Isolation and Functional Characterization of α -Smooth Muscle Actin Expressing Cardiomyocytes from Embryonic Stem Cells

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Key Words

Acta2 • ES cells • Patch-clamp electrophysiology • Cardiomyocytes • Lineage selection

Abstract

Early mammalian heart development is characterized by transient expression of α -smooth muscle actin (Acta2). To date, cardiomyocytes expressing Acta2 in the early stages of *in vivo* development have not been characterized. To functionally characterize Acta2-expressing cardiomyocytes, we used a transgenic ES cell line expressing both the puromycin acetyl transferase (Pac) and enhanced green fluorescent protein (EGFP) cassettes under the control of the Acta2 promoter. The onset of Acta2 expression occurred in parallel with the appearance of beating areas, indicating the formation of cardiomyocytes. Antibiotic selection resulted in a high yield of cardiomyocytes and smooth muscle cells. The green fluorescent beating areas stained positively for multiple cardiomyocyte markers. Comparative electrophysiological analysis including fetal and α -MHC-expressing ES cell-derived cardiomyocyte controls showed that Acta2-positive cardiomyocytes contained pacemaker-, atrial- and ventricular-like

phenotypes. Interestingly, the proportion of ventricular-like cells was much higher in the Acta2-positive cardiomyocytes population than in control α -MHC-expressing cardiomyocytes (75 % and 12 %, respectively). The findings of the present study provide a novel approach for the identification and enrichment of Acta2-positive cardiomyocytes, especially of the ventricular phenotype under *in vitro* conditions.

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Introduction

Actin is one of the most abundant and conserved eukaryotic proteins occurring in four tissue-specific and two ubiquitous isoforms [1, 2]. The actin isoforms are highly conserved at the amino acid level and only differ at their N-termini [2, 3]. The specific NH₂-terminal sequence Ac-EEED of α -smooth muscle actin (Acta2) plays an important role in the regulation of polymerization *in vitro* and *in vivo* [4]. Between the two smooth muscle cell (SMC)-specific actin isoforms (α and γ), the contractile isoform Acta2 is the predominantly expressed isoform in vascular SMC differentiation during vascular

development in the mouse embryo [5]. In the early stages of heart development, Acta2 is expressed but turned off in later stages of heart development [6, 7]. Expression of Acta2 is one of the most critical parameters of cardiomyocyte maturation in the prenatal rat heart and its expression is most extended in the ventricular conduction system, making it a convenient marker for the ventricular conduction system [8]. Acta2 expression begins at embryonic day (ED) 9 and gradually increases in all compartments of the heart until ED14. Ventricular Acta2 expression is first observed at ED10 and later at ED11 in aorta. Acta2 is transiently expressed in rat embryonic cardiac and skeletal muscles and it disappears shortly after following birth [9]. The temporal expression pattern of Acta2 during the embryonic heart development suggests that the early cardiomyocyte progenitors express Acta2.

Due to the practical difficulties of isolating Acta2-expressing cardiomyocytes during early embryonic development *in vivo*, the nature and phenotype of these cells have not been characterized. One attractive approach to identify and characterize early Acta2-expressing cardiomyocytes is the differentiation of murine embryonic stem cells (ESCs) *in vitro*. Numerous studies have proven its usefulness for the recapitulation of embryonic development *in vitro* [10, 11].

We recently generated a transgenic ES cell line expressing both the puromycin acetyl transferase (Pac) and enhanced green fluorescent protein (EGFP) cassettes under the control of the Acta2 promoter, isolated functional, non-dividing contractile SMCs using a monolayer differentiation approach and characterized these cells in detail, including their electrophysiology [12]. In the current study, we developed a new efficient method promoting cardiomyogenic differentiation, and described a novel approach to derive functional cardiomyocytes expressing Acta2 from ES cells and characterized this previously inaccessible cardiomyocyte population.

Materials and Methods

Materials

Cell culture media and fetal bovine serum (FBS) were obtained from Invitrogen (Karlsruhe, Germany), Leukemia inhibitory factor (LIF) was purchased from Millipore (Billerica, MA, USA). Carbachol and reagents for the electrophysiological buffer solutions were purchased from Sigma Aldrich (Deisenhofen, Germany). Primary mouse vascular smooth muscle cells (VSMCs) were kindly provided by Dr. Gary K Owens, Department of Molecular Physiology & Biological

Physics, University of Virginia School of Medicine, Charlottesville, VA 22908.

Generation of Acta2 ES cell line

Using BAC recombineering 5.5 kb promoter region upstream of the translation initiation site of the Acta2 gene was isolated and then subcloned into the ES cell reporter construct pPuroIRES2-EGFP [12]. For the generation of stable Acta2 ES cell line the construct was linearized and then electroporated into CGR8 ES cells (500 μ F, 2.4 kV/cm) using a Bio-Rad Gene Pulser™ (Bio-Rad, Hercules, CA, USA). After electroporation cells were incubated on ice for 10 min and then seeded onto gelatinized 10 cm dishes using culture medium. Starting from two days after transfection the cells were always maintained in culture medium containing 400 μ g/ml neomycin. Neomycin selection was discontinued only during differentiation. Well isolated clones were picked 9-11 days after transfection using inversion microscope and frozen for further experiments.

Culture of ES cells and *in vitro* differentiation of embryoid bodies

Transgenic murine CGR8 (European Collection of Cell Cultures 95011018) Acta2 ES cells and control α -myosin heavy chain (α -MHC) ES cells [10, 12] were cultured without feeder cells in Glasgow Minimum Essential Medium (GMEM) supplemented with 10 % FBS, 2 mM L-glutamine, 50 μ M β -mercaptoethanol (β -ME) and 100 units/ml LIF in 0.2 % gelatine coated flasks as described previously [10, 12]. Cells were passaged every second day and confluence was kept below 70 %. ESCs were differentiated as three-dimensional multicellular embryoid bodies (EBs) using the hanging drop method. Briefly, upon reaching ~ 70% confluence, ES cells were trypsinized and a single cell suspension of 2.5×10^4 cells/ml was prepared in EB medium consisting of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20 % FBS, 1 % non-essential amino acids (vol/vol), 2 mM L-glutamine, and 100 μ M β -ME. ESCs were aggregated in hanging drops containing 500 cells/20 μ l suspension on the inner surface of the lid of a 10 cm bacteriological dish filled with 5 ml of phosphate buffered saline (PBS) to prevent the evaporation of the drops and placed in an incubator. After two days the multicellular aggregates were transferred into a new bacteriological dish using EB medium and cultured in suspension until day 7. EBs were transferred to dishes coated with 0.2 % gelatine on day 7 and cultured for a further 8 days as adherent EBs. From day 10 until day 15, EBs were cultured in the presence of 5 μ g/ml puromycin. On day 15, EBs were enzymatically dissociated using 1 mg/ml collagenase B (Roche, Mannheim, Germany) and the single cell suspension was used for experiments.

Preparation of embryonic cardiomyocytes and single cells derived from ES cell lines

For harvesting embryonic cardiomyocytes from murine atria, mice of the strain HIM:OF1 were bred using superovulation for precise staging [13]. To harvest murine embryonic hearts, mothers were sacrificed by cervical dislocation at developmental age ED17.5. Atria and ventricles

Gene	Forward Primer	Reverse Primer	Number of Cycles
MLC2v	AAAGAGGCTCCAGGTCCAAT	TCAGCCTTCAGTGACCCTTT	26
Acte1	TCGGGACCTCACTGACTACC	CGCCATCTCGTTCTCAAAAT	28
Acta2	ATCACCAACTGGGACGACAT	CATCTCCAGAGTCCAGCACA	26
Mef2c	AGCACTGACATGGATAAGGTGTT	GGTGAGTGCATAAGAG GAGTCAG	30
Nkx2-5	CCACTCTCTGCTACCCACCT	CCAGGTTTCAGGATGTCTTTGA	29
Nppa	TCTTCTGCTGGCTCCTCACT	TCTGCCCCACTCTGGTTTCTT	30
GAPDH	GGTGCTGAGTATGTCGTGGA	CGGAGATGATGACCCTTTTG	28

Table 1. Primer sequences and cycle numbers used for RT-PCR analysis of total RNA.

were dissected and incubated with 300-600 µl collagenase B for 30 min at 37 °C. Isolated cardiomyocytes were plated on gelatin-coated 22x22 mm glass cover slips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % fetal calf serum (FCS), 1 % Minimal Essential Medium (MEM), 2 mM Glutamax, 50 units/ml penicillin and streptomycin (all from Invitrogen, Karlsruhe, Germany), and 50 µM β-ME (Sigma Aldrich, Deisenhofen, Germany). After 12 hrs of incubation, single cardiomyocytes attached to the surface of the glass cover slips and started spontaneous rhythmical contractions. Beating cardiomyocytes were used in the functional studies after 24-36 h of cultivation. Puromycin-enriched EBs at day 15 from both Acta2 and α-MHC ESC lines were dissociated as described for the embryonic heart tissue.

Patch-Clamp Electrophysiological Measurements

The whole-cell configuration of the patch-clamp technique was used. All experiments were performed at 37 °C. Patch pipettes with a resistance between 2-4 MΩ were prepared from borosilicate glass capillaries with filament using a puller from Zeitz (München, Germany). Glass pipettes were filled with internal solutions containing (in mM): KCl 50, K-aspartate 80, MgCl₂ 1, MgATP 3, glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 10, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) 10, pH adjusted to 7.4 with KOH. Extracellular solution contained (in mM): NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, glucose 10, HEPES 10, pH adjusted to 7.40 at 37 °C with NaOH.

Membrane potential (MP) and spontaneous action potentials (APs) were recorded in the current clamp mode using previously published approaches [14]. The whole cell current was recorded by a ramp pulse depolarizing from -100 mV to -50 mV in 150 ms. Signals acquired from cardiomyocytes were magnified by an EPC 9 amplifier at a sampling rate of 10 kHz, stored on hard disk and analyzed off-line using the Pulsefit (Heka, Lambrecht, Germany) analysis package and AP analysis software programmed by Philipp Sasse (University of Bonn, Germany).

Immunocytochemistry

Adherent EBs at different time points were washed with PBS, and fixed using methanol, and blocked with 5 % bovine serum albumin (BSA) in PBS for 1 hour at room temperature. Cells were incubated with 1 % BSA in PBS containing respective primary antibodies overnight at 4 °C. Cells were washed with

PBS five times. The following antibodies were used for primary detection: anti sarcomeric actinin (clone EA-53, immunogen is purified rabbit skeletal α actinin, 1:400; Sigma-Aldrich), anti cardiac troponin T (clone 13-11, immunogen is purified rabbit cardiac troponin T isoform, 1:100; Labvision), anti myosin light chain (clone F109.3E1, immunogen is human ventricular myosin light chains, 1:10, Alexis) and anti GFP (rabbit polyclonal, 1:50; Santa Cruz Biotech).

Secondary antibodies (Molecular Probes, Eugene, OR, USA) were diluted 1:1000 in 1 % BSA and incubated with the cells for 2 hours at room temperature. Cells were washed with PBS and then incubated with Hoechst 33342 dye (1:5000 in PBS; Molecular Probes) for 1 hour. Coverslips were embedded using ProLong Gold mounting medium (Invitrogen) and investigated using an Axiovert 200 fluorescence microscope (Carl Zeiss, Jena, Germany).

Semiquantitative RT-PCR Analysis

Total RNA was extracted using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). 2 µg RNA was treated with DNase I (Amplification grade, Invitrogen Life Technologies, Carlsbad, CA, USA) prior to reverse transcription (RT) into cDNA by SuperscriptTM II Rnase H-free Reverse Transcriptase (Invitrogen), using random hexamer primers according to the manufacturer's instructions. The polymerase chain reaction (PCR) amplification was done in 20 µl reactions containing 10 µl JumpStart REDTaq ReadyMix (Sigma-Aldrich, St. Louis, MO, USA), 0.3 µl of synthesized cDNA and primers at a final concentration of 0.15 µM each. All amplifications were done in an Eppendorf Mastercycler under conditions optimized for each target sequence (for primers and conditions see Table 1). The genes of interest were normalized against the housekeeping gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), according to the following conditions: an initial step of 94 °C for 2 min followed by 25-30 cycles of 94 °C for 35 sec, 60 °C for 45 sec, 72 °C for 45 sec, and a final extension of 72 °C for 10 min. Reverse transcription polymerase chain reaction (RT-PCR) products were analyzed using agarose gel electrophoresis.

Data analysis

Results are shown as mean ± standard error of the mean (SEM). Statistical analysis was done using paired and unpaired Student's t-test where applicable. P-values of <0.05 were considered significant.

Fig. 1. Schematic representation of the derivation of functional Acta2⁺ cardiomyocytes from transgenic murine Acta2 ESCs using the hanging drop method, and their subsequent enrichment using puromycin for five days starting from day 10. The presence of EGFP-expressing beating areas within a treated EB indicates the enriched population of cardiomyocytes. Scale bar, 100 μ m.

Results

Experimental protocol for enrichment and isolation of Acta2⁺ cardiomyocytes from the transgenic Acta2 ES cells

For the induction of differentiation, undifferentiated Acta2 ESCs were cultured in hanging drops leading to aggregation and EB formation (see Fig. 1). EGFP-expressing cells were observed on day 7 and significantly increased in number in the following days, indicating Acta2 promoter activity. Beating areas were first observed on day 7 with concomitant EGFP expression indicating the presence of Acta2-expressing cardiomyocytes. Puromycin treatment of EBs from day 10 to day 15 allowed the selection of viable EGFP-expressing cardiomyocytes and SMCs. Spontaneously beating cardiomyocyte clusters in 15-days old EBs are shown in the video file (please see Supplemental video file, <http://www.uni-koeln.de/med-fak/physiologie/np/Video.wmv>). The average beating frequency was 66 beats/min.

Characterization of the Acta2⁺ cardiomyocytes

In day 15 puromycin-enriched populations, colonies with distinct cellular morphologies characteristic of

cardiomyocytes and SMCs were observed (Fig. 2A). RT-PCR analysis of beating cell clusters indicated a maximal expression level of the cardiac-specific genes myosin light chain 2v (MLC2v), actin, alpha, cardiac muscle 1 (Actc1), myocyte enhancer factor 2C (Mef2c), NK2 transcription factor related, locus 5 (Nkx2-5), natriuretic peptide precursor type A (Nppa) and alpha-smooth muscle actin (Acta2) in day 15 puromycin-treated Acta2⁺ cells as compared to undifferentiated Acta2 ESCs and to 15-days old differentiated Acta2 ESCs (Fig. 2B). MLC2v, a canonical marker for ventricular cardiomyocytes, is highly expressed in day 15 enriched populations whereas it is hardly detectable in the non-enriched cell population, collectively indicating the selective enrichment of ventricular cardiomyocytes. The derivation of Acta2⁺ cardiomyocytes was verified by immunostainings (inserts shown in Fig. 2C) showing α -actinin- and EGFP-positive cells. The striated anti- α -actinin staining pattern at Z-bands and intercalated discs and their co-localization with EGFP further confirmed that the cardiomyocytes do express Acta2. Immunostaining of the day 15 puromycin-selected cells with antibodies against cardiac troponin T and myosin light chain (Fig. 2D) indicated structurally intact cardiomyocytes.

Fig. 2. Morphological observation of EGFP-expressing cells and their characterization by RT-PCR and immunohistochemistry. A, On day 15 after puromycin treatment for 5 days two different subsets of EGFP-expressing cells were visible with morphologies characteristic of cardiomyocytes and SMCs (top and bottom panels, respectively). B, RT-PCR analysis of cardiac-specific genes in Acta2-ESCs, in 15-days old EBs without puromycin treatment and 15-days old EBs with puromycin treatment (RT-PCR conditions and primers, see Table 1). Genes characteristic of cardiomyocytes and smooth muscle actin were highly enriched in day 15 puromycin-treated cells. C, Immunohistochemical characterization of the EBs at different time points. 7- to 15-days old whole EBs were stained with anti-GFP and anti-α-actinin, representing the specific expression of Acta2 and sarcomeric proteins, respectively. Detection of α-actinin striated patterns at Z-bands and intercalated discs is indicative of cardiomyocytes in day 12 and 15 puromycin-treated EBs. D, Cardiac-specific markers were co-expressed with EGFP. Immunostaining of the puromycin-selected cells with antibodies against cardiac troponin T and myosin light chain indicated structurally intact cardiomyocytes and their co-expression with EGFP further indicated their derivation from Acta2 ESCs. Nuclei are counterstained with Hoechst dye (blue). Scale bar: 50 μm.

Electrophysiological characterization of Acta2⁺ cells

At day 15, two cellular phenotypes with distinct characteristics were observed (Fig. 2A). Upon experimentation the cells were determined to be either SMC-like or cardiomyocyte-like. Besides the outward currents, the spontaneously beating phenotype (n=54 cells), had multiple inward currents (Fig. 3A) similar to the control fetal cardiomyocytes (Fig. 3B), while the other

phenotype (n=43) had one inward current (Fig. 3A), similar to the control primary SMCs (Fig. 3B). The cardiomyocytes exerted spontaneous action potential spikes (Fig. 3C) as compared to the smooth muscle-like cells (Fig. 3D) where only a few slow waves were observed. In the puromycin-enriched Acta2-positive population, the frequency of cardiomyocytes was 56 % and 44 % for SMCs. Acta2⁺ cardiomyocytes exhibited a more negative MP (-60.8 ± 1.4 mV, Fig. 3C) than the

Fig. 3. Electrophysiological characterization of the Acta2⁺ cardiomyocytes and Acta2⁺ SMCs isolated after dissociation of 15 days-old EBs. A, representative cardiomyocyte- and SMC-like whole cell currents. B, representative whole cell currents of fetal cardiomyocytes and primary SMCs. C, representative APs of the Acta2⁺ cardiomyocytes. D, representative APs of the Acta2⁺ SMCs.

Fig. 4. Effects of carbachol and isoprenaline on the Acta2⁺ cardiomyocytes. A, B, Cch induced a decrease of AP frequency (by 73.9±10.4 %, n=5, p<0.01) without significant changes in membrane potential in the Acta2⁺ cardiomyocytes. C, Cch decreased AP frequency (by 57.0±8.4 %, n=6, p<0.01) in fetal ventricular cardiomyocytes without change in MDP (n=6, p>0.05). D, Cch induced significant hyperpolarization in MDP of fetal atrial cardiomyocytes as well as strong negative chronotropies (by 57.6±6.3 %, n=6, p<0.01). E, F, Isoprenaline (ISO, 1 μM) increased AP frequency of Acta2⁺ cardiomyocytes (by 245.0±55.5 %, n=7, p<0.01).

Acta2⁺ SMCs (-38.0±1.29 mV, Fig. 3D). Carbachol (Cch) at a concentration of 1 μM exerted distinct effects on

these two cellular phenotypes. In the Acta2⁺ cardiomyocytes, Cch led to a decrease of AP frequency

	MDP (mV)	Frequency (beats/min)	Vmax (V/s)	Vdd (V/s)	APD90 (ms)	APD90/50
Acta2 LDS P like	-54.7±1.4	166±31	11.2±3.2	0.087±0.012	110±18	1.69±0.14
Acta2 LDS V like	-61.1±1.2	128±17	17.3±2.0	0.059±0.008	114±13	1.62±0.47
Acta2 LDS A like	-65.7±1.4	109±15	22.7±5.1	0.073±0.023	70±15	4.15±1.30
α -MHC LDS P like	-59.4±5.0	176±41	8.9±3.3	0.11±0.03	86±10	1.82±0.13
α -MHC LDS V like	-63.5±2.8	138±35	21.6±4.9	0.06±0.02	126±17	1.65±0.05
α -MHC LDS A like	-64.9±1.4	133±18	25.5±0.7	0.047±0.007	82±3	3.6±0.41
LDS fetal V	-66.6±1.5	132±16	28.9±2.1	0.058±0.007	117±9	1.4±0.3
LDS fetal A	-59.6±3.3	271±40	24.9±1.7	0.108±0.018	60±1.8	1.81±0.2

Table 2. AP parameters for Acta2⁺ cardiomyocytes, α -MHC⁺ cardiomyocytes and native fetal cardiomyocytes.

Fig. 5. Spontaneous action potential recordings and analysis of the Acta2⁺ derived cardiomyocytes and the α -MHC⁺ cardiomyocytes isolated from 15 to 16-days old EBs. Cardiomyocytes derived from Acta2 transgenic ES cells displayed predominantly ventricular like action potentials. Out of 81 spontaneously beating Acta2⁺ cardiomyocytes, 46 (75,4 %), 10 (16,4 %) and 5 (8,2 %) displayed ventricular-like APs (A), atrial-like (B) and pacemaker-like (C) cardiomyocytes, respectively. Out of 41 α -MHC⁺ cardiomyocytes, 5 (12,2 %), 33 (80,5 %), and 3 (7,3 %), displayed ventricular-like (D), atrial-like (E) and pacemaker-like APs, respectively. G, H, representative APs of fetal ventricular-like and atrial-like cardiomyocytes, respectively. I, Percentages of Acta2⁺ cardiomyocytes and α -MHC⁺ cardiomyocytes displaying ventricular-like, atrial-like and pacemaker-like APs.

(by 73.9±10.4 %, n=5, p<0.01) without significant changes in MP (Fig. 4A) and the normalized frequency is shown in Fig. 4B. This finding was similar to fetal ventricular cardiomyocytes (Fig. 4C) due to the low expression of IK_{ACh} . In contrast to fetal atrial cardiomyocytes which showed a significant hyperpolarization (Fig. 4D). The β 1 adrenergic agonist isoprenaline increased the AP frequency of Acta2⁺ cardiomyocytes (Fig. 4E-F).

Characterization of spontaneous APs in Acta2⁺ cardiomyocytes

We further investigated the cardiomyocyte phenotypes derived from Acta2 ESCs. At day 15 the majority of spontaneously beating cells (75.4 %) displayed ventricular-like APs (n=46, Fig. 5A), which were characterized by a long 90 % repolarization of AP duration (APD90, 114±13 ms) and a long plateau (APD90/50:

1.62±0.47). Atrial-like APs (Fig. 5B, n=10) showing typical triangle-like APs (with short APD90, 70±15 ms) and no plateau (APD90/50 4.15±1.30) and pacemaker-like APs (Fig. 5C, n=5) displaying shoulder-like APs with high velocity of diastolic depolarization (V_{dd}) (0.087±0.012 V/s), less maximum velocity of depolarization (V_{max}) (11.2±3.2 V/s), high frequency and less negative minimal diastolic potential (MDP) (-54.7±1.4 mV) were found in 16.4 % and in 8.2 % of cells, respectively (Fig. 5I). The control α -MHC⁺ cardiomyocytes predominantly displayed atrial-like APs (80.5 %, n=33, Fig. 5E), and a few cardiomyocytes exhibited ventricular-like (12.2 %, n=5, Fig. 5D) and pacemaker-like (7.3 %, n=3, Fig. 5F) APs. The phenotype of each cell was defined as observed in cells from fetal ventricles (Fig. 5G) and atria (Fig. 5H). The basic AP parameters in these two cell lines and the control fetal cardiomyocytes are listed in Table 2, showing that the Acta2⁺ cardiomyocytes displayed the features of fetal ventricular cardiomyocytes.

Discussion

Acta2 is transiently expressed during the development of embryonic heart, and its spatial and temporal expression pattern during cardiomyocyte differentiation of mouse ESCs has recently been described. Among the six mammalian actin isoforms, Acta2 is expressed first in the peripheral cells of plated EBs followed by skeletal and cardiac α -actins. Knockdown of Acta2 in ESCs results in a decrease of the percentage of beating EBs, indicating its role in cardiomyogenesis *in vitro* [15]. Due to the complexity of cardiovascular development *in vivo* and the practical difficulties of isolating definitive cellular phenotypes, mammalian Acta2⁺ cardiomyocytes have not been isolated and characterized. We developed a novel method for the derivation of Acta2-expressing cardiomyocytes from differentiating transgenic ESCs. Acta2 promoter-driven EGFP expression allowed us to monitor the onset of Acta2 expression, which occurred in parallel with the appearance of beating cardiomyocytes. Addition of puromycin at this time point resulted in a high yield of cardiomyocytes. To compare the Acta2⁺ cardiomyocytes with the α -MHC⁺ cardiomyocyte phenotypes, we performed electrophysiological characterization of both cell populations. While we observed all three cardiomyocyte phenotypes, pacemaker-, atrial- and ventricular-like, the proportion of ventricular-like cells was much higher in Acta2-expressing cardiomyocytes than in the α -MHC⁺ cardiomyocytes

(75 % and 12 %, respectively). These observations are in close agreement with the expression profiles of the respective genes. The early appearance of green fluorescent areas indicating Acta2 expression within a beating area of an EB also stained positively for the sarcomeric protein α -actinin, which is characteristic of cardiomyocytes further indicating that Acta2 can be used as an early cardiac marker in ES-derived cardiomyocytes.

Recently several studies suggested the existence of the multipotent cardiovascular progenitor cells during ESC differentiation and embryonic development (for review see [16]). Our results suggest that the Acta2⁺ population contain a novel subpopulation of cardiomyocytes. In this context, there is evidence that the expression of Acta2 may mark primitive mesodermal cells capable of generating cardiovascular cells [17]. Interestingly, using an Acta2-GFP reporter, an osteoprogenitor population has been identified, indicating the potential of Acta2⁺ cells to differentiate towards the mesoderm derived cell populations [18]. Our findings are in agreement with these reports, suggesting the existence of an Acta2-positive mesodermal population and multiple roles for these cells in different tissues and developmental stages. Moreover, the present findings suggest that the Acta2⁺ cardiomyocytes are present in the early developmental stage of the heart. This is supported by a report that the re-expression of Acta2 in Acta2-negative and highly differentiated cultured adult rat cardiomyocytes resulted in a less differentiated fetal cardiomyocyte phenotype [19].

Expression of Acta2 in adult cardiomyocytes has also been observed under pathophysiological conditions. In this regard, the expression has been detected in cardiac myofibroblasts during right ventricular pressure overload in the rabbit [20]. In biopsy specimens from the left ventricle of patients with dilated cardiomyopathy, high levels of Acta2 mRNA were found, suggesting its use as a pathological marker to classify and evaluate prognosis in dilated cardiomyopathy [21].

CMs are characterized by either occurring APs or multiple inward currents [22], while SMCs have relatively stable MP [23]. Seldomly in special tissues APs are observed with much slower depolarization velocity [24] or displaying as slow waves [25]. Till now Ca²⁺ currents are the only inward current that has been well described in SMCs [26]. By comparison, we clearly demonstrated the coexistence of CMs and SMCs in Acta2⁺ cells derived from differentiated ES cells. Our findings are in agreement with previous reports, suggesting that the expression of

contractile protein Acta2 in early cardiomyocytes might implicate its involvement in myofibrillogenesis and also in the generation of cellular tension.

The Acta2 ES cell line allows not only reproducible generation of an unlimited number of contractile SMCs but also Acta2⁺ cardiomyocytes, thus offering a novel source of cardiomyocytes. In addition, the predominantly ventricular phenotype of Acta2⁺ cardiomyocytes indicates functional properties similar to fetal ventricular cardiomyocytes that are capable of integration and

functional maturation after transplantation [27].

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References

- 1 Vandekerckhove J, K Weber: Mammalian cytoplasmic actins are the products of at least two genes and differ in primary structure in at least 25 identified positions from skeletal muscle actins. *Proc Natl Acad Sci U S A* 1978;75:1106-1110.
- 2 Kashina AS: Differential arginylation of actin isoforms: the mystery of the actin N-terminus. *Trends Cell Biol* 2006;16:610-615.
- 3 Vandekerckhove J, K Weber: At least six different actins are expressed in a higher mammal: an analysis based on the amino acid sequence of the amino-terminal tryptic peptide. *J Mol Biol* 1978;126:783-802.
- 4 Chaponnier C, M Goethals, PA Janmey, F Gabbiani, G Gabbiani, J Vandekerckhove: The specific NH2-terminal sequence Ac-EEED of alpha-smooth muscle actin plays a role in polymerization in vitro and in vivo. *J Cell Biol* 1995;130:887-895.
- 5 Skalli O, P Ropraz, A Trzeciak, G Benzonana, D Gillesen, G Gabbiani: A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol* 1986;103:2787-2796.
- 6 Ruzicka DL, RJ Schwartz: Sequential activation of alpha-actin genes during avian cardiogenesis: vascular smooth muscle alpha-actin gene transcripts mark the onset of cardiomyocyte differentiation. *J Cell Biol* 1988;107:2575-2586.
- 7 Woodcock-Mitchell J, JJ Mitchell, RB Low, M Kieny, P Sengel, L Rubbia, O Skalli, B Jackson, G Gabbiani: Alpha-smooth muscle actin is transiently expressed in embryonic rat cardiac and skeletal muscles. *Differentiation* 1988;39:161-166.
- 8 Ya J, MW Markman, GT Wagenaar, PJ Blommaert, AF Moorman, WH Lamers: Expression of the smooth-muscle proteins alpha-smooth-muscle actin and calponin, and of the intermediate filament protein desmin are parameters of cardiomyocyte maturation in the prenatal rat heart. *Anat Rec* 1997;249:495-505.
- 9 Kumar A, K Crawford, L Close, M Madison, J Lorenz, T Doetschman, S Pawlowski, J Duffy, J Neumann, J Robbins, GP Boivin, BA O'Toole, JL Lessard: Rescue of cardiac alpha-actin-deficient mice by enteric smooth muscle gamma-actin. *Proc Natl Acad Sci U S A* 1997;94:4406-4411.
- 10 Doss MX, J Winkler, S Chen, R Hippler-Altenburg, I Sotiriadou, M Halbach, K Pfannkuche, H Liang, H Schulz, O Hummel, N Hubner, R Rottscheldt, J Hescheler, A Sachinidis: Global transcriptome analysis of murine embryonic stem cell-derived cardiomyocytes. *Genome Biol* 2007;8:R56.
- 11 Sachinidis A, BK Fleischmann, E Kolossov, M Wartenberg, H Sauer, J Hescheler: Cardiac specific differentiation of mouse embryonic stem cells. *Cardiovasc Res* 2003;58:278-291.
- 12 Potta SP, H Liang, K Pfannkuche, J Winkler, S Chen, MX Doss, K Obernier, N Kamisetti, H Schulz, N Hubner, J Hescheler, A Sachinidis: Functional characterization and transcriptome analysis of embryonic stem cell-derived contractile smooth muscle cells. *Hypertension* 2009;53:196-204.
- 13 Fleischmann M, W Bloch, E Kolossov, C Andressen, M Muller, G Brem, J Hescheler, K Addicks, BK Fleischmann: Cardiac specific expression of the green fluorescent protein during early murine embryonic development. *FEBS Lett* 1998;440:370-376.
- 14 Horn R, A Marty: Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J Gen Physiol* 1988;92:145-159.
- 15 Clement S, M Stouffs, E Bettiol, S Kampf, KH Krause, C Chaponnier, M Jacon: Expression and function of alpha-smooth muscle actin during embryonic-stem-cell-derived cardiomyocyte differentiation. *J Cell Sci* 2007;120:229-238.
- 16 Kattman SJ, ED Adler, GM Keller: Specification of multipotential cardiovascular progenitor cells during embryonic stem cell differentiation and embryonic development. *Trends Cardiovasc Med* 2007;17:240-246.
- 17 Colas JF, A Lawson, GC Schoenwolf: Evidence that translation of smooth muscle alpha-actin mRNA is delayed in the chick promyocardium until fusion of the bilateral heart-forming regions. *Dev Dyn* 2000;218:316-330.

- 18 Kalajzic Z, H Li, LP Wang, X Jiang, K Lamothe, DJ Adams, HL Aguila, DW Rowe, I Kalajzic: Use of an alpha-smooth muscle actin GFP reporter to identify an osteoprogenitor population. *Bone* 2008;43:501-510.
- 19 Eppenberger-Eberhardt M, I Flamme, V Kurer, HM Eppenberger: Reexpression of alpha-smooth muscle actin isoform in cultured adult rat cardiomyocytes. *Dev Biol* 1990;139:269-278.
- 20 Leslie KO, DJ Taatjes, J Schwarz, M vonTurkovich, RB Low: Cardiac myofibroblasts express alpha smooth muscle actin during right ventricular pressure overload in the rabbit. *Am J Pathol* 1991;139:207-216.
- 21 Adachi S, H Ito, M Tamamori, M Tanaka, F Marumo, M Hiroe: Skeletal and smooth muscle alpha-actin mRNA in endomyocardial biopsy samples of dilated cardiomyopathy patients. *Life Sci* 1998;63:1779-1791.
- 22 Schram G, M Pourrier, P Melnyk, S Nattel: Differential distribution of cardiac ion channel expression as a basis for regional specialization in electrical function. *Circ Res* 2002;90:939-950.
- 23 Tolsa JF, M Marino, AC Peyter, JL Beny: Role of membrane potential in endothelium-dependent relaxation of isolated mouse main pulmonary artery. *J Cardiovasc Pharmacol* 2006;47:501-507.
- 24 Hashitani H, AF Brading: Ionic basis for the regulation of spontaneous excitation in detrusor smooth muscle cells of the guinea-pig urinary bladder. *Br J Pharmacol* 2003;140:159-169.
- 25 Hotta A, Y Kito, H Suzuki: The effects of flufenamic acid on spontaneous activity of smooth muscle tissue isolated from the guinea-pig stomach antrum. *J Smooth Muscle Res* 2005;41:207-220.
- 26 Marks TN, SW Jones: Calcium currents in the A7r5 smooth muscle-derived cell line. An allosteric model for calcium channel activation and dihydropyridine agonist action. *J Gen Physiol* 1992;99:367-390.
- 27 Halbach M, K Pfannkuche, F Pillekamp, A Ziomka, T Hannes, M Reppel, J Hescheler, J Muller-Ehmsen: Electrophysiological maturation and integration of murine fetal cardiomyocytes after transplantation. *Circ Res* 2007;101:484-492.