA at later stage of differentiation (day 4-8) failed to promote cardiogenesis in α PIG44 ESC and AT25 iPSC lines (Fig. 3D). Comparable results were also obtained with CGR8 ESCs (data not shown). Taken together, these data show that among five tested compounds only the AA applied during the first two days of differentiation efficiently enhanced the yield of CMs in three different murine PSC lines. The most pronounced, consistent and reproducible cardiogenic effect of AA was observed with CGR8 ESCs, while the effect on α PIG44 ESCs and AT25 iPSCs was inconsistent and variable between different biological replicates.

Fig. 4. Effect of AA treatment on global gene expression profiles in differentiating CGR8 EBs. A. Enhancement of cardiogenesis in the same parallel cultures of samples that were used for microarray analysis as determined by counting the percentage of beating EBs in untreated and AA-treated EBs in three independent biological experiments at day 8 of differentiation (left panel). Quantitative RT-PCR analysis of expression levels of α -actinin transcripts (*Actn2*) in AA-treated and control group (right panel). Numbers indicate the relative mRNA expression levels normalized to undifferentiated CGR8 ESCs. Data are shown as means $\pm$ S.D. of one biological experiment run in triplicate (* $p < 0.05$). B. Principal component analysis of 45,037 probe sets [undifferentiated ESCs (D0), and untreated and AA-treated day 2 EBs (D2 and D2+AA), day 3 EBs (D3 and D3+AA), and day 5 EBs (D5 and D5+AA)] compared with reference time kinetic data set (DX-TK) from study of Gaspar et al. [27] after removing batch effect which is introduced due to different date of array processing. C. The number of differentially expressed transcripts was determined by considering only those probe sets with $p < 0.05$, 2.0-fold change after controlling for false discovery rate in various intergroup comparisons. D. The number of differentially expressed transcripts ($p < 0.05$, 1.5-fold change) in various intergroup comparisons. E. Quantitative RT-PCR validation of selected genes that were found to be upregulated after AA treatment on differentiation day 5 in microarray study. F. Expression levels of the same genes as found in the microarray study. In both panels data are shown as fold change in expression level relative to untreated control group.

periment run in triplicate (* $p < 0.05$). B. Principal component analysis of 45,037 probe sets [undifferentiated ESCs (D0), and untreated and AA-treated day 2 EBs (D2 and D2+AA), day 3 EBs (D3 and D3+AA), and day 5 EBs (D5 and D5+AA)] compared with reference time kinetic data set (DX-TK) from study of Gaspar et al. [27] after removing batch effect which is introduced due to different date of array processing. C. The number of differentially expressed transcripts was determined by considering only those probe sets with $p < 0.05$, 2.0-fold change after controlling for false discovery rate in various intergroup comparisons. D. The number of differentially expressed transcripts ($p < 0.05$, 1.5-fold change) in various intergroup comparisons. E. Quantitative RT-PCR validation of selected genes that were found to be upregulated after AA treatment on differentiation day 5 in microarray study. F. Expression levels of the same genes as found in the microarray study. In both panels data are shown as fold change in expression level relative to untreated control group.

Global transcriptional profiling of AA-induced genes

We next performed a global gene expression analysis to better understand the mechanism of AA-induced cardiac differentiation in CGR8 ESCs. Total RNA was prepared from undifferentiated ESCs as well as control and AA-treated EBs on day 2, 3 and 5 of differentiation from batches in which AA significantly enhanced cardiac differentiation efficiency at day 8 of differentiation (Fig. 4A). The raw microarray data is stored at NCBI GEO database and can be found under the accession number GSE54242. Principal component analysis (PCA) performed with the entire normalized data set of 45,037 probe sets on a microarray showed that samples cluster in four groups corresponding to a) undifferentiated ESCs, b) day 2 EBs, c) day 3 EBs and d) day 5 EBs (Fig. 4B). The transcriptional profiles of undifferentiated ESCs were more related to those of day 2 EBs than to those of day 3 or day 5 EBs. The AA-treated EBs clustered together with untreated EBs of the same stage. Comparison of our

dataset with the reference time kinetic data that was recently reported by Gaspar et al. for pluripotent and differentiated CGR8 ESCs at different stages of differentiation [27] revealed that ESCs and EBs from our and Gaspar's experiments cluster together on the corresponding time points (Fig. 4B), confirming the high reproducibility of our differentiation protocols and reliability of results.

Analysis of differentially expressed genes (p-value < 0.05; > 2.0-fold change, with raw p-values adjusted for false discovery rate) revealed that none of the variable transcripts significantly differed in their expression levels between AA-treated and untreated EBs on day 2 and 3 of differentiation and that only 9 unique transcripts were differentially expressed on day 5 of differentiation (Fig. 4C). In contrast, in this analysis we detected 762, 1478, 3363 transcripts that were differentially expressed between undifferentiated ESCs and untreated EBs on day 2, day 3 and day 5 of differentiation, respectively, reflecting the steadily increasing developmental distance between pluripotent ESCs and differentiating EB cell populations (Fig. 4C). Since we were unable to identify a significant number of AA induced genes after stringent statistical analysis, we repeated the analysis by considering probe sets as significantly differentially expressed when $p < 0.05$ and an expression change higher than 1.5-fold without adjusting the raw p-values for the false discovery rate. Under these conditions we found that 61 unique transcripts were differentially expressed between AA-treated and untreated day 2 EBs of which 51 unique genes were

Table 2. List of 51 unique differentially expressed genes UP-regulated upon ascorbic acid (AA) treatment of day 2 EBs compared to untreated day 2 EBs (CTRL). Expression changes higher than 1.5-fold with $p < 0.05$ were considered significant. Redundant and unknown genes are removed. Signal intensities are shown as log2-transformed values. Genes were considered to be expressed when the signal intensity was higher than 6.5 (the log2-transformed value)

Probe Set ID	Gene Symbol	Mean CTRL	Mean AA	FC	p value
1436291_at	Dpys	6.12	7.54	2.7	0.0014
1450943_at	Magohb	7.62	8.95	2.5	0.0030
1427277_at	Six1	5.62	6.93	2.5	0.0496
1419540_at	Fthl17	6.00	7.27	2.4	0.0011
1438390_s_at	Pttg1	10.54	11.77	2.3	0.0016
1452670_at	Myl9	6.54	7.69	2.2	0.0134
1423582_at	Dmrt1	8.20	9.34	2.2	0.0044
1424713_at	Calm14	5.70	6.83	2.2	0.0104
1423627_at	Nqo1	7.37	8.50	2.2	0.0024
1420357_s_at	Xlr3a	5.60	6.70	2.1	0.0386
1427284_a_at	Ttpa	7.15	8.22	2.1	0.0237
1417027_at	Trim2	6.99	8.03	2.1	0.0073
1451335_at	Plac8	7.90	8.94	2.1	0.0009
1453133_at	Slc25a31	5.65	6.69	2.1	0.0154
1417077_at	Bcap29	6.40	7.44	2.0	0.0389
1417482_at	Tex19.1	8.66	9.66	2.0	0.0450
1419021_at	Mcf2	7.33	8.32	2.0	0.0167
1416332_at	Cirbp	7.26	8.23	2.0	0.0208
1448406_at	Eid1	6.74	7.66	1.9	0.0007
1416904_at	Mbnl1	7.12	8.04	1.9	0.0050
1435532_at	Rimklb	6.53	7.44	1.9	0.0236
1433523_at	Radil	7.15	8.06	1.9	0.0358
1442878_at	Prdx6	6.09	7.00	1.9	0.0146
1423065_at	Dnmt3a	8.97	9.87	1.9	0.0239
1434817_s_at	Rprd2	7.53	8.42	1.9	0.0049
1448265_x_at	Mpzi2	7.03	7.92	1.8	0.0027
1429776_a_at	Dnajb6	8.14	9.01	1.8	0.0401
1434292_at	Snhg11	7.16	8.03	1.8	0.0217
1436714_at	Lpp	6.41	7.27	1.8	0.0053
1438824_at	Slc20a1	6.53	7.36	1.8	0.0260
1454120_a_at	Pcgf6	8.48	9.31	1.8	0.0412
1415904_at	Lpl	7.55	8.39	1.8	0.0248
1426787_at	Sfi1	5.87	6.70	1.8	0.0317
1427912_at	Cbr3	9.87	10.70	1.8	0.0057
1424176_a_at	Anxa4	6.92	7.74	1.8	0.0014
1438349_at	Zfp229	7.45	8.26	1.7	0.0505
1416308_at	Ugdh	8.95	9.74	1.7	0.0047
1453957_a_at	Igf2bp3	6.92	7.71	1.7	0.0009
1421277_at	Spna1	6.36	7.14	1.7	0.0208
1450781_at	Hmga2	6.45	7.24	1.7	0.0142
1441917_s_at	Tmem40	7.42	8.21	1.7	0.0042
1450037_at	Usp9x	5.79	6.57	1.7	0.0065
1421011_at	Hsd17b11	6.12	6.90	1.7	0.0099
1421816_at	Gsr	8.63	9.41	1.7	0.0044
1435127_a_at	Osgepl1	7.12	7.90	1.7	0.0357
1417777_at	Ptgr1	9.35	10.11	1.7	0.0119
1434949_at	Armcd8	7.09	7.85	1.7	0.0033
1456505_at	Braf	7.27	8.03	1.7	0.0429
1436871_at	Srsf7	8.39	9.14	1.7	0.0419
1425108_a_at	Smagp	7.56	8.31	1.7	0.0160
1419402_at	Mns1	7.08	7.83	1.7	0.0242

up-regulated and 10 down-regulated (Fig. 4D). At day 3, only 21 unique transcripts were differentially expressed with 11 genes being up-regulated and 10 down-regulated. Similarly, only 77 unique transcripts were affected by AA in day 5 EBs of which 68 genes were up-regulated and 9 down-regulated (Fig. 4D). All transcripts that were differentially up- or down-regulated by AA in day 2 and day 5 EBs are listed in Tables 2-5.

*Gene ontology analysis
of genes up-regulated in
AA-treated EBs*

Table 3. List of 10 unique differentially expressed genes DOWN-regulated upon ascorbic acid (AA) treatment of day 2 EBs compared to untreated day 2 EBs (CTRL). Expression changes higher than 1.5-fold with $p < 0.05$ were considered significant. Redundant and unknown genes are removed. Signal intensities are shown as log₂-transformed values. Genes were considered to be expressed when the signal intensity was higher than 6.5 (the log₂-transformed value)

Probe Set ID	Gene Symbol	Mean CTRL	Mean AA	FC	p value
1452318_at	Hspa1b	8.94	7.53	2.7	0.0030
1441429_at	Irs4	8.34	7.19	2.2	0.0065
1418536_at	H2-Q7	7.46	6.36	2.1	0.0376
1450079_at	Nrk	7.07	5.98	2.1	0.0066
1418649_at	Egln3	8.63	7.58	2.1	0.0213
1439148_at	Pfkfb	9.75	8.82	1.9	0.0057
1434875_at	Hmgn3	8.10	7.24	1.8	0.0024
1417355_at	Peg3	9.65	8.85	1.7	0.0440
1424850_at	Map3k1	6.85	6.07	1.7	0.0230
1422470_at	Bnip3	10.31	9.52	1.7	0.0325

In order to identify the functional categories enriched by AA we have next performed a gene ontology analysis of unique transcripts that were found to be up-regulated in AA-treated EBs (Tables 2-5) using DAVID software [31]. The main GO-terms that were differentially up-regulated or down-regulated by AA in day 2 and day 5 EBs are shown in Tables 6 and 7, respectively. Among genes up-regulated in AA-treated group on day 5 of differentiation we detected increased expression of genes involved in angiogenesis (*Gata2*, *Tek*, *Runx1*), blood vessel development (*Elk3*, *Sox18*, *Tnfrsf2*, *Cdh5*) and hematopoiesis/erythropoiesis (*Klf1*, *Tal1*, *Nfe2l3*, *Heph*, *Trim10*, *Runx1*, *Irf1*). Two cardiac specific transcripts *Mef2c* and *Myl7* and the early mesodermal marker *T-brachyury* were also induced by AA in day 5 EBs (Table 4) but these transcripts were not significantly enriched as a GO-term in DAVID analysis (Tables 6 and 7). The expression levels of selected genes (*Col5a*, *Mef2c*, *Ets2*, *Klf1*, *Gata2* and *Elk3*) were validated by RT-qPCR analysis which confirmed their up-regulation in microarray analysis (Fig. 4E, F). Among the unexpectedly small number of differentially expressed transcripts in EBs on day 2 and 3 of differentiation we did not detect any early lineage specific mesendodermal markers in AA-treated cultures despite the pronounced enhancement of cardiac differentiation in these same cultures at the later stage of differentiation as shown in Fig. 4A. This data suggest that AA induces only minor transcriptional changes in early stage EBs that are sufficient for enhancement of cardiogenesis in later stages of differentiation.

QS11 enhances AA-mediated induction of cardiomyogenesis

We observed high efficiency and reproducibility of AA-induced cardiac differentiation in CGR8 ESC line while other two ESC and iPSC lines frequently yielded inconsistent CM yield upon application of AA despite its well documented role in promoting cardiac differentiation of different PSC lines [7, 22, 37, 38]. The involvement of Wnt/ β -catenin signaling pathway in cardiac development is well established in the literature [15, 16, 39-42]. Therefore, we tested whether QS11, a small molecule reported to activate the Wnt/ β -catenin signaling pathway in the presence of Wnt3a [16], is able to enhance the cardiogenic effect of AA. In these experiments CGR8 ESCs were treated with AA during day 0-2 and with QS11 during day 4-9 of differentiation as shown in Figure 1. Under these conditions cardiac differentiation of CGR8 ESCs was strongly enhanced: QS11+AA generated 6-fold higher number of TnnT2-positive CMs ($7.2 \pm 1.3\%$) than in untreated group (1.2 ± 0.7) and 2.5-3 fold higher than in AA-only ($3.6 \pm 1.1\%$) or QS11-only group ($3.0 \pm 1.0\%$, Fig. 5A). In addition, AA and QS11 also increased expression of several cardiospecific transcripts in CGR8 ESCs on day 12 of differentiation (Fig. 5B). Compared to control group, AA alone, QS11 alone and AA+QS11 induced significantly higher expression of *TnnT2*, cardiac α -actinin (*Actn2*), α -myosin heavy chain (*Myh6*), atrial

Table 4. List of 68 unique differentially expressed genes UP-regulated upon ascorbic acid (AA) treatment of day 5 EBs compared to untreated day 5 EBs (CTRL). Expression changes higher than 1.5-fold with $p < 0.05$ were considered significant. Redundant and unknown genes are removed. Signal intensities are shown as log₂-transformed values. Genes were considered to be expressed when the signal intensity was higher than 6.5 (the log₂-transformed value)

natriuretic peptide (*Nppa*) and *Nkx2.5* transcripts. However, compared to AA group alone, the combined treatment of CGR8 ESCs with AA+QS11 resulted in stronger induction of *Actn2*, *Nppa* and *Nkx2.5* but not of *TnnT2* and *Myh6* transcripts. AA and QS11 also induced higher yields of eGFP- and *TnnT2*-positive CMs in AT25 iPSC (Fig. 5C,D) and α PIG44 ESC cultures (Fig. 5E,F). This effect was not seen when QS11 was applied during differentiation days 0-9 or 0-2 (Fig. 6A-C), indicating that its action is limited to a specific time window. However, the effect of AA and QS11 was variable (Fig. 5C-F and 6A,B) and the basal differentiation efficiency was much lower in some experiments reflecting the inherent instability in cardiac differentiation of AT25 iPSC and α PIG44 ESC cell lines (Fig. 5E). These data indicate that cardiogenic potential of some PSC lines can be enhanced by small molecules but batch-to-batch variability in CM yield cannot be completely abolished by combined application of AA and QS11 or by each drug alone.

Probe Set ID	Gene Symbol	Mean CTRL	Mean AA	FC	p value
1450736_at	Hbb-bh1	9.15	12.46	9.9	0.01122
1436823_x_at	Hbb-y	5.13	8.02	7.4	0.02981
1436853_at	Snca	4.58	6.92	5.1	0.00302
1450943_at	Magohb	5.93	8.13	4.6	0.00096
1456014_s_at	Fermt3	5.77	7.97	4.6	0.01532
1418788_at	Tek	7.36	9.18	3.5	0.01362
1450194_at	Myb	5.13	6.90	3.4	0.00475
1449071_at	Myl7	7.65	9.37	3.3	0.04878
1424105_at	Pttg1	8.75	10.39	3.1	0.00067
1449389_at	Tal1	7.95	9.59	3.1	0.00957
1426340_at	Slc1a3	5.48	7.03	2.9	0.00912
1423110_at	Col1a2	5.74	7.26	2.9	0.01457
1422437_at	Col5a2	6.34	7.78	2.7	0.02401
1438020_at	Hapln1	7.80	9.20	2.6	0.00333
1440244_at	Erg	6.23	7.60	2.6	0.02577
1433512_at	Fli1	7.22	8.57	2.5	0.04257
1416904_at	Mbnl1	7.36	8.69	2.5	0.02249
1422864_at	Runx1	5.25	6.56	2.5	0.02301
1416842_at	Gstm5	8.04	9.35	2.5	0.01694
1452352_at	Ctla2b	6.10	7.39	2.4	0.04710
1420872_at	Gucy1b3	7.06	8.32	2.4	0.01873
1454086_at	Lmo2	8.67	9.90	2.3	0.00330
1455607_at	Rspo3	8.84	10.04	2.3	0.01004
1438933_x_at	Rasgrp2	5.45	6.62	2.3	0.04464
1427196_at	Wnk4	5.95	7.11	2.2	0.00637
1448449_at	Ripk3	5.76	6.87	2.2	0.03151
1423213_at	Plxnc1	5.96	7.06	2.1	0.01364
1438855_x_at	Tnfrsf2	7.10	8.19	2.1	0.01831
1423091_at	Gpm6b	5.58	6.67	2.1	0.02133
1448696_at	Heph	5.89	6.98	2.1	0.03078
1452001_at	Nfe2	5.64	6.73	2.1	0.00199
1448600_s_at	Vav3	6.18	7.23	2.1	0.01881
1449135_at	Sox18	6.68	7.72	2.1	0.02692
1422756_at	Slc32a1	6.08	7.12	2.1	0.00499
1418600_at	Klf1	7.10	8.13	2.0	0.05029
1434528_at	Aard	6.36	7.39	2.0	0.00381
1450744_at	Ell2	7.41	8.44	2.0	0.01717
1435454_at	BC006779	5.50	6.52	2.0	0.02383
1425643_at	Gypa	6.24	7.25	2.0	0.01802
1416564_at	Sox7	6.86	7.83	2.0	0.01874
1449232_at	Gata1	6.26	7.24	2.0	0.01336
1433956_at	Cdh5	6.05	7.02	2.0	0.03140
1423222_at	Cap2	6.70	7.67	2.0	0.02520
1421027_at	Mef2c	7.40	8.34	1.9	0.00345
1448194_at	H19	7.63	8.57	1.9	0.00212
1415948_at	Creg1	7.49	8.39	1.9	0.00808
1423878_at	Gypc	6.57	7.45	1.9	0.02534
1450716_at	Adamts1	6.15	7.02	1.8	0.04417
1419311_at	Trim10	5.69	6.56	1.8	0.03931
1451867_x_at	Arhgap6	6.00	6.86	1.8	0.01929
1450333_at	Gata2	7.53	8.39	1.8	0.03977
1448471_at	Ctla2a	5.87	6.73	1.8	0.02382
1436538_at	Ankrd37	6.49	7.34	1.8	0.02101
1433695_at	Cnrip1	7.22	8.06	1.8	0.00931
1422644_at	Sh3bgr	6.50	7.33	1.8	0.04257
1455618_x_at	Tspan33	6.12	6.95	1.8	0.04497
1448797_at	Elk3	7.68	8.50	1.8	0.03221
1423465_at	Frrs1	6.76	7.58	1.8	0.00332
1419120_at	Lyl1	6.40	7.21	1.8	0.04110
1434875_at	Hmgcn3	7.67	8.47	1.7	0.00402
1419304_at	T	7.48	8.28	1.7	0.00386
1436312_at	Ikzf1	5.96	6.76	1.7	0.02110
1430640_at	Prkar2b	8.05	8.83	1.7	0.00773
1418892_at	Rhoj	6.74	7.53	1.7	0.03182
1422670_at	Rnd2	9.72	10.49	1.7	0.00457
1416268_at	Ets2	9.29	10.06	1.7	0.01059
1434170_at	Dcaf12l1	7.63	8.38	1.7	0.01037
1420965_at	Enc1	8.59	9.34	1.7	0.02960

Table 5. List of 10 unique differentially expressed genes DOWN-regulated upon ascorbic acid (AA) treatment of day 5 EBs compared to untreated day 5 EBs (CTRL). Expression changes higher than 1.5-fold with $p < 0.05$ were considered significant. Redundant and unknown genes are removed. Signal intensities are shown as log₂-transformed values. Genes were considered to be expressed when the signal intensity was higher than 6.5 (the log₂-transformed value)

Probe Set ID	Gene Symbol	Mean CTRL	Mean AA	FC	p value
1417928_at	Pdlim4	9.66	8.69	2.0	0.0027
1449534_at	Sycp3	6.95	5.97	2.0	0.0284
1420565_at	Hoxa1	8.64	7.77	1.8	0.0250
1419123_a_at	Pdgfc	7.82	7.03	1.7	0.0443
1424797_a_at	Pitx2	8.93	8.12	1.8	0.0193
1452730_at	Rps4y2	10.56	9.75	1.8	0.0069
1422870_at	Hoxc4	6.89	6.10	1.7	0.0000
1418733_at	Twist1	9.53	8.74	1.7	0.0315
1435892_at	Asxl3	6.64	5.89	1.7	0.0207

Table 6. Gene ontology analysis of genes UP-regulated in ascorbic acid-treated day 2 EBs and day 5 EBs. Processes found to be induced by AA are presented for categories: biological processes (BP), molecular function (MF), and cellular component (CC). Only significantly enriched GO-terms are shown ($p < 0.05$) with a threshold of at least 3 gene counts. The percentage indicates the number of genes involved in the respective GO-term / total number of genes in this GO-term. The p-values associated with each annotation term represent a modified Fisher Exact P-value as defined in DAVID software

EB stage	Category	Term	%	p-value	Genes
Day 2	BP	oxidation-reduction	13.7	9.8E-3	Nqo1, Ugdh, Cbr3, Hsd17b11, Ptgr1, Gsr, Prdx6
	BP	regulation of RNA metabolic process	17.6	4.3E-2	Dnmt3a, Dnajb6, Eid1, Dmrt1, Mbnl1, Pcgf6, Hmga2, Six1, Zfp229
	BP	M-phase	7.8	5.0E-2	Mns1, Pttg1, Hmga2, Usp9x
	MF	zinc ion binding	23.5	2.8E-2	Braf, Dnmt3a, Lpp, Osgepl1, Dpys, Dmrt1, Mbnl1, Pcgf6, Ptgr1, Trim2, Zfp229
Day 5	BP	regulation of RNA metabolic process, regulation of transcription, regulation of angiogenesis	25.4	1.5E-4	Ets2, Elk3, Fli1, Gata1, Gata2, Ikzf1, Klf1, Sox18, Sox7, Tal1, Erg, Ell2, Lyl1, Creg1, Mbnl1, Myb, Mef2c, Nfe2, Runx1
	BP	erythrocyte differentiation / hematopoiesis	6.0	9.3E-4	Gata2, Adamts1, Tek, Runx1
	BP	regulation of myeloid cell differentiation	6.0	1.1E-3	Klf1, Tal1, Heph, Trim10
	BP	regulation of myeloid cell differentiation	9.0	5.5E-3	Ikzf1, Tal1, Runx1
	BP	hemopoietic / lymphoid organ / immune system development	9.0	6.0E-3	Ikzf1, Klf1, Tal1, Heph, Runx1, Trim10
	BP	vasculature / blood vessel development	7.5	2.2E-2	Elk3, Sox18, Cdh5, Mef2c, Tnfrsf2
	CC	extracellular matrix	4.9	4.8E-3	Adamts1, Col1a2, Col5a2, Slc1a3, Hapln1
	MF	transcription regulator activity, DNA binding	25.4	8.7E-5	Ets2, Elk3, Fli1, Gata1, Gata2, Ikzf1, Klf1, Lmo2, Sox18, Sox7, Tal1, Erg, Hmgn3, Lyl1, Myb, Mef2c, Nfe2, Runx1, Arhgap6, Pttg1, Sh3bgr

Table 7. Gene ontology analysis of genes DOWN-regulated in ascorbic acid-treated day 2 EBs and day 5 EBs. Only significantly enriched GO-terms are shown ($p < 0.05$) with a threshold of at least 3 gene counts. The percentage indicates the number of genes involved in the respective GO-term / total number of genes in this GO-term. The p-values associated with each annotation term represent a modified Fisher Exact P-value as defined in DAVID software

EB stage	Category	Term	%	p-value	Genes
Day 2	BP	Apoptosis	40.0	2.0E-3	Egln3, Map3k1, Peg3, Bnip3
	BP	Cellular response to stress	30.0	2.2E-2	Nrk, Hspa1b, Map3k1
	BP	ATP-binding	40.0	3.0E-2	Nrk, Hspa1b, Map3k1, Pfkf
Day 5	BP	Homeobox / DNA-binding / embryonic organ development / transcription / pattern specification process	33.3	2.6E-3	Asxl3, Hoxa1, Hoxc4, Pitx2, Pdgfc, Sycp3, Twist1

Functional properties of cardiomyocytes obtained by AA+QS11 treatment

Electrophysiological analysis by the whole-cell patch-clamp method was used to examine the action potential (AP) properties of murine AT25 iPSC-derived CMs obtained after spontaneous differentiation without drugs and after combined administration of AA and QS11. For these analyses, CMs were purified with puromycin from day 9-16 of differentiation and dissociated single CMs were analyzed on day 30 of differentiation. These analyses

Fig. 5. Effect of QS11 on cardiac differentiation of different murine PSC lines. A. Effect of AA, QS11 or combined administration of AA and QS11 on CM yield of murine CGR8 ESCs. AA was added during days 0-2 and QS11 during days 4-8 of differentiation and the fraction of TnnT-positive CMs was determined by flow cytometry on day 12 of differentiation. Data are shown as mean $\pm$ S.E.M. of 4 independent

experiments (* $p < 0.05$). B. Quantitative RT-PCR of transcripts encoding for indicated cardiac structural proteins in AA, QS11 and AA+QS11-treated EBs on day 12 of differentiation. mRNA expression levels were normalized to untreated control which received the value of 1. Error bars represent S.D. from one biological experiment run in triplicate (* $p < 0.05$). C-F. Effect of combined administration of AA and QS11 on CM differentiation of murine AT25 iPSC (C,D) and αPIG44 ESC line (E,F). The fraction of eGFP-positive and TnnT-positive CMs was determined by flow cytometry on indicated days of differentiation. Data are shown as mean $\pm$ S.E.M. of three independent experiments (panels C,E), two independent experiments (panel D) or one experiment (panel F), each run in triplicates (* $p < 0.05$, n.s. – non-significant). The symbols shown in panel B also apply for all other panels.

revealed that AA and QS11 did not affect the functional properties of CMs. Both control and AA+QS11-induced CMs exhibited typical AP characteristics of immature embryonic CMs and responded similarly to the β -adrenergic receptor agonist isoproterenol (Iso) and the muscarinic receptor agonist carbachol (CCh) (Fig. 7 and Table 8). In both cell groups, Iso (1 μ M) significantly increased the spontaneous AP frequency whereas CCh (1 μ M) caused a stop of beating (Fig. 7). Differences observed between control CMs and AA+QS11 CMs were not significant, suggesting that small molecule induced CM are functionally intact.

Fig. 6. QS11 has no cardiac enhancing effect when applied during days 0-2 or 0-9 of differentiation. Effect of QS11 applied at different time intervals on CM yield of α PIG44 ESC and AT25 iPSC lines (A) and CGR8 ESCs (B). The fraction of eGFP-positive CMs in panel A was determined by flow cytometry on day 11 of differentiation. Data are shown as mean $\pm$ S.E.M. of one experiment run in triplicate (* $p < 0.05$, ** $p < 0.01$). Level of *TnnT2* mRNA expression in panel B was determined by quantitative RT-PCR and shown relative to untreated control which received the value of 1. Data is shown as mean $\pm$ S.D. of one experiment run in triplicate, * $p < 0.05$, n.s. – non-significant. The Fig. legend in panel A also applies for panel B. C. The fraction of TnnT2-positive CMs was determined by flow cytometry on day 10 of differentiation. Data is from one experiment with CGR8 cell line.

Fig. 7. iPSC-derived CMs induced with combined administration of AA and QS11 are functionally intact. A. Current-clamp characterization of control and AA+QS11 treated-CMs. Action potential recording traces showing the effect of Iso (1 μ M) and CCh (1 μ M) on drug selected untreated CMs (top panel) and AA+QS11-treated CMs (bottom panel). B,C. Effect of Iso and CCh on the spontaneous AP frequency in untreated (B) and AA+QS11-treated CMs (C) that were purified by puromycin selection using AT25 iPSC line. Data are shown as means $\pm$ S.E.M (the number of analyzed cells in each group is indicated in parentheses, * $p < 0.05$).

Discussion

Pluripotent ESCs and iPSCs represent an attractive source of clinically useful CMs because they are easily accessible and expandable in culture, have broad developmental

Table 8. Parameters of action potentials in untreated and AA+QS11-treated groups of murine iPSC-derived puromycin selected cardiomyocytes.* Data are presented as mean $\pm$ S.E.M. N indicates the total number of cells tested from 3 independent differentiations. *Abbreviations:* A-like, atrial type CM; P-like, pacemaker type CM; MDP, maximal diastolic potential; Vmax, upstroke velocity; APD90, action potential (AP) duration measured from the maximal depolarization to 90% repolarization; APD50, AP duration measured from the maximal depolarization to 50% repolarization; APD90/APD50, ratio of APD90 to APD50

AA+QS11	CM type	N	MDP [mV]	Vmax [V/s]	APD50 [ms]	APD90 [ms]	APD90/APD50
-	A-like	5	-53.2 $\pm$ 1.75	12.63 $\pm$ 3.70	44.83 $\pm$ 3.79	173.85 $\pm$ 77.3	3.38 $\pm$ 0.45
	P-like	1					
+	A-like	21	-55 $\pm$ 2.6	21.69 $\pm$ 3.88	22.73 $\pm$ 6.53	119.7 $\pm$ 44.5	5.75 $\pm$ 1.45
	P-like	4					

potential and high capacity to reproducibly differentiate into spontaneously beating cardiac cells *in vitro*. The major effort of many investigators in recent years has been to develop methods for enrichment of ES cell-derived CMs *in vitro* in order to improve their yield, purity and safety. Currently used protocols require optimization and CM yield shows high variability between cell lines. Here we show that AA has robust effect on cardiac differentiation while still there is some interline difference that cannot be overcome by addition of a single factor. We show also that cardiac enhancing effect of cAMP and dorsomorphin was not achieved in our tested cell lines, which could be due to cell-to-cell line variability and requirement for protocol optimization. It was shown that apart from blockade of BMP-mediated SMAD1/5/8 phosphorylation dorsomorphin also inhibits AMP-activated protein kinase (AMPK) and receptor tyrosine kinases for PDGF and VEGF signaling [43]. VEGF is important indirect supporter of CM differentiation [6] and regulator of Flk1 phosphorylation. We also observed that AT25 iPSC cultures with successful cardiac induction by AA had increased numbers of Flk1-positive cells (unpublished data). Thus, inhibition of VEGF pathway by dorsomorphin may explain why this substance did not promote cardiogenesis in our cell lines. We demonstrate that in ESC line CGR8 the AA is capable of inducing cardiac differentiation in a highly consistent and efficient manner, which was less frequently observed with α PIG44 ESC and AT25 iPSC lines. Recent studies attribute the mechanism of action of AA to enhanced collagen synthesis or pro-oxidant properties of AA in culture [7, 37, 38]. Cao et al have shown that AA (vitamin C) applied during day 2-10 of murine iPSC differentiation increases proliferation of cardiac progenitor cells via the MEK-ERK1/2 pathway through promoting collagen synthesis. Sato et al demonstrated the same effect in murine ESC [38]. Bartsch and coworkers demonstrated that AA enhanced cardiac differentiation by upregulation of the NADPH oxidase isoforms NOX2 and NOX4, phosphorylation of endothelial nitric oxide synthase, and cyclic GMP formation, indicating that reactive oxygen species (ROS) as well as nitric oxide may be involved in cardiomyogenesis [37]. However, Crescini and coworkers did not detect any relationship between the redox activity of AA and enhanced CM differentiation in *Fgfr1*^{+/+}, *Fgfr1*^{-/-}, AA-treated *Fgfr1*^{-/-}, and hFGFR1-overexpressing *Fgfr1*^{-/-} murine ESCs [44]. In addition, AA was shown to stimulate the CM differentiation in EphB4 knockout EBs [45], thus suggesting that the compound is acting downstream of both FGFR1 and EphB4.

In order to reveal the mechanism of cardiogenic action of AA in a more detail we performed for the first time the global gene expression profiling of AA-treated and untreated CGR8 ESCs at different days of differentiation – on day 2 immediately after the completion of AA treatment and on days 3 and 5 of differentiation after further cultivation of EBs for 1 or 3 days in the absence of AA, respectively. When replicates of these same cultures were further incubated we could verify that AA robustly enhanced the percentage of beating EBs as well as the expression of α -actinin mRNA in these cultures. However, to our surprise, this analysis revealed only minor changes in gene expression profiles in EBs upon addition of AA. Microarray analyses performed with day 2 EBs suggest that AA induces the transcripts involved in regulation of oxidation-reduction and transcription and reduces the apoptotic

processes. The up-regulation of genes playing a role in oxidation-reduction corroborates other studies showing that ROS mediate stimulation of ESC-derived cardiomyogenesis [46]. However, we could not detect the induction of transcripts for NADPH oxidase isoforms NOX2 and NOX4 that were suggested to mediate the AA enhanced cardiac differentiation of murine ESCs [37].

Further analyses with day 3 EBs that were treated with AA for the first 2 days of differentiation and then cultivated for one day in the absence of AA showed even more reduced changes in gene expression profiles between treated and untreated EBs. This is surprising, because the number of differentially expressed genes increased again on day 5 of differentiation. Most likely the AA induced subtle changes that were seen in day 2 EBs require some time to cause changes in gene expression profiles that ultimately result in induction of cardiac specific transcripts and subsequently higher CM yields at later days of differentiations. It is possible that AA first acts at the epigenetic level. It was shown previously that AA can cause epigenetic remodeling in human ESCs and induce broad demethylation of genes that are associated with cancer, cellular growth, proliferation and tissue development [47, 48]. In addition, AA was shown to accelerate and increase somatic cell reprogramming with exogenous factors by overcoming cell senescence through enhancement of histone demethylase activity and promotion of cell cycle [49, 50]. Blaschke and coworkers also showed that AA induces Tet-dependent DNA demethylation and a blastocyst-like state in ESCs [51]. In our microarray analysis we observed that epigenetic regulators, such as DNA methyltransferase 3A (*Dnmt3a*), inhibitor of EP300 and CBP histone acetyltransferase activity (*Eid1*), high mobility group AT-hook 2 (*Hmga*), polycomb group ring finger 6 (*Pcgf6*) and muscleblind-like splicing regulator 1 (*Mbnl1*) that is involved in regulation of cardiac troponin-T (TNNT2) pre-mRNA processing, were among the most up-regulated genes in day 2 AA-treated group. Most likely, these factors were responsible for up-regulation of genes that were detected as differentially expressed in AA-treated group on day 5 of differentiation. For example, we have detected increased expression of collagen genes *Col1a2* and *Col5a2* while the expression of *Col4*, which has been shown to facilitate the development of cardiovascular Flk1-positive cells from murine ESCs [52], remained unchanged. Thus this data support the conclusion from a recent study that AA facilitates cardiac differentiation through enhanced collagen synthesis [7, 38] which is known common effect of vitamin C action as its deficiency causes lack of extracellular collagen assembly, leading to scurvy. In addition, on day 5 of differentiation several mesodermal and cardiac-specific transcripts, such as *Mef2c*, *T-brachyury* and *Myl7*, were significantly increased in AA-treated day 5 EBs. The expression of cardiac muscle α -actin and *TNNT2* transcripts also showed tendency to be increased in AA-treated day 5 EBs but due to a variable signal in one replicate the difference did not reach statistical significance. On the other hand, we couldn't detect the increase of other known mesoderm inducing (*Mesp1*) and early cardiac markers (*Nkx2-5*, *myocardin*), indicating that AA exerts selective effect on promoting cardiac differentiation of ESCs. However, AA strongly up-regulated a number of other factors involved in transcriptional regulation in day 5 EBs. Of interest is also the up-regulation by AA in day 5 EBs of GO-terms erythrocyte differentiation, hematopoiesis, angiogenesis and blood vessel development. This may be the result of AA-mediated stimulation of these processes independently of its cardioinductive properties but it is also possible that these share common pathways that act in a specific molecular context to promote differentiation of different cell types toward different specific cell lineages. Indeed, media used for ESC differentiation to hematopoietic precursors regularly contain ascorbic acid in addition to other specific factors. In addition, Chen and coworkers showed that endothelial cells regulate CM development from murine ESCs [45].

Although AA strongly and reproducibly induced cardiac differentiations of murine CGR8 ESCs in this study, its effect on other murine ESC lines was less potent and consistent, suggesting that intrinsic molecular constellation and cell culture conditions exert a strong modifying effect on the ability of an ESC line to differentiate to CM lineage. We demonstrate that QS11, a synergist of Wnt/ β -catenin pathway and inhibitor of ARFGAP, GTPase activating proteins that regulate ADP-ribosylation factors (ARF) [16], increases the efficiency of

cardiac differentiation and has enhancing effect in all three ESC lines when applied at days 4-9 of differentiation after initial treatment with AA. As QS11 is shown to synergise with Wnt3a to activate canonical Wnt/ β -catenin signaling we assume that its activity in our system is mediated through this pathway [53]. However, it is also possible that QS11 also modulates other biological processes mediated by ARFGAPs in PSC-derivatives. It has been shown also that inhibition of canonical Wnt/ β -catenin pathway in murine ESCs can induce cardiac differentiation [13]. Active Wnt/ β -catenin pathway is also required for successful cardiac differentiation in P19 teratocarcinoma cell line [10]. On the other hand, activation of Wnt/ β -catenin by BIO during later stages of differentiation induced the proliferation of Isl1- and Nkx2.5-positive cardiac progenitors and increases cardiac differentiation efficiency [53]. Activation of Wnt/ β -catenin pathway by recombinant Wnt3a ligand induced cardiac differentiation in human ESC line [12]. However, precise role of this pathway is still unclear, since development of another population of cardiovascular progenitors, Flk1⁺/CXCR4⁺/VE-cadherin⁺, is inhibited by Wnt3a and redirected towards hematopoietic lineage and application of Dkk-1 was shown to promote cardiac differentiation of Flk1⁺/CXCR4⁺/VE-cadherin population [52, 54, 55]. Mesp1-positive earliest cardiac progenitor cells were shown to differentiate towards cardiac lineage thorough Dkk-1 mediated inhibition of Wnt/ β -catenin pathway in murine ESCs [56]. Small molecule inhibitors of Wnt/ β -catenin pathway were shown to efficiently induce cardiac mesoderm population from human ESCs and iPSCs [40, 57, 58]. Synergetic cardiac enhancing effect of BMP-4 together with IWR-1, which acts as an Wnt-inhibitor, points out toward a cross-talk between these signaling pathways in cardiac differentiation of human ESCs [59]. In this study QS11 had no effect on cardiac differentiation of murine PSCs when applied during days 0-2 or days 0-9 but it enhanced the cardiogenesis when applied on later stages of differentiation (days 4-9) albeit with variable efficiency. This indicates that QS11 promotes cardiomyogenesis after the stage of mesodermal progenitor development and is in agreement with the reported biphasic effect of Wnt/ β -catenin pathway on *in vivo* cardiac development and *in vitro* cardiogenesis [60]. Previously, it has also been shown that AA regulates ubiquitination-mediated proteosomal degradation pathway [61], which indicates that the effect of AA on ESC cardiomyogenesis also may occur through influence on protein stability rather than gene expression level. Thus, it would be interesting to perform more detailed proteome studies in order to elucidate whether AA acts at the post-translational level by affecting the stability or modifications of specific proteins in CMs or neighboring cells that support their development.

In conclusion, we demonstrate that the cardiogenic potential of some previously reported compounds is difficult to reproduce and that different murine PSC lines exhibit different capacity to reproducibly respond to small molecule inducers of cardiogenesis. We also show that the combined use of AA and QS11 enhanced cardiac differentiation of murine ESCs and iPSCs although still with high batch-to-batch variability in CM yield. We revealed for the first time that murine ESCs show graded transcriptional response to AA with different biological processes being affected at different stages of differentiation and different days upon withdrawal of AA. Most evident response to AA that was administered for the first two days of differentiation was visible in day 5 EBs and was accompanied by up-regulation not only of cardiac-specific transcripts but also of transcription factors involved in angiogenesis, hematopoiesis and vasculature development. Increasing our understanding of the mechanism of action of cardiogenic compounds and combined use of small molecules acting on different signaling pathways at different time windows during differentiation may allow production of sufficient amounts of CMs for research, drug screening and potential therapeutic applications in regenerative medicine.

Acknowledgements

This work was funded by the Federal Ministry of Education and Research to T.Š. (grant 01GN0947), Köln Fortune Program to T.Š., and the Seventh Framework Program of the European Commission to J.H. (grant 222995).

Disclosure Statement

Authors have no conflicts of interest.

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