Cardiomyogenesis of embryonic stem cells upon purinergic receptor activation by ADP and ATP

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Abstract Purinergic signaling may be involved in embryonic development of the heart. In the present study, the effects of purinergic receptor stimulation on cardiomyogenesis of mouse embryonic stem (ES) cells were investigated. ADP or ATP increased the number of cardiac clusters and cardiac cells, as well as beating frequency. Cardiac-specific genes showed enhanced expression of α -MHC, MLC2v, α -actinin, connexin 45 (Cx45), and HCN4, on both gene and protein levels upon ADP/ATP treatment, indicating increased cardiomyogenesis and pacemaker cell differentiation. Real-time RT-PCR analysis of purinergic receptor expression demonstrated presence of P2X1, P2X4, P2X6, P2X7, P2Y1, P2Y2, P2Y4, and P2Y6 on differentiating ES cells. ATP and ADP as well as the P2X agonists β , γ -methylenadenosine 5'-triphosphate (β , γ -MetATP) and 8-bromoadenosine 5'-triphosphate (8-Br-ATP) but not UTP or UDP transiently increased the intracellular calcium concentration ($[Ca^{2+}]_i$) as evaluated by the calcium indicator Fluo-4, whereas no changes in membrane potential were observed. $[Ca^{2+}]_i$ transients

induced by ADP/ATP were abolished by the phospholipase C- β (PLC- β) inhibitor U-73122, suggesting involvement of metabotropic P2Y receptors. Furthermore, partial inhibition of $[Ca^{2+}]_i$ transients was achieved in presence of MRS2179, a selective P2Y1 receptor antagonist, whereas PPADS, a non-selective P2 receptor inhibitor, completely abolished the $[Ca^{2+}]_i$ response. Consequently, cardiomyocyte differentiation was decreased upon long term co-incubation of cells with ADP and P2 receptor antagonists. In summary, activation of purinoceptors and the subsequent $[Ca^{2+}]_i$ transients enhance the differentiation of ES cells toward cardiomyocytes. Purinergic receptor stimulation may be a promising strategy to drive the fate of pluripotent ES cells into a particular population of cardiomyocytes.

Keywords Cardiomyocyte differentiation · Purinergic receptors · Intracellular $[Ca^{2+}]_i$ signaling · Embryonic stem cell · ATP · ADP

Introduction

Differentiation of ES cells to the cardiac lineage provides a unique system to investigate mechanisms and genes involved in the earliest steps of cardiomyogenic differentiation [1]. Various substances and stimuli have been used to enhance in vitro cardiomyogenesis from ES cells. These include erythropoietin [2], oxytocin [3], retinoic acid [4], ascorbic acid [5], fibroblast growth factor-2 (FGF-2), transforming growth factor- β (TGF- β) [6], insulin-like growth factor (IGF) [7], bone morphogenic proteins (BMPs) [8], and low concentrations of reactive oxygen species (ROS) [9–14]. Although cardiomyogenic differentiation was substantially increased by different stimuli, a universal recipe for efficient and large-

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scale generation of cardiomyocytes from ES cells is still elusive [15]. Emerging evidence points toward the role of $[Ca^{2+}]_i$ as a major second messenger in directing the fate of ES cells into cardiomyocytes, patterning of the heart during early heart development and regulation of cardiac transcription cascades and myofibrillogenesis [16, 17]. Intracellular $[Ca^{2+}]_i$ exerts multiple functions in the process of cardiac cell differentiation and early heart development depending upon the amplitude, space, and time of $[Ca^{2+}]_i$ signaling. Decoding the molecular mechanism of the multifaceted roles of $[Ca^{2+}]_i$ signaling will therefore not only contribute to decipher molecular pathways of cardiogenesis but also help to elaborate protocols for highly efficient cardiomyocyte differentiation from ES cells [18–21]. During embryonic heart formation, a couple of cardiogenic factors turn on Ca^{2+} -dependent signaling pathways in cardiac progenitor cells. All cardiogenic factors are integrated into transcriptional networks, among which $[Ca^{2+}]_i$ plays a principal role [16, 17, 22]. Purinergic receptors, also known as purinoceptors, belong to the first group of receptors which stimulate intracellular $[Ca^{2+}]_i$ signaling during the early time of embryonic development as well as cardiogenesis [23–25]. Extracellular nucleotides (purines and pyrimidines) activate different subtypes of purinergic receptors. These receptors are classified based on their agonist specificity and pharmacological properties in P1 and P2. Metabotropic P1 receptor subtypes are selective for adenosine while P2 receptors are principally activated by ATP and ADP and are subdivided into P2X and P2Y subtypes based on their structural characteristics. P2X receptors are ATP-activated ligand-gated cationic channels, unselectively permeable to Na^+ , K^+ , and Ca^{2+} . The mammalian G-coupled metabotropic P2Y receptor family contains eight receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14) based on phylogenetic similarity [25, 26]. P2Y receptors are stimulated by a range of purine and pyrimidine nucleotides (ATP, ADP, and to a lesser extent UTP, UDP, or UDP-glucose) [23, 26, 27]. The P2Y1 receptor is a major subtype expressed generally in all cell types, and it mainly involves in regulating cell proliferation and differentiation. Interestingly, the principal physiological agonist for P2Y1 is ADP rather than ATP [25, 28].

Purinoceptor signal transduction is mediated through various numbers of signaling mediators to exert unique roles in cells [29, 30]. Metabotropic P2Y receptors are mostly coupled to Gq/11 proteins activating phospholipase C- β (PLC- β) and the phosphoinositide cascade, with subsequent IP3 formation and $[Ca^{2+}]_i$ release from intracellular stores. $[Ca^{2+}]_i$ mobilization plays a central role in the regulation of gene transcription. For instance translocation of Mef2c, a cardiac-specific transcription factor, into the nucleus is a calcium-dependent process in the regulation of cardiac cell differentiation [22, 25, 27].

While short-term purinergic signaling is involved in events such as neurotransmission, neuromodulation, secretion,

chemoattraction, acute inflammation, and platelet aggregation, long-term (trophic) signaling regulates cell proliferation, differentiation, migration and death in embryonic development, regeneration, homeostatic maintenance of tissue remodeling, cancer and aging [23, 26, 27, 30–32]. Dysregulation of purinergic signaling contributes to pathological conditions in different organs [32]. In the heart, enhanced stimulation of purinergic signaling involves in pathological conditions such as pressure or volume overload, ischemia, or myocardial infarction [33]. Although acute stimulation of purinergic signaling contributes to cardiac remodeling and wound healing following myocardial infarction, dysregulation of this signaling pathway may play a key role in cardiac fibrosis (i.e., excessive scarring and stiffness) [33, 34]. Considering the importance of purinergic signaling in stem cell proliferation, differentiation, and recruitment, we were interested to evaluate the role of purinergic receptor stimulation mediated by ADP/ATP in ES cells as an *in vitro* cellular model for cardiac cell commitment.

Materials and methods

Culture of ES cells

The ES cell line CGR8 was derived from the inner cell mass of preimplantation blastocyte-stage mouse male embryos of the strain 129 (ECACC, UK). Undifferentiated CGR8 cells were maintained on 0.1 % gelatine-coated tissue flasks without feeder cells in Glasgow minimal essential medium (GMEM) (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 10 % heat-inactivated (56 °C, 30 min) fetal bovine serum (Sigma-Aldrich, Taufkirchen, Germany), 1.8 mM L-glutamine (Biochrom, Berlin, Germany), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich), and 10^3 U/ml leukemia inhibitory factor (LIF) (Chemicon, Hampshire, UK) in a humidified environment containing 5 % CO_2 at 37 °C. ES cells were differentiated *in vitro* into three-dimensional multicellular structures termed embryoid bodies (EBs) using spinner flask bioreactors (Integra Biosciences, Fernwald, Germany) [35]. In brief, 1×10^7 cells were seeded into a siliconized spinner flask containing 125-ml Iscove's medium supplemented with the same additives as described above, but devoid of LIF. The spinner flask medium was stirred at 22 rpm using a stirring system (Integra Biosciences).

Induction of cardiomyogenesis upon purinergic receptor stimulation

Upon day 5 of differentiation Iscove's medium (without LIF) was supplemented with either 5 μ M ADP or 10 μ M ATP until day 15, and cells were further cultivated until day 18. Treatment with ATP/ADP was performed twice per day (every 12 h). During the differentiation time, the number and

frequency of contracting clusters were evaluated at days 10, 15 and 18 using a Zeiss Telaval 31 inverted microscope (Carl Zeiss Jena, Germany). P2 receptor antagonists, i.e., MRS2179 (100 μ M), PPADS (100 μ M), and TNP-ATP (100 μ M) were applied between day 5 and day 15. The chronotropic response of cardioactive drugs including 10 μ M propranolol as a negative and 10 μ M epinephrine or atropine, as positive chronotropic agents was investigated in contracting clusters of differentiated 18-day-old cardiomyocytes and control groups.

Intracellular $[Ca^{2+}]_i$ signal imaging

$[Ca^{2+}]_i$ was measured in 5-day-old single cells loaded with fluo-4, AM (Invitrogen, Darmstadt, Germany). Briefly, 4-day-old EBs were enzymatically dissociated using collagenase II (2 mg/ml) (Boehringer Ingelheim, Germany) and were seeded at a density of 20,000 cells/well on coverslips coated with 0.1 % gelatine in 24-multiwell tissue culture plates. Twenty-four hours later, cells were incubated with 2 μ M fluo-4, AM in serum-free GMEM at 37 °C for 30 min. Dye-loaded cells were washed twice with Tyrode buffer containing (in mM) $CaCl_2$ 2, NaCl 135, KCl 6, Na_2HPO_4 0.33, Na^+ pyruvate 5, $MgCl_2$ 1, glucose 10 and HEPES 10 (pH 7.4). In some experiments, $[Ca^{2+}]_i$ free conditions were ensured with tyrode buffer containing 0.5 μ M EGTA instead of $CaCl_2$. $[Ca^{2+}]_i$ imaging was performed using a confocal laser scanning microscope (cLSM 510, Carl Zeiss Jena, Germany), equipped with an argon ion laser (458, 477, and 488 nm). Different concentrations of ADP, ATP, UDP, UTP, β , γ -MetATP, and 8-Br-ATP as indicated were applied on cells and the respective $[Ca^{2+}]_i$ response was detected. To unravel molecular mechanisms of $[Ca^{2+}]_i$ signaling triggered by ADP or ATP, $[Ca^{2+}]_i$ imaging was performed in presence of different pharmacological substances (30 min at 37 °C), i.e., U-73211 (1 μ M), clotrimazol (10 μ M), apamin (100 nM), PPADS (100 μ M), MRS2179 (100 μ M), and NTP-ATP (100 μ M).

Intracellular $[Ca^{2+}]_i$ signals were recorded during times as indicated, and the amplitude and duration of signals were analyzed for single responding cells. The amplitude of the $[Ca^{2+}]_i$ signal stimulated with ADP/ATP was determined as followed: amplitude (I) = maximum fluorescence intensity (I_{max}) – resting fluorescence (I_{rest}). Resting fluorescence (I_{rest}) of a single cell was obtained as an average of intensity recorded during a period of 10 s prior to application of the stimulating substance on the cells. For calculation of the signal duration (π), two time points of the $[Ca^{2+}]_i$ signal reaching $1/e$ of the maximum amplitude intensity were selected, and the time difference between these two points was defined as signal duration. Of note, the number e is Euler's number.

Di-8-ANEPPS membrane potential imaging

Cells were loaded with Di-8-ANEPPS (2 μ M) (Life Technologies, Darmstadt, Germany) in serum-free medium for 25 min at 37 °C. Cells were washed with tyrode buffer and incubated with dye-free tyrode buffer for 10 min at 37 °C prior to cLSM examination, and then excited at 488 nm with the argon laser. Membrane potential-dependent fluorescence emission of Di-8-ANEPPS was measured by two spectral bands: band pass filter (BP) 530–600 nm; low pass filter (LP) >615 nm. The ratio signal was calculated as follows: ratio signal = BP signal / LP signal [36, 37].

RNA isolation and RT-PCR

Total RNA was extracted from cells at days 5, 7, 10, 15, and 18 with 0.5 ml TRIzol[®] reagent according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μ g total RNA using the M-MLV reverse transcriptase and random primer (Invitrogen GmbH, USA) method. Semiquantitative RT-PCR was performed using 1 μ l of synthesized cDNA, 5 μ l of Red Load Taq Master 5 $\times$ (Jena Bioscience, USA), 2 μ l of the primer mix consisting of 240 nM of forward and reverse primers and 17 μ l of RNase- and DNase-free water by following conditions: initial denaturation at 95 °C for 3 min and per cycle: 30 s for 95 °C, 40 s at the respective annealing temperature and 72 °C for 30 s, finalized by extension at 72 °C for 5 min. The primers were designed by following sequences (Sigma Geonysis, Germany): α -MHC, sense 5'-CTG CTG GAG AGG TTA TTC CTC G-3', antisense 5'-GGA AGA GTG AGC GGC GCA TCA AGG-3'; MLC2v, sense 5'-AAA GAG GCT CCA GGT CCA AT-3', antisense 5'-CCT CTC TGC TGT GTG GTC A-3'; HCN4, sense 5'-GAC AGC GCA TCC ATG ACT AC-3', antisense 5'-ACA AAG TTG GGA TCT GCG TT-3'; cTnI, sense 5'-TAA GAT CTC CGC CTC CAG CC-3', antisense 5'-CGG CAT AAG TCC TGA AGC TC-3'; RyR2, sense 5'-GAC GGC AGA AGC CAC TCA CCT GCG-3', antisense 5'-CCT GCA GAG AAA CTG ACA ACT GGA-3'; Cx45, sense 5'-GGC AGC TCG GAG CAA ACC T-3', antisense 5'-TCC TGG CCA GCA GCT GCA AC-3'; Cx30.2, sense 5'-GCT ACA GTC GCC GCT CGT GG-3', antisense 5'-GCC TCC TTG CTG GCC TGG TG-3'; Polr2a, sense 5'-GAC AAA ACT GGC TCC TCT GC-3', antisense 5'-GCT TGC CCT CTA CAT TCT GC-3'. The PCR products were separated by electrophoresis on a 1.5 % agarose gel containing 1 $\times$ TBE buffer and 1 mg/ml ethidium bromide.

Semiquantitative real-time RT-PCR analysis was done to identify messenger RNA (mRNA) expression of P2Y and P2X receptor subtypes in 5–18-day-old cells by using the QuantiFast SYBR Green kit followed by an initial denaturation step at 95 °C for 10 min and 50 cycles at 95 °C for 15 s and 62 °C for 1 min with following

primers [38]: P2X1, sense 5'-GAG AGT CGG GCC AGG ACT TC-3', antisense 5'-GCG AAT CCC AAA CAC CTT CA-3'; P2X4, sense 5'-CCC ACT GCC TGC CCA GAT AT-3', antisense 5'-ACA CTC ACC AAG GCA TAT GG-3'; P2X6, sense 5'-CCC AGA GCA TCC TTC TGT TCC-3', antisense 5'-GGC ACC AGC TCC AGA TCT CA-3'; P2X7, sense 5'-GCA CGA ATT ATG GCA CCG TC-3', antisense 5'-CCC CAC CCT CTG TGA CAT TCT-3'; P2Y1, sense 5'-CGT GCT GGT GTG CCT CAT T-3', antisense 5'-GGA CCC CGG TAC CTG AGT AGA-3'; P2Y2, sense 5'-TTC CTG CCA TTC CAC GTC A-3', antisense 5'-TTG AGG GTG TGG CAG CTG A-3'; P2Y4, sense 5'-TTG AGG GTG TGG CAG CTG A-3', antisense 5'-TGT CCT TTT CCT CAC CTG CAT-3'; P2Y6, sense 5'-CCT GCC CAC AGC CAT CTT-3', antisense 5'-GGC TGA GGT CAT AGC AGA CAG TG-3'. Experiments were performed in triplicate for each data point. Relative expression values were obtained by normalizing CT values of the P2Y receptor genes in comparison with CT values of the housekeeping gene.

Immunocytochemistry

Differentiated cardiac cells were identified by immunohistochemistry using a monoclonal anti-sarcomeric α -actinin (clone EA-53, antibody (Sigma-Aldrich, Germany)). Cells were fixed in methanol/acetone (7:3) at -20°C . Samples were incubated with permeabilization solution PBS-Triton-X100 (0.1 %) for 5 min [39]. After washing with PBS-Triton-X100 0.01 % ($3\times$ for 5 min), unspecific binding sites were blocked with 10 % milk powder solution in PBS for 60 min at room temperature. Subsequently, samples were incubated overnight with primary antibody at 1:150 dilution. Then, samples were washed ($3\times$ for 5 min) and incubated with the appropriate fluorescence-labeled secondary antibody at 1:200 dilution (60 min at room temperature). Finally, cell nuclei were stained with the fluorescence dye DAPI at 1:2500 dilution (10 min at room temperature). The expression of specific protein was evaluated by cLSM.

Flow cytometry analysis

Upon day 5 of differentiation, EBs were treated twice a day with either 5 μM ADP or 10 μM ATP until day 15, washed twice with prewarmed PBS and incubated for 10 min at 37°C with collagenase B (2 mg/ml) (Roche, Grenzach-Wyhlen, Germany). After dissociation by gentle pipetting and passing through a 40- μm cell strainer, single-cell suspensions were centrifuged at 400g for 5 min at 4°C , resuspended at 10^6 cells/ml in precooled PBS containing 2 % fetal calf serum and 2 mM EDTA and stained for 20 min at 4°C with anti α -actinin antibody (Sigma-Aldrich). As secondary antibody, Alexa488 anti-mouse (Life Technologies, Darmstadt,

Fig. 1 Purinergic receptor agonists transiently increase $[\text{Ca}^{2+}]_i$ in differentiating ES cells. **a** Quantitative evaluation of the percentage of cells reacting to different concentrations of ADP and ATP with intracellular $[\text{Ca}^{2+}]_i$ transients. Experiments were recorded in Ca^{2+} -containing ($+\text{[Ca}^{2+}]_e$) tyrode buffer ($n=5$, $*p<0.05$). **b** Representative traces of $[\text{Ca}^{2+}]_i$ transients in cells evoked by 5 μM ADP and 10 μM ATP to illustrate the average of signal amplitude and signal time duration (π). **c** Changes in intracellular fluorescence (in %) of fluo-4 mediated by ADP and ATP in $[\text{Ca}^{2+}]_i$ -containing ($+\text{[Ca}^{2+}]_e$) and Ca^{2+} -free ($-\text{[Ca}^{2+}]_e$) buffers to reveal whether the nucleotide-mediated intercellular $[\text{Ca}^{2+}]_i$ response is originating from intracellular or extracellular sources ($n=5$ for $+\text{[Ca}^{2+}]_e$ and $n=3$ for $-\text{[Ca}^{2+}]_e$) buffers. **d** Percentage of cells showing $[\text{Ca}^{2+}]_i$ signals in presence of SK_{Ca} and IK_{Ca} channel inhibitors. Cells were incubated with 2 μM fluo-4 as well as either 10 μM clotrimazole or 10 nM apamin for 30 min. Bars show the means $\pm$ SEM for $n=3$, $*p<0.05$. $[\text{Ca}^{2+}]_i$ transients in cells treated with either the P2X receptor agonist $\beta,\gamma\text{MetATP}$ (10 μM) (**e**) or 8-Br-ATP (10 μM) (**f**). In **g**, the percentage of cells showing $[\text{Ca}^{2+}]_i$ signals in presence of different concentrations of P2X agonists is shown ($n=3$). Representative traces of $[\text{Ca}^{2+}]_e$ signal in presence of **h** U-73211, a PLC- β inhibitor; **i** MRS2179, a selective P2YRs inhibitor; **j** PPADS, a non-selective P2YRs inhibitor; and **k** TNP-ATP, a selective P2XRs inhibitor after stimulating cells with 5 μM ADP. Cells were preincubated with 2 μM fluo-4 and inhibitor in $[\text{Ca}^{2+}]_e$ buffer ($n=4$). The arrows indicate the time point of ADP application

Germany) was applied. In each experiment, IgG-matched isotype controls were used. Data were acquired by FACSCaliburTM instrument (BD) and BD CellQuest Pro software, and analyzed by Flowing software (Turku Center of Biotechnology, Finland). Data are shown as number of positive-stained cells per total counted events in each experiment.

Western blot analysis

Samples were washed with PBS and lysed in 300 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 1 % Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 10 $\mu\text{g/ml}$ aprotinin, 5 $\mu\text{g/ml}$ pepstatin A, 25 $\mu\text{g/ml}$ leupeptin, 1 mM sodium vanadate) on ice for 30 min. High-power ultrasound was used to improve the cell lysis process and releasing intracellular compounds. Samples were kept on ice for 30 min at RT, and then the cell lysate was centrifuged at 10,000g (4°C) for 10 min to remove the cell debris. Protein concentration was determined with spectroscopic Bradford protein assay. A total of 30 μg protein per sample was heated (95°C for 6 min) with SDS-PAGE loading buffer, separated in SDS polyacrylamide gels, transferred to a nitrocellulose transfer membrane (pore size 0.45 μm) and blotted at 22 mV for 3–4 h using the wet transferring method. Then, the membrane was blocked with 12 % (w/v) dry fat-free milk powder in 0.1 % PBS-Tween for 8 h at 4°C . The following primary antibodies were used and incubated for overnight at 4°C : monoclonal anti- α -actinin (sarcomeric) (clone EA-54); monoclonal anti-MLC2v (clone MLM527); monoclonal anti-

Fig 1. (continued)

Cx43 as well as polyclonal anti-GAPDH. Subsequently, the membrane was washed with 0.1 % PBS-Tween buffer. For the secondary antibody reaction, the membrane was incubated with the appropriate horseradish peroxidase (HPR)-conjugated secondary antibody (Santa Cruz Biotechnology) at room temperature for 2 h. Corresponding bands were visualized using the Amersham ECL Western blotting detection kit and images captured by a digital imaging system. Quantification of chemoluminescence signals was performed by Quantity One 1-D Analysis Software (Biorad, Munich, Germany). Intensity for each protein was normalized to the intensity of GAPDH as a housekeeping protein. Relative variation for the intensity of specific proteins to housekeeping protein was compared between control and substance-treated samples.

Statistical analysis

Data in the figures are given as mean value $\pm$ SEM with “*n*” indicating the number of experiments. Statistically significant data were identified using one-way ANOVA with “Student-Newman-Keuls method” for multiple comparisons among control, as well as ADP- and ATP-treated sample groups. Significant findings are labeled with one star ($*p<0.05$) in graphical representations.

Results

ADP/ATP-mediated intracellular $[Ca^{2+}]_i$ transients

To determine the optimal concentration of extracellular ADP and ATP for in vitro stimulation of purinergic receptors, a possible physiological concentration range from 10 nM to 10 μ M of the intended nucleotides was applied on cells at day 5 of differentiation [40, 41]. For each concentration of ADP or ATP, the percentage of positive cells showing detectable $[Ca^{2+}]_i$ transients was calculated. A concentration of 5 μ M ADP and 10 μ M ATP showed the maximum percentage of positive cells approaching 35 ± 2 and 30 ± 5 % of the cell population for ADP and ATP, respectively (Fig. 1a). The means $\pm$ SEM of amplitude and time duration (π) of the $[Ca^{2+}]_i$ signal for ADP (10 μ M) were 470 ± 22 % and 20 ± 0.24 s, respectively. For ATP, the signal amplitude was 420 ± 85 % and the signal duration was 20 ± 0.14 s. Statistical analysis showed that there was no significant difference regarding amplitude and duration of the $[Ca^{2+}]_i$ response elicited by either ADP or ATP. Figure 1b shows representative traces of the intracellular $[Ca^{2+}]_i$ transient in cells evoked by 5 μ M ADP and 10 μ M ATP, respectively

To clarify whether the $[Ca^{2+}]_i$ response originated from an extracellular influx of Ca^{2+} or efflux from intracellular stores

(endoplasmic reticulum) into the cytoplasm, experiments were performed in nominally Ca^{2+} -free tyrode solution. The results show that $[\text{Ca}^{2+}]_i$ signals triggered with 5 μM ADP or 10 μM ATP may result mainly from intracellular $[\text{Ca}^{2+}]_i$ release since Ca^{2+} -free conditions did not significantly reduce the percentage of cells responding to ATP and ADP as compared to Ca^{2+} -containing buffer (Fig. 1c).

To decipher the molecular mechanism involved in ADP- or ATP-stimulated $[\text{Ca}^{2+}]_i$ transients, different pharmacological inhibitors were applied on cells and the properties of $[\text{Ca}^{2+}]_i$ responses were evaluated. Since purinoceptor-mediated $[\text{Ca}^{2+}]_i$ signaling interferes with activation of Ca^{2+} -activated potassium channels, intermediate- and small-conductance K_{Ca} channels were inhibited during $[\text{Ca}^{2+}]_i$ imaging (Fig. 1d). Intermediate-conductance K_{Ca} (IK_{Ca}) channels were inhibited with clotrimazole (10 μM) and small-conductance K_{Ca} (SK_{Ca}) were blocked selectively by the bee venom peptide apamin (100 nM). The quantitative evaluation of positive cells showed that in presence of clotrimazole, 24 ± 2 and 23 ± 3 % of cells responded to ADP and ATP treatment, respectively, which was not significantly

different to treatment with nucleotides in the absence of inhibitor. Moreover, the $[\text{Ca}^{2+}]_i$ transients elicited by ADP or ATP were not significantly inhibited in presence of 10 μM apamin.

P2X receptors may be involved in $[\text{Ca}^{2+}]_i$ signaling of differentiating ES cells. Therefore the P2X receptor agonists 8-Br-ATP (Fig. 1e) and β, γ -MetATP (Fig. 1f) were applied in concentrations of 1, 10, 50, and 100 μM . Both applied P2X agonists elicited $[\text{Ca}^{2+}]_i$ transients and dose-dependent increased the number of responding cells (Fig. 1g). The means $\pm$ SEM of amplitude and time duration (π) of the $[\text{Ca}^{2+}]_i$ signal for β, γ -MetATP (10 μM) were 205 ± 30 % and 60 ± 2 s, respectively. For 8-Br-ATP, the signal amplitude was 245 ± 13 % and the signal duration was 52 ± 9 s.

P2Y receptors activate PLC- β through G-proteins leading to hydrolysis of PIP2 and IP3-mediated release of $[\text{Ca}^{2+}]_i$ from internal stores. To confirm the involvement of PLC- β in the $[\text{Ca}^{2+}]_i$ signaling cascade, U-73122, a known PLC- β inhibitor, was applied. Intracellular $[\text{Ca}^{2+}]_i$ mobilization elicited with ADP was completely abolished in presence of U-73122 (1 μM),

Fig. 2 Effects of ADP/ATP and EBIO on the membrane potential of differentiating ES cells. **a** Two sequences of confocal images (recorded after 15, 25, 35, and 100 s) indicating changes in membrane potential upon treatment with 100 μM EBIO (as positive control) and ADP. The upper panel demonstrates changes in fluorescence intensity upon stimulation of cells with EBIO, indicating membrane potential hyperpolarization. The lower panel indicates that no changes in

fluorescent intensity occurred upon ADP stimulation. Bar=50 μm . **b** Representative traces of signal ratio intensity of Di-8-ANNEPS upon treatment with either 100 μM ADP, ATP, or EBIO. The illustration shows no detectable changes in membrane potential subsequent to ADP or ATP stimulation. Arrows mark the time point of applying substances ($n=4$)

indicating involvement of IP₃ mediated intracellular $[Ca^{2+}]_i$ release upon purinergic receptor stimulation (Fig. 1h). Comparable results were achieved when ATP was applied in presence of U-73122 (data not shown).

To unravel the type of purinergic receptors responding toward either ADP or ATP, receptor-specific pharmacologic inhibitors were applied. After incubation of cells with MRS 2179 (100 μ M), a specific P₂Y₁ receptor antagonist, the amplitude of the $[Ca^{2+}]_i$ signal amplitude was reduced by 35 % (Fig. 1i), and 28 ± 4 % of cells displayed an intracellular $[Ca^{2+}]_i$ response following stimulation of cells with 5 μ M ADP. Comparable results were achieved with ATP (data not shown). In the presence of PPADS (100 μ M), a non-selective P₂-receptor antagonist (P₂X and P₂Y), the $[Ca^{2+}]_i$ signal triggered by either ADP or ATP was completely abolished (Fig. 1j). The amplitude of $[Ca^{2+}]_i$ transients in cells treated with either ADP or ATP in presence of TNP-ATP (100 μ M) as a selective P₂X receptor antagonist was not impaired (Fig. 1k).

To determine whether other purinergic receptor agonists would induce $[Ca^{2+}]_i$ signals in differentiating ES cells, UDP and UTP were applied on cells. It was observed that even high concentrations of UDP and UTP

(200 μ M) were not able to evoke intracellular $[Ca^{2+}]_i$ signals (data not shown).

Purinoreceptor-mediated $[Ca^{2+}]_i$ transients do not induce membrane potential changes

To investigate hyperpolarization subsequent to purinoreceptor-mediated $[Ca^{2+}]_i$ signals, membrane potential measurement was performed following stimulation of the cells with ADP or ATP. The K_{Ca} channel agonist EBIO was used as positive control of changes in membrane potential [36]. The potentiometric signal detection of Di-8-ANNEPS presented a stable fluorescent signal pattern following stimulation of cells with ADP as well as ATP, indicating that the membrane potential remained in a “steady-state” during purinoreceptor-induced $[Ca^{2+}]_i$ signaling. This finding was achieved even with high (100 μ M) concentrations of ADP or ATP. In contrast, when cells were stimulated with EBIO (100 μ M) as a positive control, a rapid drop in ratio-signal intensity of Di-8-ANNEPS was observed, indicating membrane hyperpolarization upon activation of K_{Ca} channels (Fig. 2a, b).

Fig. 3 Effects of ADP/ATP on differentiation of contracting cardiac clusters, cardiac cell number, and cardiac cell function. **a** Display of the number of beating clusters (%) after incubation with either 5 μ M ADP or 10 μ M ATP. **b** The bar charts show beating frequency upon incubation of cells with ADP and ATP ($n=6$), $*p<0.05$. **c** Increase in α -actinin-positive cardiac cell number as evaluated in flow cytometry experiments ($n=6$). **d** The chronotropic response toward epinephrine, atropine, and propranolol of differentiated cardiomyocytes at day 18. Beating frequency in beats per minute (bpm) was counted before and 2 min after drug administration ($n=3$); significance was tested against the control group; $*p<0.05$

Effect of ADP and ATP treatment on the number, frequency, and chronotropic response of contracting cardiac cells

To investigate the effects of purinergic receptor stimulation on the differentiation and function of cardiac cells, long-term

treatment of cells with either 5 μ M ADP or 10 μ M ATP was performed, and the number as well as beating frequency of cardiac cell clusters was analyzed from day 10 to day 18 by microscopic examination and compared with those in the control group. Spontaneously contracting clusters were first observed at day 8 in the ADP-treated group and 1 day later in

Fig. 4 ADP/ATP increase mRNA expression of cardiac-specific genes. Expression analysis of cardiac-specific gene including: α -MHC (a), c TnI (b), MLC2v (c), HCN4 (d), Cx45 (e), and Cx30.2 (f) by semiquantitative RT-PCR. The data were plotted (in ratio) in relation to the transcript level

at d5 and were normalized against the expression of Polr2a ($n=6$). Significance was tested against the control group; * $p < 0.05$. g Representative of gene PCR products for cardiac markers as indicated

cells treated with ATP. ADP (5 μ M) and ATP (10 μ M) significantly increased the number of beating clusters at day 10 (32 ± 5 and 26 ± 3 %, respectively) in comparison with the control group (18 ± 4 %). At day 15, the number of beating clusters was further increased, approaching 48 ± 3.5 , 38 ± 3 , and 23 ± 4 % in ADP, ATP, and control groups, respectively. At day 18, the number of beating clusters was decreased; however, ADP- and ATP-treated samples still displayed a higher rate of cardiomyogenesis as compared to the untreated control (Fig. 3a). The beating frequency increased within the course of differentiation. In general, ADP or ATP treatment significantly increased the beating frequency in comparison with the control group. For instance, at day 15, the frequency of beating increased from 56 ± 5 to 89 ± 6 and 79 ± 6 beats/min in control, ADP, and ATP groups, respectively (Fig. 3b). To verify that ATP/ADP treatment increased the number of differentiated cardiac cells, flow cytometry experiments were performed. Indeed ADP (5 μ M) and ATP (10 μ M) treatment increased cardiac cell numbers by approximately 60 % on days 10, 15, and 18 of differentiation as compared to the untreated control (Fig. 3c, supplemental Fig. 1).

To evaluate function of ES cell-derived cardiomyocytes, different types of chronotropic drugs were used as agonists or antagonists of adrenoceptors. In this study, 10 μ M epinephrine, 10 μ M atropine, or 10 μ M propranolol were applied at day 18 of differentiation, and the number of beats/min was counted before as well as 2 min after addition of the drugs to evaluate their chronotropic effect. Of note, adrenoceptors play an important role in regulating heart rate and heart function, and it has been reported that late stage (unlike early) ES cell-derived cardiomyocytes are responsive to β -adrenergic stimulation [20]. In presence of the β -receptor agonist epinephrine, contracting clusters in both experimental groups showed positive chronotropic effects, i.e., the beating frequency was increased (Fig. 3d). Furthermore, treatment with epinephrine activated contractions in silent, non-contracting cardiac areas. Propranolol, a non-selective β -adrenoceptor antagonist, exerted negative chronotropic effects on differentiated cardiomyocytes, leading to decrease or even to stop of contractions in both control and treated cells (Fig. 3d). Atropine (10 μ M) as an antagonist of muscarinic acetylcholine receptors did not evoke a positive chronotropic response. Of note,

Fig. 5 ADP/ATP increase protein expression of cardiac-specific markers. **a** Representative immunohistochemical images of double stainings for cell nuclei (blue) and α -actinin (green) in ES cell-derived cardiomyocytes treated with 5 μ M ADP at d5+10. The cell nuclei were stained with DAPI (blue). Scale bar=50 μ m. **b** Statistical analysis of the α -actinin-positive cell area at d5+10 of differentiation by

immunocytochemistry. **c** Representative Western blot images of α -actinin, CX43, and MLC2v protein expression at d5+10 of differentiation; GAPDH was used as housekeeping protein. **d** Bar chart of cardiac-specific protein expression in comparison with GAPDH expression ($n=3$); $*p<0.05$

cardiac clusters in ADP- or ATP-treated groups displayed a better response to these cardioactive drugs as compared to the untreated control group, suggesting differentiation toward a more mature type of cardiomyocytes.

Effect of ADP and ATP on cardiac-specific gene expression

To further underscore the pro-cardiogenic effects of ADP/ATP, gene expression of a panel of cardiac-specific genes was assessed. mRNA expression of α -MHC, a general marker of early cardiomyocyte differentiation, was significantly upregulated at day 10 and day 15 upon incubation with ADP. Likewise, ATP treatment strongly enhanced α -MHC ~2.5-fold from day 7 to day 15 (Fig. 4a, g). In contrast, the cTnI mRNA expression level was not significantly increased by ADP/ATP as compared to the untreated control (Fig. 4b, g). MLC2v, as an early marker of ventricular cells became strongly upregulated following 10 days of ADP/ATP treatment, indicating commitment of ES cells towards ventricular-like cardiomyocytes (Fig. 4c, g). The expression of HCN4, a main isoform of pacemaker channels, was prominently upregulated at the mRNA level not only at early but also at late days of differentiation in ADP- and ATP-treated cultures (Fig. 4d, g).

In addition to HCN4, the expression of the low conductance connexins, Cx45 and Cx30.2, which are preferentially expressed in adult pacemaker cells, was evaluated at the mRNA level. After 10 days of incubation of cells with ADP/ATP, a significant upregulation of Cx45 was observed (Fig. 4e, g). In contrast to HCN4 and Cx45 as pacemaker cell markers, ADP/ATP treatment did not increase the expression of Cx30.2 mRNA as compared to the untreated control (Fig. 4f, g).

Effect of ADP and ATP on cardiomyocyte-specific proteins

Immunohistochemical staining for α -actinin, a protein located in the Z-disc of cardiac cells and patterning cross striation of sarcomeres, showed a widespread and dense network of longitudinally oriented structures in ADP or ATP treated differentiating ES cells (Fig. 5a). Notably the size of cardiac areas was significantly increased in either ADP-treated ($19.3 \pm 1.8 \mu\text{m}^2$) or ATP-treated ($15.6 \pm 2.5 \mu\text{m}^2$) cells as compared to the untreated control ($3.7 \pm 0.9 \mu\text{m}^2$) (Fig. 5b). Western blot analysis showed that protein expression of MLC2v was significantly increased in cells treated with ADP and ATP (Fig. 5c, d); this result was in agreement with the mRNA expression of MLC2v (Fig. 4c, g). Moreover, protein expression of α -actinin was strongly enhanced, suggesting stimulation of cardiomyocyte differentiation upon ADP/ATP treatment of ES cells. The protein expression of Cx43, a specific cardiomyocyte gap junction protein, did not show significantly elevated expression in ADP and ATP groups at day 15 of differentiation (Fig. 5c, d).

Inhibition of cardiac-specific gene expression upon pharmacological interference with purinoceptors

The data of the present study demonstrated that cardiomyogenesis of ES cells was increased upon treatment with ADP. To corroborate these findings, ES cells were incubated with ADP in the presence of purinergic receptor antagonists. Long-term treatment of ES cells with ADP in presence of the P2Y receptor antagonists MRS2179 and PPADS apparently decreased cardiomyocyte differentiation. RT-PCR revealed that the expression of α -MHC (Fig. 6a, c), MLC2v (Fig. 6b, d), and HCN4 (Fig. 6f, h) mRNA was significantly downregulated in cells co-cultivated with ADP and PPADS from day 5 to 15 of differentiation, whereas downregulation of Cx45 remained non-significant (Fig. 6e, g). Moreover, mRNA expression of MLC2v and HCN4 at day 15 of differentiation was significantly decreased upon treatment of cells with ADP and MRS2179 (Fig. 6b, d, f, h). The selective P2X receptor antagonist TNP-ATP [42, 43] did not exert any significant effect on cardiac gene expression, indicating that P2X receptors may not be involved in stem cell differentiation toward cardiomyocytes (data not shown).

P2Y receptor expression profiling in differentiating ES cells

Real time RT-PCR evaluation of P2Y1, P2Y2, P2Y4, and P2Y6 receptors as well as P2X1, P2X4, P2X6, and P2X7, known to be expressed in mouse heart tissue [44] was performed from day 5 to 18. Expression of P2Y1, P2Y2, P2Y4, and P2Y 6 (Fig. 7a–d) and P2X1, P2X4, P2X6, and P2X7 (Fig. 7e–h) was confirmed during the time course of differentiation. The expression level of P2X and P2Y receptors did not significantly change during the time course of differentiation. Addition of ATP/ADP to the incubation medium did not significantly affect the expression level of P2X and P2Y receptors (data not shown).

Discussion

A number of peptide growth factors, cytokines, and chemical substances have been extensively studied to target different cell receptors and signaling pathways in ES cells in order to induce cardiac restricted transcription factors and subsequently differentiation of cells into distinct populations of cardiomyocytes [18]. Purinoceptors belong to those receptors which are widely expressed in early stages of ES cell differentiation and are involved in the regulation of several basic physiological processes including stem cell proliferation, differentiation, and recruitment [23, 25].

Considering the prominent role of Ca^{2+} as a cellular second messenger in directing the fate of stem cells towards cardiac

Fig. 6 Inhibition of ADP/ATP-mediated stimulation of cardiomyogenesis upon treatment with P2-receptor antagonists. mRNA expression analysis of cardiac specific gene including α -MHC (a, c), MLC2v (b, d), Cx45 (e, g), and HCN4 (f, h) by semiquantitative RT-PCR. Samples were treated with the purinoceptor antagonist MRS219 and PPADS in either presence or absence of 5 μ M ADP from d5 to d15. The data were plotted (in ratio) in relation to the mRNA expression level at d5 and were normalized against the expression of Polr2a ($n=4$); significance was tested against the control group; $*p<0.05$. The lower panel shows representative blots of gene PCR products for cardiac markers as indicated

Fig. 7 Evaluation of P2Y1, P2Y2, P2Y4, and P2Y6 (a–d) and P2X1, P2X4, P2X6, and P2X7 (e–h) receptor subtype mRNA expression in EBs during cardiomyogenesis. The expression of P2YRs was compared with the expression of Polr2s ($n=3$)

cells, targeted activation of $[Ca^{2+}]_i$ signaling in ES cells may confer great hope in stem cell biology and cardiac cell regeneration [19–21]. We postulated that one of the critical targets for induction of $[Ca^{2+}]_i$ signaling involved in cardiomyogenesis could be the activation of purinergic receptors. In this study, we focused on trophic effects of extracellular nucleotides on stem cell differentiation as well as their underlying molecular mechanisms.

P2Y receptors are stimulated by a range of purine and pyrimidine nucleotides [23, 27]. ADP/ATP as purines as well as UDP/UTP as pyrimidines were selected to evaluate $[Ca^{2+}]_i$ transients in 5-day-old cells. Different concentrations of ADP and ATP induced transient $[Ca^{2+}]_i$ signals, whereas UDP and UTP did not evoke $[Ca^{2+}]_i$ release even in high concentration (200 μ M). This observation showed that P2 receptors expressed on 5-day-old ES cells do use pyrimidines as principal agonists for receptor activation. Treatment of cells with ADP elicited $[Ca^{2+}]_i$ signals in a higher percentage of cells as compared with ATP which may indicate that ADP has a higher rank order potency compared to ATP (ADP>ATP) to stimulate P2 receptors. Thus, ADP treatment was expected to be more effective than ATP to stimulate cardiomyocyte differentiation from ES cells.

It is reported that both P2YRs (G-protein coupled metabotropic receptors) and P2X receptor (ligand-gated cationic channels) subtypes exert trophic actions in physiological cell processes via increase of cytosolic $[Ca^{2+}]_i$

concentration [45]. In the present study, the P2X receptor agonists β,γ -MetATP and 8-Br-ATP elicited $[Ca^{2+}]_i$ transients, indicating presence of P2X receptors which was confirmed by RT-PCR experiments. However, the duration of the $[Ca^{2+}]_i$ signals was significantly longer as compared to ATP/ADP, which may indicate that the concentration of ATP/ADP used in the present study did exclusively activate P2Y receptors. In line with this observation the P2X antagonist TNP-ATP did not impair ADP/ATP-mediated $[Ca^{2+}]_i$ transients. Moreover, inhibition of PLC- β by U-73122 totally abolished the ATP/ADP-induced $[Ca^{2+}]_i$ signal. PLC- β is a membrane enzyme connected with G-protein coupled receptors such as P2YRs to cleave PIP2 into IP3 and DAG upon receptor stimulation in order to transduce extracellular signals into the cytosol [46, 47]. These data were corroborated by the observation that partial inhibition of $[Ca^{2+}]_i$ signaling was observed in presence of MRS2179, a selective P2Y1 receptor antagonist. Moreover, in presence of PPADS, a non-selective P2 receptor antagonist, intracellular $[Ca^{2+}]_i$ mobilization was completely blocked. Of note, it is reported that the P2Y1 subtype has a widespread expression and is involved in blood platelet aggregation, vasodilatation and neuromodulation [23, 28]. RT-PCR evaluation of P2Y receptors revealed P2Y1, P2Y2, P2Y4, and P2Y6 receptor expression during the time course of ES cell differentiation. Notably knockout of single P2Y receptors, e.g. P2Y1, does not result in a cardiac phenotype [48], which may indicate that loss of single P2Y receptors may be compensated by others.

Membrane hyperpolarization subsequent to purinoceptor-mediated $[Ca^{2+}]_i$ signaling as well as ATP release from the cytosol into the extracellular compartment have been reported in several preparations. There are controversial reports whether membrane hyperpolarization occurs in downstream signaling of P2 receptor stimulation and $[Ca^{2+}]_i$ release or whether it is a stimulator for releasing ATP upstream of purinergic receptor activation [27, 49]. In this study, potentiometric fluorescent dye-dependent measurements showed no changes in membrane potential upon stimulation of the cells with ADP as well as ATP.

An increase in the number of beating clusters, cardiac cell numbers as well as beating frequency upon long term treatment of cells with ADP or ATP was the first indication that nucleotide-mediated purinergic receptor stimulation might have positive effects to improve cardiomyocyte differentiation and maturation in vitro. Since ADP/ATP treatment enhanced the chronotropic effects of epinephrine on differentiated cardiomyocytes, we assumed that treatment with ADP and ATP promoted differentiation of cardiomyocytes with improved functional properties. It is reported that adrenoceptors involved in the regulation of cardiomyocyte contraction belong to those receptors that are expressed in the late stage of differentiation [50]. The results of this study were in agreement with previous reports in which different types of chronotropes were applied on differentiated cardiomyocytes [50, 51].

Myofibrillogenesis in differentiating cardioblasts can be triggered during developmental processes mainly by P2 receptors and agonists like ADP or ATP [16]. Immunohistochemical staining for α -actinin illustrated well organized sarcomeric structures after treatment with ADP/ATP for 10 days, whereas this tissue-like and cross-striated structure was not detected in the control group. Apparently, ADP and ATP treatment led to an increase in α -actinin expression and subsequently development of high-order assemblies and more rigid actin bundles in differentiated cardiomyocytes. Myosin is a hexameric enzyme composed of two heavy chains (~220 kDa each) and four light chains (20 kDa each) [52, 53]. During heart development, the α -MHC mRNA level increases in both atrial and ventricular muscle cells and it is considered as a general marker for cardiomyocytes with no specificity to distinguish between different cardiac cell populations. Monitoring the expression of α -MHC demonstrated a significant increase of its mRNA levels following addition of ADP and ATP to the cell culture medium. The α -MHC isoform is characterized by a higher ATPase activity [53] and faster-shorter speed of contraction rather than the β -MHC isoform [54]. Therefore, cardiomyocytes rich in α -MHC protein upon treatment with ADP and ATP would have high intrinsic contractile potential [55]. In other words, the significantly increased cardiac beating frequency in the ADP/ATP experimental group may

correlate with high expression of the α -MHC isoform in these groups. MLC2v mRNA is highly expressed and spatially restricted to the ventricular part of the mouse heart tube and the outflow tract even at early stages, with no detectable expression in the atria [56]. Exposure of ES cells to ADP/ATP for 10 days led to significant increase in MLC2v mRNA and protein levels. This result indicates an enriched population of ventricular-like cardiomyocytes under stimulation with ADP or ATP (especially ADP). Spontaneous activity or rhythmicity of the heart is generated in pacemaker cells located in sinoatrial node and atrioventricular node regions. To assess whether ADP/ATP treatment would increase the pacemaker cell population in ES cell-derived cardiac areas, the expression of HCN4, Cx45, and Cx30.2 was evaluated. These genes are expressed specifically in pacemaker cells [57]. Our data showed that ATP/ADP treatment resulted in elevated HCN4 mRNA levels. Interestingly, the expression of HCN4 was high even at day 18. Furthermore, it seems that treating the cells with 10 μ M ATP was slightly more effective than ADP regarding pacemaker cell differentiation.

Connexin gap junction channel proteins are known to be necessary for impulse propagation through the heart. Different isoforms of connexin proteins are expressed in different types of cardiac cells. For example Cx43, a fast conductance protein is expressed in the working myocardium, whereas Cx45 and Cx 30.2 as low conductance velocity connexin proteins are preferentially expressed in the cardiac conduction system [58]. If the differentiation of sinus node-like cells is upregulated after exposure of cells to ADP or ATP, an enhancement of corresponding gap junction proteins was expected. The molecular analysis of Cx45 mRNA expression showed a significant increase in ADP/ATP-treated cells. In contrast, Cx30.2 mRNA expression did not show any significant difference between treated and untreated cells. However, it has been reported that the function of certain cardiac connexins could be compensated or substituted by other connexins [59]. This means that in ES cell-derived cardiomyocytes, Cx45 may be the principal connexin rather than Cx30.2.

Conclusion In the present study, it was demonstrated that stimulation of purinergic receptors with ADP and ATP increased cardiomyogenesis of ES cells. Conversely, inhibition of purinergic receptor signaling by their antagonists has as a suppressive effect on differentiation of cardiomyocyte in vitro. These findings shed light on the importance of purinoceptor signaling during cardiomyogenesis. It is suggested that ADP/ATP-mediated P2 receptor stimulation and the subsequent trophic intracellular $[Ca^{2+}]_i$ transients in ES cells may be a prominent trigger for driving the fate of pluripotent ES cells into the cardiac cell lineage with a high percentage of pacemaker cells. Acquiring a pure cell population of cardiomyocytes would facilitate stem cell therapeutic approaches in various cardiovascular diseases.

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