***In vitro* Developed Spontaneously Contracting Cardiomyocytes from Rainbow Trout as a Model System for Human Heart Research**

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Key Words

Drug testing • Cardiac • Electrophysiology • Action potential • Trout

Abstract

Background/Aims: Cellular models are an interesting tool to study human heart diseases. To date, research groups mainly focus on mouse models, but important murine physiology is different from human characteristics. Recently, scientists found that the electrophysiology of fish cardiomyocytes largely resembles that of humans. So far, cardiomyocyte models were generated using differentiation medium, were stimulated electrically or, when contracting spontaneously, only did so over a short time period. We established an *in vitro* spontaneously, long-term beating heart model generated from rainbow trout, with the potential to be used as a new human heart model system because of its electrophysiology. **Methods:** Spontaneously contracting 3D cell layers from rainbow trout were generated *in vitro* and analyzed using PCR and immunochemistry. Further, electrophysiology was measured via intra – and extracellular recordings. **Results:** Contracting cardiomyogenic aggregates were generated without differentiation medium and

were beating autonomously for more than one month. Electrophysiological measurements exhibit that the action potential properties of fish cardiomyocytes in part resemble the characteristics of human cardiomyocytes. The sensitivity of the beating cell aggregates to drugs could also be confirmed. **Conclusion:** Spontaneously contracting cardiomyogenic cell aggregates from rainbow trout generated *in vitro* are suitable for human heart research and pharmacology.

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Introduction

The interest in using simple and low cost models for the human heart is steadily increasing. Model systems, devoid of the existing endogenous influences in the organism, are suitable tools for studying physiological mechanisms *in vitro* for the first testing period [1]. They are therefore useful *in vitro* systems to examine adaptive mechanisms of cardiomyocytes to varied environmental influences. To investigate human heart diseases, researchers are currently focusing on murine models.

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Here, the common tools for research with murine models are isolated heart models [2], slices from heart tissue [e.g. 3], cardiac explant derived cells [4], iPS cells [e.g. 5, 6] and embryonic stem cells [7] as well as embryoid bodies [e.g. 8]. However, limits are set in several aspects of murine biology for using mouse as a model for human research (e.g. cardiovascular ion channel composition). Furthermore, research groups also study the ability of human embryonic stem cell-derived cardiomyocytes (hESC-CM) to be used as a stem cell source for cardiac recovery [9]. Also here mouse models are indispensable, e.g. for first implantation experiments. Research groups reported that the electrophysiology of hESC-CM is significantly heterogeneous within a population and is mostly similar to fetal rather than adult cardiac cells, as indicated by their depolarization resting membrane potential [9-11]. Besides murine models, also chicken is being used within heart research. In most cases embryonic chicken heart cells or tissues were used for developmental [e.g. 12, 13] and electrophysiological studies [14]. To our knowledge, there is no study that uses chicken embryonic cardiomyocytes for human heart studies or as a human heart model, respectively.

Several research groups found out that fishes can be useful model animals, as they have a high potential for application in physiology, regeneration biology, biotechnology and pharmaceutical research [15, 16]. The use of fishes for regenerative studies of some tissues such as fin, retina, maxillary barbel and heart has been already well developed [17-21]. Those observations motivated researchers to use fish cell lines for biomedical purposes. In 2008 and 2009 it was already reported, that fish cells are an important model for investigating cardiac physiology or for human heart research [e.g. 22, 23]. Brette et al. 2008 [23] and Shiels et al. 2006 [24] described that the heart physiology of mice differs a lot from that of human beings while it has been revealed that the cardiac physiology of fish is much closer in their action potential characteristics to human heart. One striking similarity is the action potential of fish cells, which exhibits a long duration and a plateau phase. Additionally, Milan et al. 2006 [25] found out that the action potential duration and QT-interval in the electrocardiogram in fish depend upon the beating frequency as well as it is observed in larger mammals. Zebrafish were often used in those studies, but also rainbow trout, as a robust common teleost in limnological and marine systems of the northern hemisphere, was a useful model. However, in all studies heart models had to be maintained with differentiation medium [26] or obtained electrical stimulation to achieve contrac-

tion. Other cardiac models were beating spontaneously, though just for a period of two weeks or even less. As a result of the above mentioned facts, we were motivated to establish a novel *in vitro* model for heart research from fish cardiomyocytes that is stable, exhibits long-term spontaneously contracting cell aggregates (SCCs) and whose electrophysiological properties are comparable to the human heart. As many other research groups, we have focused on rainbow trout as it is a fish of high economic interest [e.g. 24, 27-29].

An earlier study done by our research group [30] demonstrated that long-term spontaneously, autonomously and rhythmically beating cardiomyocytes from larvae of rainbow trout can be differentiated from progenitor cells *in vitro*. For this approach no co-culture system, differentiation medium or electrical stimulation was used. In the present study we characterized the cells from the spontaneously contracting cell aggregates by immunochemistry and PCR. We also analyzed the electrophysiological properties, which are of importance for the application as a human model system in pharmacology. The action potential was analyzed by performing intracellular measurements using sharp electrodes and the overall excitability of the tissue was measured using multielectrode arrays (MEA's). Furthermore, first pharmaceutical tests were performed in order to investigate the pharmacological properties of the system.

Conclusively, our present study shows that the differentiated 3D SCCs from rainbow trout exhibits cardiomyocytes and have several advantages over human heart models applied to date:

1. We developed cell aggregates which are beating autonomously for more than one month and longer without the influence of differentiation medium or electrical stimulation.
2. It could be shown that it is possible to generate up to three SCCs out of one fish larva and that the propagation of SCCs is possible [30].
3. The electrophysiological characteristics of the cells demonstrate similarities to cardiomyocytes from human hearts and initial pharmacological tests show that the SCCs are sensitive to drugs.

Materials and Methods

Cell isolation and cell culture

Protocols have undergone an ethical review process by the German animal welfare law §8a (ethic committee: Ministerium für Landwirtschaft, Umwelt & ländliche Räume of Schleswig-Holstein; permit number: 41/A01/09). Rainbow trout

(*Oncorhynchus mykiss*) from eye-point-eye stadium till the end of yolk sac stadium were obtained from the fishery farm in Lütjenberg (Germany). The fish were raised indoors at 10 °C by a continuous flow of fresh water. For anesthesia the carefully manually perforated eggs or the larvae were incubated 5 minutes in a 20 % MS222 (methanesulfonic acid salt; Sigma Aldrich, Germany) solution dissolved in aquarium water. That followed the dissection of the larvae with scissors and digestion in 100 µl 0.1 % trypsin (PAA Laboratories, Austria) for one till two minutes. Digestion was stopped by addition of the triple volume of DMEM (Dulbeccos Modified Eagle Medium; Gibco Germany) supplemented with 20 % FCS (fetal calf serum) and followed by centrifugation for five minutes at 130 g. The cell pellets were resuspended in DMEM-FCS 20 % and seeded into 6 well culture plates (TPP, Switzerland).

The developed cardiomyocytes cell aggregates were isolated and used for characterizations via Immunocytochemistry and electrophysiological examinations via intracellular recordings and Multielectrode Array.

Immunocytochemistry

Using immunocytochemistry to determine the expression and intercellular organisation of cardiac specific structural proteins in the SCCs we chose primary antibodies which were tested positive for proteins special for heart from [30] and [31].

SCCs were cut out of the cell layer with a scalpel, seeded on chamber slides and cultured for one day. After fixing with methanol:acetone (7:3) containing 1 mg ml⁻¹ DAPI (Roche, Switzerland) for 5 min at +20 °C, cells were washed three times in PBS. After incubation with 1.7 % normal goat serum at room temperature for 20 min, the samples were incubated with trout cross-reactive primary antibodies in a humid chamber for 1 h at 37 °C. Anti-troponin I (mouse monoclonal 1:50; Acris, Germany), anti-β-catenin (1:50; BD Biosciences, Germany), anti-α-actinin (mouse monoclonal 1:50; Sigma, Germany), anti-N-Cadherin (1:50; BD Biosciences, Germany), anti-l1&2-Desmoplakin (Progen, Germany), anti-HCN4 (1:100; abcam, Germany) and anti-Connexin 45 (1:1000; abcam, Germany). After rinsing three times in PBS, slides were incubated for 1 h at 37 °C with the secondary antibody (FITC-labelled anti-mouse (IgG 1:200; Dianova, Germany) or CY3-labelled anti-mouse (IgG 1:400; Dianova, Germany). In one sample the actin-labelling reagent phalloidin dissolved at a concentration of 6.6 µmol/ml methanol (1:40; Interchim, Germany) were added. Slides were washed three times in PBS, covered in Vectashield mounting medium (Vector, USA) and analyzed with a laser scanning microscope (Zeiss, Germany), respectively.

For a positive control frozen tissue sections of the heart from an adult rainbow trout were chosen. For anesthesia the fish was incubated 5 minutes in a 20 % MS222 (methanesulfonic acid salt; Sigma Aldrich, Germany) solution dissolved in aquarium water and killed through decapitation. The heart was dissected from rainbow trout and was instantly frozen in isopentane for 30 seconds up to a minute. Afterwards it was transferred to liquid nitrogen for a minute. The heart was cut into 12 µm thick tissue sections with a Cryostat, fixed to coated cover glasses and cut into millimetre-sized fragments, so called BioChips™ (Euroimmun, Lübeck, Germany). The BioChips™

were then arranged in the reaction field (BioChip Mosaics™, Euroimmun, Lübeck, Germany). For Immunofluorescence the slides were handled with the same protocol as described above.

mRNA Isolation and reverse-transcription polymerase chain reaction (RT-PCR)

Messenger RNA was isolated with a Dynabeads mRNA Direct Kit (Invitrogen Dynal, Norway) according to the manufactures protocol. Complementary DNA was synthesised using Quantitect Transcriptase Kit (Qiagen, Germany) following manufactures protocol.

PCR was carried out with 1 µl cDNA in 50 µl reaction volume using Dream-Taq DNA polymerase (Fermentas, Germany) and intron-spanning primers, if possible. The reaction was performed in 40 cycles. Control (PCR-water) runs without reverse transcription were done to test RNA preparation for contamination with genomic DNA. No amplification products and for that reason no contamination was showed. To control the cDNA concentration in the RT samples, we measured also the relative expression of a housekeeping gene – elfa.

The PCR conditions were as follows:

The primer sequences, expected fragment size and optimal PCR annealing temperature used were as follows: elfa (elongation factor alpha)- 5/: AGG CTG ATT GCG CTG TGC, 3/: CCC CAT GCC AAC CAG AGA T (272bp, 58°C); α-actin – 5/: CAT CAG GTAGT CAGT CAG G, 3/: GAT GGT GTG ACC CAC AAC (102 bp, 53°C); cardiac muscle myosin heavy chain 6 alpha (myh6) – 5/: GCA CTA CGG CAA CAT GAG, 3/: CAA TGC ACC GAG GGA ATA (211 bp, 60°C); connexin 43 (cx43) – 5/: TCA TGC TGG TGG TCT CTC TG, 3/: AGT GGT GGA CAG ATC CTT GG (161 bp, 58°C). Automated gel electrophoresis was carried out with QIAxcel (QIAGEN, Germany) capillary gel electrophoresis system. The QIAxcel utilizes a preassembled cartridge (DNA screening cartridge) to simultaneously run samples and collect data. The gel-matrix in the gel cartridge consists of proprietary linear polymer with ethidium bromide dye. The system was used according to the manufacturer's operation manual: 15 µl of the PCR products were placed in the instrument sample tray and were automatically injected into the capillary channel and subjected to electrophoresis by selecting the AM420.mtd method from the BioCalculator software. The sample injecting voltage was 5 kV with sample injection time of ten s followed by separation voltage of five kV and separation time of 420 s. Data were analyzed on a PC running BioCalculator software, which labels the integrated peak area automatically, saves the data collected by the unit and allows size analyses after collection. This method exhibits an accuracy of 20 bp.

MEA

To obtain multi-electrode array (MEA, Multi Channel Systems, Reutlingen, Germany) recordings, MEA's with an inter-electrode distance of 200 µm, an electrode diameter of 30 µm and internal reference were used.

SCCs were transferred to the MEA, by the use of a pipette and after 3 days when the SCCs attached to the electrodes, MEA recordings were performed. For cultivation Dulbecco's Modified Eagle Medium (DMEM, Gibco® Invitrogen, Germany) supplemented with 20% fetal calf serum (FCS), 1 U/ml penicillin

Fig.1. Identification of a spontaneously contracting cardiomyocyte cell aggregate (SCC), which formed in the primary culture after one week. Scale bar indicates 100μm.

(Biochrom AG, Germany) and 10 mg/ml streptomycin (Biochrom) was used. The recordings ran 5 minutes per measurement, without external stimulation and special measurement medium. These spontaneous field potentials were recorded by means of a MEA 60- system (60 recording electrodes) and were analyzed with special MEA analyzing program (McRack 3.8.0).

Action potential recordings

Intracellular potential changes were measured with glass microelectrodes filled with 2 M KCl (5-40 MΩ). Resulting signals were recorded with a SEC-05L amplifier (npi electronics, Hamm, Germany), digitalized (EPC9, HEKA) and acquired with Pulse 8.8 (HEKA). Using the analysis software Chart 5 (ADInstruments) the properties of stable action potentials were determined. Pharmacological substrates were added to the medium in denoted concentrations and periods (Isoproterenol: Sigma-Aldrich, Germany and Rilmakalim, Sanofi-Aventis, Germany). Statistical analysis and graphs were made with Prism 4.03 (GraphPad) and Igor Pro 5.01 (Wavemetrics) respectively. Only cells with a RMP of less than -40mV were analyzed. The action potentials of the SCCs were recorded at room temperature.

Results

Cell aggregate morphology, formation ratio and beating frequency

In a previous study we described that generally the SCCs could be found in primary cell cultures from rainbow trout larvae after one week [30]. Meanwhile, we could verify this method for five other fish species (sea trout (*Salmo trutta trutta*), Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*), maraena (*Coregonus maraena*), zebrafish (*Danio rerio*) and Atlantic salmon (*Salmo salar*). However, our following study focuses on

Fig. 2. Immunochemical staining with heart specific antibodies. The left column shows fluorescent labelling of the heart specific antibodies on contracting elements differentiated *in vitro* (SCC). The right column shows the same antibody staining on frozen heart tissues sections from an adult rainbow trout as a positive control. (a) Detection of troponin I (green) and actin filaments labelled with phalloidin (red) for staining the troponin I-actin-complex. (b) Detection of α-actinin. Staining of the gap junction proteins, (c) β-catenin, (d) N-cadherin and (e) 1&2-desmoplakin. The nuclei were stained with DAPI (blue). Scale bars: 10μm.

Fig. 3. RT-PCR analysis of expression of cardiac markers (connexin43, cx43 161 bp; myosin heavy chain 6 alpha, myh6: 211 bp; alpha actin: 102 bp) in isolated spontaneously contracting cell-aggregates (SCC1, SCC2, SCC3), isolated heart tissue as the positive control (PC) and non-beating isolated cells from rainbow trout larvae from passage 3 (experiment control, EC). The housekeeping gene elfa (elongation factor alpha) was used as an internal reference. Generated PCR products were separated by capillary gel electrophoresis (QIAxcel; Qiagen).

the cells of rainbow trout. The morphology of the contracting structures was mostly ball-like or two-centre shaped, as shown in Fig. 1. The diameter of the SCCs in primary cultures varied between 25 μ m and 55 μ m with a thickness of around 4 cell layers. Furthermore, our data demonstrates that the formation ratio of SCCs is on average 0.92 ± 0.25 . Up to three SCCs could be generated from one larva. Each of the contracting areas exhibited constant contractions while the frequencies of the different contracting areas were heterogeneous. During one exemplary microscopic observation over 30 days the cell aggregate beat spontaneously with an average rate of 73 ± 20 beats per minute ($n=14$). During monitoring, the frequency of the cell aggregate was essentially constant while daily aberrations could be observed. Altogether, the activity of the SCC could be monitored up to 50 days in culture. Additionally, a one month observation of about 70 prepared larvae revealed that on average 5 % of the SCCs formed in less than seven days after preparation. After 14 days, already 70 % and after 22 days 96% of the total SCCs could be found.

Detection of cardiac-specific proteins in SCCs

In our previous study electron microscopic examinations confirmed the existence of cardiomyocytes in the SCCs [30]. In this study, we evidenced the expression of heart specific proteins in these cells with immunochemistry. Staining of the actin filaments with Phalloidin and detecting of heart specific troponin I (Fig. 2a) revealed cells with well-organized cross-striated actin-tropomyosin in the SCCs. The z-lines of each sarcomere in the cardiomyocytes were made visible using an antibody against the actin-binding protein α -actinin (Fig. 2b). We further aimed to determine the presence and properties of gap junctions within the contracting cell aggregates, because their number, size, and distribution are important determinants of conduction during physiological and pathological conditions [32]. The staining showed that the

three *fascia adhaerens* (resp. the gap junction) marker β -catenin (Fig. 2c), N-cadherin (Fig. 2d) and desmoplakin (Fig. 2e) are equally distributed between the cells of the SCCs. All tested proteins showed a similar expression pattern in the SCCs as in frozen tissue sections of a heart from an adult rainbow trout (positive control). Additionally, the RT-PCR analyses revealed the expression of cardiomyocyte-specific genes, cardiac muscle myosin heavy chain 6 alpha (myh6), α -actin and connexin 43 (Fig 3). Especially the detection of connexin 43 as a standard marker for the gap junction system in the myocardium supports the idea that SCC exhibit a gap junction system. Distinct bands were detected in all three samples from different SCCs and also in the positive control. In comparison to these samples, in the control specimen (cultivated non beating cells from rainbow trout larva from passage 3) only the housekeeping gene was amplified.

Furthermore, in this study also pacemaker channels were analyzed via immunochemistry. Harzheim et al. 2008 [33] described the HCN4 as an important cardiac pacemaker marker and elevation of HCN4 channel activity by cAMP is essential for viability. In the generated SCCs the antibody against HCN4 could be detected (Fig. 4a). Also Connexin 45, a gap junction protein, is likely to act as a constituent of the pacemaker system [34] and could be detected in our SCCs (Fig. 4b). Nevertheless, the expression of these proteins could not be found in the whole aggregate. Only a distinct population of cells contains connexin 45 indicating that these cells exhibit pacemaker cell activity that might activate the whole SCC.

Conduction properties of SCCs via MEA measurements

To evaluate the applicability of the SCCs as a human heart model, their electrophysiological integrity has to be investigated. The conductive velocity of single SCCs was analyzed and excitation maps were generated by seeding cardiomyogenic aggregates on MEAs. Exem-

Fig. 4. Immunofluorescence staining with pacemaker specific antibodies. (a) Detection of HCN4. (b) Detection of connexin 45 as a gap junction marker in pacemaker cells. Signals could only be detected in a small part of the aggregates. The nuclei were stained with DAPI (blue). Scale bars: 10 μ m.

plary, the culture of two SCCs from different larvae, seeded on one single array, is shown. Three days after plating on the MEA dish, both SCCs started to contract rhythmically and developed spontaneous electrical activity. This activity is indicated by a typical cardiac extracellular field potential (FP) shape (Fig. 5a). During the first days the upper and larger SCC showed clear FPs on 15 electrodes. The lower and smaller SCC had FPs on two electrodes only. In the following days, the growth of both SCCs was observed and their FPs could be measured on more electrodes. Besides, the smaller SCC migrated in the direction of the bigger SCC (Fig. 5b). On day 49, already more than 40 electrodes out of 60 detected FPs (Fig. 5c). During the recordings the beating frequency in both aggregates decreased over time (Fig. 6). The monolayer of cells around the aggregates seemed to impede the contractions. Furthermore, it could be observed, that the smaller SCC (right column) had a lower field potential than the bigger SCC (left column). The different heights of the FPs might be due to non-uniform interspaces between the electrodes and the cells. Although at the half of the testing period (day 28) cells from both SCCs were laying adjacent to each other, their FPs were still separated (Fig 5b and 6d). As usually one pacemaker triggers a functional syncytium, formed by gap junctions, it can be assumed that at this time point, the two aggregates were still independent from each other. After 49 days, however, the beating frequency of the smaller SCC fell in line with the frequency of the bigger SCC, thus building one functional syncytium (Fig. 5c).

Fig. 5. Extracellular recordings of two SCCs plated on a MEA plate. The left pictures illustrate the spontaneously beating tissue and the right picture the electrical activity of all 60 electrodes on day 3 (a), day 28 (b) and day 49 (c) after plating.

Fig. 6. Representative recordings from the two SCCs plated on the MEA. Left column shows the changes in the extracellular recordings of the bigger SCC (electrode number: 23) and the right column the smaller SCC (electrode number 38) on day 3 (a), day 28 (b) and day 49 (c) after plating. On day 28 the overlap of the two SCC on one electrode (number 25) is visible (Fig. 5d). FPs from both aggregates can be measured on the same electrode. The illustration shows the independence of both aggregates. The frequency of the smaller aggregate is higher (arrows) and the field potential lower than of the bigger SCC.

Fig. 7. Intracellular action potential recordings from cells of SCCs. (a) Example of an action potential from an *in vitro* cultivated spontaneously contracting cardiomyogenic aggregate. (b) Correlation of the APD₉₀ and the period of time (n=19).

Table 1. AP parameters of control SCCs. Action potential was measured with sharp electrodes in single cells of SCCs from rainbow trout at room temperature. RMP, Resting membrane potential; APA, action potential amplitude, dV/dt_{max} , maximum depolarization velocity; APD₅₀ and APD₉₀, action potential duration at 50% and 90% of repolarisation; period, time between two action potentials, reciprocal of beating frequency.

Parameter	Control (n=25)
RMP (mV)	-52,08±0,2
APA (mV)	61,9±0,2
dV/dt_{max} (V/s)	18,7±2,6
APD ₅₀ (ms)	82,8±7,6
APD ₉₀ (ms)	104,8±7,7
Period (s)	1,3±0,3

AP recordings

A fundamental prerequisite for the application of the SCCs from fish as pharmacological human heart model system is a functional similarity in the the physiology of the cardiomyocytes of both species. The similarity must be reflected in comparable intracellular action potential recordings (resting membrane potential (RMP); action potential amplitude (APA), maximum depolarization velocity (dV/dt_{max}); action potential duration at 50% and 90% of repolarisation (APD₅₀ and APD₉₀) and the period). Similar to the action potential found in human ventricular cells, recordings from single cells of the SCC showed a rapid upstroke followed by a long-lasting plateau phase that ended in a phase of rapid terminal repolarisation (Fig. 7a). In the illustrated example the resting membrane potential is -65 mV. Average AP parameters from the *in vitro* cultivated SCCs of rainbow trout larva are summarized in Table 1. In total, measurements of the SCCs (n=25) revealed a resting membrane potential of -53 mV, a maximal upstroke velocity of 19 V/s and an amplitude of 62 mV. Furthermore, the duration of the AP was measured at 50 % and 90 % of repolarisation (83 ms and 105 ms, respectively). The mean period between two APs was 1.3 s. In our experiments, the cells recorded from SCCs had a stable resting membrane potential in between the action potentials. This indicates that these cells do not act as pacemaker cells well known for their spontaneous depolarisations between action potentials.

Another important factor for intact cardiac electro-

physiology is the correlation between the APD₉₀ and the frequency. In mammals, an increase of the beating frequency usually follows a decrease of the APD₉₀. The same correlation could also be observed in the *in vitro* developed SCCs (Fig.7b).

Effects of β -adrenergic agonists Isoproterenol

One of the most critical determinants of normal cardiac electrophysiology is the intact response to hormones and transmitters of the central nervous system. For that reason, we studied the effects of isoproterenol, a well known sympathomimetic drug, on the action potentials of the SCCs. We applied isoproterenol as a β -phenylethylamin derivate [35]. It is a standard agonist of the β_1 - and β_2 -adrenergic signalling cascade, like adrenaline. Isoproterenol acts by phosphorylating L-type calcium channels via a cAMP- dependent kinase [36]. As a result, isoproterenol increases the contraction intensity (positive inotropic) and the beating frequency (positive chronotropic) in the mammalian heart.

In Figure 8a the effects upon addition of 10 μ M isoproterenol are illustrated. In comparison to the control (cells from non-treated SCC) treated cells show a faster upstroke during the depolarisation phase indicating a change in sodium channel conductance. Furthermore, at the peak of the curve the short influx of potassium is better visible than in the control. Additionally, the plateau phase was prolonged, which could be a possible explana-

Fig. 8. Effect of β -phenylethylamin derivate isoproterenol (10 μ M). (a) The action potential of a control cell is shown in comparison to the AP of a cell treated with isoproterenol. (b): Average extension of action potential duration 90 (APD₉₀) in treated (n=5) SCCs compared to control (n=5) SCCs. Data are mean values $\pm$ SEM. *p < 0.05 significantly different from control.

tion for the positive inotropic effect that might be related to an increase in Ca^{++} -influx mediated by L-type Ca^{++} -channels. The L-type calcium channels are open for a longer period. For that reason, the potassium channels are opening delayed. On average, the APD₉₀ of cells from the isoproterenol treated SCCs is about 200 ms longer than in the control (Fig. 8b).

Effects of potassium channel opener Rilmakalim

The effects of rilmakalim as a benzopyran potassium channel opener in higher vertebrates were studied by Linz et al. in 1992 [37]. Rilmakalim presents many characteristics of a reversible ATP-sensitive potassium channel activator in cardiac tissue. Also Riccioppo Neto et al. 1997 [38] described that the most significant effect of rilmakalim on rabbit myocardium was a concentration-dependent reduction of the APD. This effect was also described by Bouchard et al. 1995 [39]. To verify whether homologous potassium channels exist in cells of the fish SCCs and whether they show a similar response to the application of rilmakalim, APs were measured in cells before and after the addition of 10 μ M rilmakalim (Fig. 9). As shown previously (Fig. 7b), the increase in frequency and decrease of the period, respectively, are most likely the indirect result of the reduction of the APD₉₀ induced by rilmakalim.

Fig. 9. Effect of the potassium channel opener rilmakalim. (a) The frequency of the action potentials of the cells from SCCs (n=3) increases significantly after addition of 10 μ M rilmakalim. (b) Reduction of period between two APs and the action potential duration 90 (APD₉₀) in treated (n=3) compared to untreated (n=3) SCCs. Mean $\pm$ SEM. *p < 0.05, #p < 0.27, indicates significant difference compared to controls.

Discussion

Myocardial infarction is a major cause of morbidity and mortality worldwide. The limited ability of progeny of cardiac cells to survive an ischemic attack results in a

damaged heart and heart failure [40]. Consequently, models for the human heart research are of great importance. In the past, research groups have studied the electrophysiology of murine, chicken, fish and also human cardiomyocytes from whole organs, heart slices or *in vitro* differentiated cells. In our present study, a new *in vitro* test system as a model in human heart research for the use in medical and pharmacological studies was established. The model consists of a primary cell culture which exhibits long-term spontaneously contracting cardiomyogenic cell aggregates (SCC) generated from rainbow trout larva.

In this study the 3D SCCs were beating over a period of up to 50 days. Furthermore, the formation ratio is, with up to three SCCs developing from the primary culture of one larva, very high (0.92 ± 0.25 , $n=77$) This indicates that the technique is easy and reproducible at any time.

In addition, we have proven the existence of cardiomyocytes in the SCCs via immunochemistry and RT-PCR. Besides specific heart muscle proteins (Troponin I, α -actin and cardiac myosin) also proteins of gap junctions (β -catenin, N-Cadherin, connexin 43) could be verified.

Normal heart muscle cells need an exogenous stimulus such as a change in the electrical field to depolarise the cell membrane. By this, the threshold potential is reached, followed by the AP [41]. In comparison to that, pacemaker cells do not need a stimulus to change their membrane permeability, but undergo spontaneous depolarisation mediated by certain ionic currents including the HCN4 mediated ones and generate an AP without external influence. These pacemaker cells are responsible for the autorhythmic heart activity.

The spontaneous electrical activity observed within the SCC's indicates the presence of pacemaker cells within the tissue. To verify the existence of these cells immunostainings with antibodies specific against HCN4 and connexin 45 were performed. Immunoreactive cells were present only in a small, distinct part of the aggregates. It is very likely that these cells activate the contraction in the whole SCC which would reflect the situation in normal mammalian and fish hearts. The validation of these two antibodies was proven via blast search in ncbi (<http://blast.ncbi.nlm.nih.gov>). Here, the sequences of HCN4 and connexin 45 in fish (zebrafish) and mammals (human, mouse, rat) showed a high similarity [up to 75 %; data not shown]. Further we described that the two antibodies marked only distinct spots in the SCCs. Therefore, we are convinced

that also in trout HCN4 and Connexin 45 can be used as a marker for pacemaker cells.

In agreement with the existence of pacemaker activity, during measurements via multi electrode arrays a consecutive field potential could be observed. One important determinant of electrical load distribution and the conduction properties of cardiomyocytes are the cell – cell - connections by gap junctions [32]. Our results demonstrate that the developed SCCs do have this determinant. This fact, the detection of gap junction proteins and the evidence that two previously independent SCCs grew together, exhibiting only one shared FP recording after 49 days in culture (see Fig. 5c), render obvious that a functional syncytium was formed *in vitro*. (To our knowledge, it is the first time that a long-term autonomously organ-like syncytium out of heart cells could be developed).

Additionally, the measurements showed, that during the 49 days of observation the FP of the two aggregates did not change a lot. In both aggregates a high and a smaller peak are present. We assume that the *in vitro* activity of the SCCs is not changing over the time. Moreover, the SCC expanded by cell division. Also new cells exhibited the same FP, which show that heart cells were generated. For that reason, we assume that the phenotype of these trout cells is not changing over the time. This point has to be proven in further studies.

For pharmacological examinations, the response to drugs in a functional cell cluster is very important.

Intracellular potential recordings of the SCCs reveal that some properties of the action potential are similar to human cardiomyocytes and hESC-CMs, in contrast to the AP pattern of mice [22, 23, 42]. The resting membrane potential exhibited a voltage of down to -65 mV, while the average was -53 mV. In comparison resting membrane potential of mammalian myocytes exhibit -80 mV. We assume that the RMP of the SCCs are depending on the age of the prepared fish larva or the age of the *in vitro* developed SCC, as it could be observed in embryonic cardiomyocytes. Also, freshly isolated ventricular cells from an adult rainbow trout exhibit a RMP of around -75 mV [27]. Due to these statements, we assume that the SCC of older larvae might reach a RMP around -75 mV, too. This statement has to be examined for the SCCs in further studies.

Besides the RMP, especially the APD_{90} revealed similarities to human cardiac physiology. Nemtsas et al. 2009 [22] measured the APD_{90} in the ventricle and the atrium of adult zebrafish, human and adult mice preparations (Tab.2). He stated that the APD_{90} in the atrium and

	SCC	Atrium*			Ventricle*		
		Zebrafish	Humans	Mouse	Zebrafish	Humans	Mouse
Preparations (n)	25	16	28	7	16	5	6
APA (mV)	61,9±0,2	100±2	97±2	104±1	99±2	101±4	95±2
RMP (mV)	-52,8±0,2	-74±2	-74±1	-81±2	-72±2	-77±3	-74±1
dV/dt _{max} (V/s)	18,7±2,6	129±9	246±22	239±10	92±5	180±10	166±11
APD ₅₀ (ms)	82,8±7,6	46±2	148±8	9±1	110±4	165±12	9±3
APD ₉₀ (ms)	104,8±7,7	62±2	316±7	42±4	132±4	242±15	82±4

Table 2. Action potential parameters in SCC compared with atria and ventricles of the adult zebrafish, mouse and human. Action potentials were measured with sharp electrodes. *In vitro* generated SCC and heart tissue of zebrafish (kept at 28°C) were beating spontaneously while human and mouse tissues were electrically stimulated at 1 Hz and kept at 37°C. APA, action potential amplitude, RMP, Resting membrane potential; dV/dt_{max}, maximum depolarization velocity; APD₅₀ and APD₉₀, action potential duration at 50% and 90% of repolarisation. *Data from Nemtsas et al. 2009 [22].

ventricle of mice is 7.5 times and three times shorter than in human beings, respectively. The APD₉₀ in the atrium of zebrafish was five times and in ventricle two times shorter than in adult human cardiomyocytes. Measured values in human ventricle were 242 ms and 316 ms in the atrium. In our study, the *in vitro* developed SCCs exhibit APD₉₀s which are only twice (compared to the data of ventricle) to thrice (compared to the data of atrium) shorter than in humans and very similar to hESC-CMs (APD₉₀ ~ 100 ms, [42]). Here, it is necessary to mention, that the measurements of the fish cardiomyocytes have been conducted at lower temperatures (25-28°C), while those of human and mice were measured at 37°C. Shiels et al. 2002 [43] described that the cardiac contractility is influenced by temperature in all vertebrates. The group showed that increased temperature causes a decrease in intracellular calcium concentration in trout atrial myocytes. Therefore, we suppose that the differences in APD between SCCs and the heart tissues of zebrafish, human and mouse are in fact much larger than illustrated in table 2. Due to this, the influence of the temperature on the APD in the SCC should be considered in further studies.

In contrast, the maximum depolarization velocity (dV/dt) and the action potential amplitude (APA) of SCCs are lower than in adult cardiomyocytes of zebrafish, humans and mice [22]. We deduce that the overall shape of the SCC AP exhibits similarities to that of the human heart and that the AP of the generated SCCs is in parts closer to human cardiomyocytes than mouse and zebrafish.

We assume that our SCCs consist of larval cardiomyocytes, where, similar to embryonic cardiomyocytes, calcium channels are responsible for a gentler slope. Calcium currents are slower compared to sodium currents, which are activating the initial rapid depolarisation in adult cardiomyocytes [42]. Another explanation for the low dV/dt-value could be the voltage sen-

sitivity of sodium channels that are at least partly inactivated at a resting membrane potential of about -60 mV. In several measurements of SCC cells, the membrane potential was less negative. Therefore, some of the fast sodium channels might have been in an inactivated state, thus causing a lesser response to excitation of the cell membrane.

In addition, we find several similarities in the shape of the action potential between our data and the action potentials described for hESC-CMs [9, 42]. Our data show that the AP parameters of SCCs are closer to adult human cardiomyocytes than to hESC-CM, maybe because of their maturity. The SCCs were established using fish larvae of eye-point-eye stadium or yolk-sack stadium, presenting a more matured system than that of hESC-CMs [42]. An evidence for the advanced development of cardiomyocytes in our *in vitro* model was also given in a former publication [30]. Electron microscopy of cardiomyocytes from SCCs cultured for two to four weeks revealed fully developed striated muscle cells with differentiated sarcomers, in contrast to cardiomyocyte progenitor cells from 170 degree-day old *Oncorhynchus mykiss* larva. However, this hypothesis will be further substantiated in following studies.

Although, the shape of the action potential is similar to human, the underlying ion currents might be different. For that reason, first drug tests were performed in this study. For the drug analyzes, we chose isoproterenol as a standard agonist of the β_1 -adrenergic signalling cascade and rilmakalim as a potassium channel opener. The electrophysiology of the SCCs reacts in the same way like expected in human heart. This indicates that our developed test-system might reveal the same ion channel properties like expected in human heart cells. The last years, significant differences in ion channel composition of the heart between fish and mammals [24, 44] were described.

Further, differences were also characterized for the stage of heart development in vertebrates [45] and for atrial and ventricular myocytes of trout heart [46]. Especially the sodium (voltage-gated-sodium channels, Nav) and the potassium channels (e.g. Erg channel) seem to be different in their composition between the three mentioned comparisons [47, 48]. For that reason, further analyzes of ion channel composition (especially I_{Na} , I_K) have to be done on the SCC in future.

Recapitulating, our data show that the action potential characteristics from *in vitro* cultured cardiomyocytes of rainbow trout in part resemble those of humans resp. hESC-CMs. These results are consistent with other publications, which prove that fish cells are better suited as a human model than mouse cells because of their electrophysiology (see introduction). In addition to that, the fact that the SCCs are beating spontaneously for a long time prove that they are a potential alternative to existing human models.

SCC's might provide an experimental system to explore the molecular mechanisms of the addition of drugs or cardiomyogenic development and complement mouse studies which are limited by their action potential characteristics. Further improvement of the system, especially the resting membrane potential of the SCCs, which is important in human cardiac physiology, might be achieved

by ion channel related mutation screenings. As sequence data accumulates for many fish species and genetic tools and reagents are developed, the importance of fish models will continually increase in importance for studies of development, toxicology, evolution and human diseases [15]. Additionally, we believe that this *in vitro* heart system could also be a useful model for studying physiology (e.g. ion channel composition and ion concentration against temperature), pathology, development and also regeneration of heart in fish.

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