Journal of Cell Science & Therapy
ISSN: 2157-7013

Grunow et al., J Cell Sci Ther 2012, 3:1
DOI: 10.4172/2157-7013.1000116

Research Article Open Access

Development of an *In vitro* Cultivated, Spontaneously and Long-term Contracting 3D Heart Model as a Robust Test System

Bianka Grunow[1]*, Maren Schmidt[1], Matthias Klinger[2] and Charli Kruse[1]

[1]*Fraunhofer Research Institution for Marine Biotechnology, Fraunhofer Society, Paul Ehrlich Str. 1-3, 23562, Lübeck, Germany*
[2]*University of Lübeck, Institute of Anatomy, Ratzeburger Allee 160, 23562, Lübeck, Germany*

Abstract

The study deals with an *in vitro* heart model established from rainbow trout larvae. This model is based on spontaneously contracting cell aggregates (SCC) exhibiting fully developed cardiomyocytes connected via gap junctions and building up a functional syncytium with a pacemaker centre. The cellular structure and the electrophysiological properties of the cardiomyocytes resembled that of human heart cells. It was possible to generate more than one SCC from one fish larva. The SCCs contracted spontaneously over several weeks with a stable contracting frequency similar to human heart. Our analysis supports the use of this model as a high-throughput test system in cardiac research with the potential to complement and reduce animal testing.

Keywords: Heart model; Cardiomyocytes; Cardiomyogenic contraction; Test system; Cell culture; Animal reduction; 3Rs

Abbreviations: ATU: Accumulated Thermal Units; CF: Contracting Frequency; DMEM: Dulbeccos Modified Eagle Medium; EM: Electronic Microscopy; FCS: Fetal Calf Serum; RT: Room Temperature; SCC: Spontaneously Contracting Cell Aggregate

Introduction

Despite the fact that invasive/non-invasive therapies of cardiovascular disease have advanced dramatically over the last two decades, heart disease is still a major cause of morbidity and mortality in industrialized countries [1,2]. Many research groups focus on the development of cardiomyogenic models to dissect how damaged heart muscle may be repaired. Further, neovascularization of infracted myocardial tissue as well as drug treatment are topics of major importance in cardiology, as regeneration capacity of cardiomyocytes in mammals is generally low [3,4]. Any improvement of drug treatment in heart diseases will rely on the development of specific cardiac test systems.

Currently, the development of one new cardiac drug costs around 400 million US dollars [5] to ensure quality, efficacy and safety. Although there are several cardiomyogenic *in vitro* and *in vivo* models available including cell cultures [1], the testing of a new compound in high throughput test systems relies on the best option for analyzing the effects on proteins, the specificity and efficacy of a drug and also the toxicity caused by a drug. Each drug is tested on cardiomyocytes for analyzing the effect on heart activity. Therefore, it is important to know that in addition to the communication between cardiomyocytes an interaction with surrounding non-cardiomyogenic cells is present [6]. For that reason, any model for testing drugs should be complex and involve intercellular interactions as well as contain human analogue characteristics.

Currently available cardiac *in vitro* models are primarily cell monolayers or single cells. For that reason, during initial drug tests an analysis of influence on cell-cell interactions are not present and a limited aspect of the *in vivo* situation is presented [7]. Another problem is the use of test systems generated from marine organisms as several publications show that the electrophysiological properties of cardiomyocytes from mouse and rat differ significantly from human characteristics.

We developed an *in vitro* model that is based on spontaneously contracting cell aggregates (SCC) generated from rainbow trout larvae (*Oncorhynchus mykiss*, Walbaum). This 3D *in vitro* heart model contains fully developed heart muscle cells with cell-cell connections and a pacemaker centre. Furthermore, the use of these SCC as a human drug test system has been described as their electrophysiological properties and their reaction to drugs are similar to human cardiomyocytes [8,9]. Fish cardiomyocytes could be an alternative because of their analogy to human heart electrophysiology [10-12].

In this study, we optimized the method of SCC generation for use as a high-throughput *in vitro* test system. Furthermore, in line with previous studies, we examined additional structures described to be important for the evidence of cardiac functionality [13]. Such models should represent 3D cardiac tissue with interconnected cardiomyocytes exhibiting a synchronous contraction, due to complete electrical couplings.

Materials and Methods

Cell isolation

Rainbow trout larva (*Oncorhynchus mykiss*, Walbaum) from the beginning of the eye-point-eye stadium (at 252 ATU; ATU - accumulated thermal units; day degree) till the end of yolk sac stadium (462 ATU) were obtained from a fish farm located in Luetjenburg (Germany). ATU was calculated as the summation of the daily mean temperature of the larvae. The larvae were kept indoors at 6°C by a continuous flow of fresh water (0.15 m sec^{-1}). The cell isolation was performed

***Corresponding author:** Bianka Grunow, Fraunhofer Research Institution for Marine Biotechnology, Fraunhofer Society, Paul Ehrlich Str. 1-3, 23562, Lübeck, Germany, Tel: +49-451-384448-16; Fax: +49-451-384448-12; E-mail: bianka.grunow@emb.fraunhofer.de

Received January 19, 2011; **Accepted** February 14, 2012; **Published** February 16, 2012

Citation: Grunow B, Schmidt M, Klinger M, Kruse C (2012) Development of an *In vitro* Cultivated, Spontaneously and Long-term Contracting 3D Heart Model as a Robust Test System. J Cell Sci Ther 3:116. doi:10.4172/2157-7013.1000116

based on a procedure described by Grunow et al. [9]. For anaesthesia the larva or the carefully manually perforated egg was incubated in a 20% MS 222 (methanesulfonic acid salt; Sigma Aldrich, Germany) solution dissolved in aquarium water for 5 minutes. The larvae were dissected with scissors and digested in 500 µl 0.1% trypsin (PAA Laboratories, Austria) for one minute. Digestion was ended by addition of the triple volume of culture medium and centrifugation for five minutes at 130 g. The resulting cell pellet was resuspended in ~2 ml culture medium, seeded into 6 well culture plates (TPP, Switzerland) and cultivated at 20°C and 2.0% CO_2. The culture medium was composed of DMEM (Dulbeccos Modified Eagle Medium; Gibco Germany) supplemented with 20% FCS (fetal calf serum) and 100 U/ ml Penicillin and 0.1 mg/ ml Streptomycin (PAA Laboratories, Austria). All protocols have undergone an ethical review process by the German animal welfare law §8a (ethic committee: Ministerium für Landwirtschaft, Umwelt & laendliche Raeume of Schleswig-Holstein; permit number: 41/A01/09).

Electron microscopic analysis (EM)

The aggregates were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h. For post fixation the SCCs were kept in 1% OsO_4 in 0.1 M cacodylate buffer for 2 h. The samples were then dehydrated with ethanol and embedded in araldite (Fluka, Switzerland). Ultrathin sections were stained with uranyl acetate and lead citrate (Ultrostainer Carlsberg System, LKB, Sweden) and were examined with a Philips electron microscope EM 400 at 60 kV (Philips, The Netherlands).

Experimental procedure

Primary cultures from the SCCs were cultivated at 20°C and 2.0% CO_2. During a cultivation period from three weeks up to three months medium exchange was carried out the first time two days post preparation (day 2) and then every three or four days.

To determine the rate of SCC generation as a function of developmental stage, larvae were prepared at six different time points. The developmental ratio (formation ratio) of the SCCs was determined via the relation (division) of number of developed SCCs and number of prepared larvae. At each preparation time 18 larvae were taken. The age of the prepared larvae ranged from eye-point stage (252 ATU, 294 ATU and 335 ATU) to yolk-sac stage (378 ATU, 420 ATU and 462 ATU). At around 350 ATU the rainbow trout larva is hatching. For the period of three weeks post preparation the culture dishes were microscopically checked for SCCs twice a week (Axiovert 40CFL; Germany). At the end of this observation period, the total number of SCCs was illustrated as bars. The total number of SCCs has been summed up and set up as 100%. In the bar the percental relation of cell cultures with 0, 1, 2, 3, 4 or 5 SCCs is shown. All SCCs were photographed with AxioVision (Zeiss, Germany) and filmed for 30s with Powershot A640 (Canon) via microscope adapter directly after development. The contraction frequency (CF) was calculated for one minute. The area representative for the size of the SCCs was determined via the software of AxioVision.

Temperature dependence of the beating frequency was determined over the range of 16°C to 25°C in four SCCs prepared at 350 ATU. In the experiment the starting temperature was 20°C with 2.0% CO_2 incubation. This was lowered to 16°C with 1.8% CO_2 and then raised to 18°C and 1.9% CO_2, 20°C and 2.0% CO_2, 25°C and 2.2% CO_2. At the end of the experiments the CF at 20°C with 2.0% CO_2 was recorded. CO_2 incubation was changed for having a pH value of 7.8-7.9 in the cell culture, which was the pH of the prepared fish larva. The cells were kept for one hour at each temperature for adaptation to the new conditions. The starting CF of SCCs kept at 20°C equaled 100% and the aberration from the initial temperature was calculated as a percentage.

Statistical analysis

Data are presented as mean ± SE. Statistical analysis was performed using R-Statistik and Graph Pad Prism5. The results were tested for normality via Kolmogorov-Smirnov Test. The exclusion of a normality ($p > 0.05$), a Kruskal-Wallis-signed-rank-test was performed. Based on a significant effect ($p < 0.05$) the differences between the groups were controlled and calculated via Wilcoxon-signed-rank-test.

Results

Cell aggregate morphology and evaluation by electron microscopy

EM analysis demonstrated the SCCs were composed of fully developed cardiomyocytes. These groups of cells took on 2 primary gross structures: ball shaped (Figure 1a) or a shape that was rounded with 2-centres (Figure 1b).

Additionally, the analysis of the cardiomyogenic structures showed high concentration of mitochondria in the cytoplasm of the cardiomy-

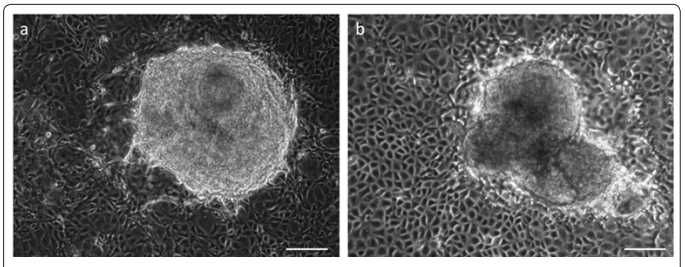

Figure 1: Identification of spontaneously contracting cell aggregates (SCC) in cell cultures from rainbow trout larvae (*Oncorhynchus mykiss*) from 350 ATU. The form of the SCC was mostly (a) ball shape or (b) two centre-shaped. Additionally, non-contracting cells were building a monolayer in the cell dish. Scale bar indicates 100 µm.

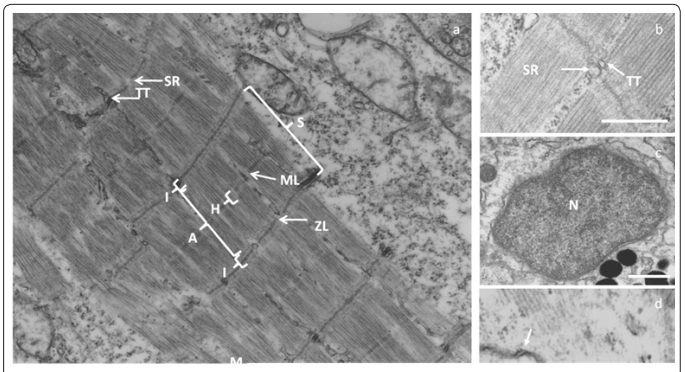

Figure 2: Electron micrographs of structures in cardiomyocytes of a SCC generated from 350 ATU old larvae. (a) Sarcomeres (S) which are bordered by z-Lines (ZL) and subdivided in I-Band (I: isotrop) and A-Band (A: anisotrop) with H-Zone (H) and M-Line (ML: M: middle of a sarcomere). High concentration on mitochondria (M) is present. Visible is also the Cell membrane (CM) and sarcoplasmic reticula (SR) and T-tubuli (TT). (b) Co-location of SR and TT in the z-Line. (c) Detection of nuclei (N). (d) The transversally orientated sections of the intercalated disk consist of *fascia adhaerens* (arrows). Scale bar indicates 1 µm.

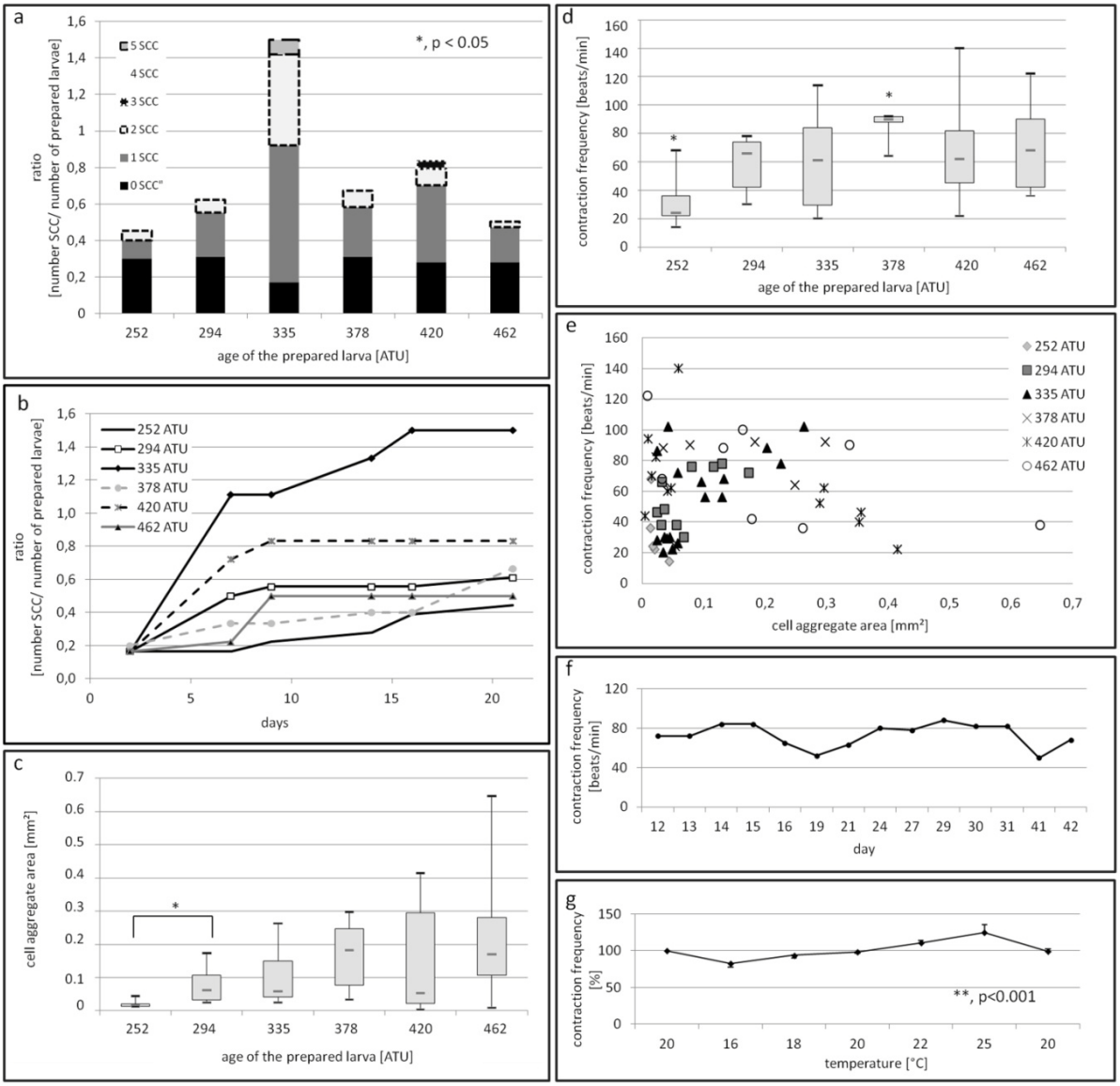

Figure 3: Analysis of the SCCs at different ages of the prepared larvae [ATU – accumulated thermal unit, day degree]. (a) Formation ratio as the relation number of developed SCC and prepared larvae (18 larvae at each preparation time) and the relation of larvae which developed 1 to 5 SCC. The distribution exhibits normality ($p < 0.05$; Kolmogorov-Smirnov test). (b) Formation ratio of SCCs in relation to the time. (c) Box-Whisker-plot of the size of the SCCs in dependence of the age of the prepared larvae. Illustrated are minimum and maximum (crossbars at the end), median (grey bar) and upper and lower quartile (borders of the bar). Significant differences between the group of 252 ATU and 294 ATU ($p < 0.05$, Kruskal-Wallis-signed-rank-test). The size of the SCCs rises significantly with the age of the larvae ($p < 0.05$, Wilcoxon signed-rank-test). (d) Box-Whisker-plot of the contraction frequency of the SCCs in dependence of the age of the prepared larvae. Illustrated are minimum and maximum (crossbars at the end), median (grey bar) und upper and lower quartile (borders of the bar). Significant differences between the groups ($p < 0.05$, Kruskal-Wallis-signed-rank-test) exist. The beating frequency was lower at 252 ATU and at 378 ATU higher than at the other groups ($p < 0.05$ Wilcoxon signed-rank-test). (e) Contraction frequency in dependence of the aggregate size shows no significantly correlation ($p = 0.8$, t-test). (f) Illustration of contraction frequency of a SCC prepared from a 350 ATU old larva during 30 days observation. Frequency was between 50 and 88 beats/ min. (g) Contraction frequency in dependence of the temperature (n=4, 350 ATU). A highly significant correlation between temperature and contraction frequency was present ($p < 0.001$, t-test). Higher temperature evokes higher contraction frequencies and *vice versa*.

ocytes (Figure 2a). Furthermore, the T-Tubuli structures in the cells of the SCCs were located in the z-bands and next to the sarcoplasmic reticula (Figure 2b). Moreover; in the centre of every cardiomyocyte one nucleus was located (Figure 2c). The EM analysis verified structures of cell-cell connections (Figure 2d) within the SCCs.

Formation ratio, size and contractility of the SCCs

Analysis of the developmental ratio showed that the total number of SCCs followed a normal curve of distribution ($p < 0.05$; Kolmogorov-Smirnov Test; 252 ATU: 8 SCC; 294 ATU: 11 SCC; 335 ATU: 27 SCC; 378 ATU: 10 SCC; 420 ATU: 15 SCC; 462 ATU: 9 SCC), Figure 3a. The maximum value had a ratio of 1.5 at 335 ATU (short time before hatching), Figure 3a. At this time up to five SCC could be generated out of one fish larva. The analyses of the time of SCC development revealed that in all groups after two days (after the first medium exchange) SCCs with a ratio of 0.2 were present (Figure 3b). After nine days 75 up to

100% of all SCCs in a three-week-observation could be detected.

Another important factor for the use of a model system as a high through-put test system is the time of preparation. For example, in a 15 minute preparation the cell cultivation of six larvae was possible which means an average generation of 9 SCCs. A 96 well plate having one SCC per well could be managed in a preparation time of 2.6 hours (not included the SCC-developing time).

Size determination of the SCCs revealed a significant difference between the groups at 252 ATU and 294 ATU ($p < 0.05$; Kruskal-Wallis-signed-rank-test), Figure 3c. The area of the SCC increased with the age of the larva. At 252 ATU the SCCs exhibit an area of 0.02 mm^2. In comparison, at 462 ATU the SCCs area was four times bigger. In the other SCC-groups no significant difference between the sizes could be calculated.

To evaluate the applicability of the SCCs as human heart model, their CF has to be investigated. The studies showed a significant difference in the CF between the SCC groups of the diverse larval ages in relation to different preparation times ($p < 0.05$; Kruskal-Wallis signed-rank-test), Figure 3d. Comparison of the groups revealed that at 252 ATU the CF was significantly lower and at 378 ATU significantly higher compared to the other groups ($p < 0.05$; Wilcoxen- signed-rank-test), Figure 3d. The CF of the SCCs from other preparations did not show a significant difference ($p > 0.05$). Superficially, the contraction frequencies from 294 up to 462 ATU was similar or equal to the human heart frequency (60 – 80 beats/min). An analysis of the comparison between CF and size of the SCCs showed no significance ($p=0.8$; t-test; Figure 3e).

Another aspect for the evaluation as a test system is the stability of the frequency over a long time period. For that reason, the frequencies of SCCs from a larva at the age of 350 ATU were measured over 30 days. The SCC started to contract 12 days after preparation. The contraction of the SCC was synchronous and constant during the measurements. However, small variations between the measurements were recorded. The SCCs exhibited a frequency of 50 to 88 beats per minute (Figure 3f).

Figure 3g depicts the variance of CF of the SCCs with increasing and decreasing temperature. The CF correlated highly with temperature ($p<0.001$; t-test). A steady and significant increase in their frequency with increasing temperature and *vice versa* was observed. The maximal frequency was achieved at 25°C. The variance of the CF at 20°C did not differ between the three measurements at 20°C (at the beginning, middle and end of the temperature experiment).

Discussion

This study reports an *in vitro* heart model (SCC – spontaneously contracting cell aggregate) from rainbow trout with human analogue properties, which can be generated in high numbers. For the application as a test system in e.g. pharmacology the ratio of the generation of these SCCs and the time of the generation is economically important. Furthermore, for an application in human research the characteristics of the cells should be similar to human heart cells.

The detailed electron microscopical analysis revealed fully developed cardiomyocytes which exhibit myofilaments with the typical tightly packed and highly organized sarcomer structure, mitochondria in high amounts, sarcoplasmic reticulum and in the cell centre a nucleus. The high number of mitochondria indicates high metabolic activity of the cells, as they assure the aerobic metabolism.

Furthermore, we could show a co-location of the T-tubulus localization and the sarcoplasmic reticulum system similar to human heart cells (Figure 2b), [14]. This structure is important for ion exchange between cells and ion influx into the cells for generation of action potentials, however in this study this structure is only reported in small amounts. Eschenhagen et al. [13] described that in newborn rats this system is absent. We assume that in our model the larval age of the prepared fish (beginning of yolk sac stage) might be the reason for this small number. Immunocytochemical analysis published in [8,9] revealed the existence of the typical contractile proteins actin, troponinI and myosin and that the cells differentiated from progenitor cells *in vitro* [8]. Also, proteins of intercalated disk like β-catenin, N-cadherin and 1 & 2 desmoplakin and the gap junction proteins connexin 43 and connexin 45 in pacemaker cells could be detected. Most of the important features mentioned by Eschenhagen et al. [13] for fully developed adult cardiomyocytes are also present in the SCCs. Even if the cells of the SCC are not homologous to the human heart in every detail, it could be stated that the SCCs contain cardiomyocytes which are connected via cell-cell-contacts like adherens junctions, desmosomes and gap junctions [9] and are building up a functional syncytium which is similar to human heart tissue. The contractile function in the SCC activated by a pacemaker centre is synchronous and as a human analogue, e. g. action potential as well as effects of drugs as important for a model system are present in the SCCs [9,15-17].

Further, the study showed that SCC could be generated during the whole larval time with a maximal generation capacity immediately before hatching (325 ATU, Figure 3a,3b). At the age of 325 ATU in average 1.5 and up to five SCC were generated out of one fish larva. This result shows the possibility to generate more SCCs than animals were used. Consequently, this *in vitro* model has a high potential for the use as a reduction method for animal experiments and follows the 3R concept [18]. Furthermore, the preparation time of this high throughput system is possible in a very short time (96 well in 2.6 h). Another important factor for test systems is the economical costs. The preparation of a larvae and generation of SCCs from one larvae costs less than 1€. To our knowledge there is no model system in heart research available which can be generated in less time and at a low cost.

Attention should also be paid to the developmental period. From an economical point of view the generation of test system should be easy and possible in a short period. Analyses revealed that after nine days post preparation 75 rsp. 100% of all SCCs are present which were found during a three-week-observation (Figure 3b). This shows that the cultures can be used after nine days post preparation, because the development of further SCCs during the following days is economically low (labour, material and salary).

A further advantage of this model system is the 3D structure. Effects of drugs could be analyzed in cell formation and not only in single cells. This is a further step towards an *in vivo* like situation [7]. The area of the SCCs is dependent on the age of the prepared larva. The area of a SCC is significantly increasing from SCCs prepared from larvae at 252 ATU and 294 ATU. For other preparation times a larger aggregate size with increasing age could be measured but significance could not be proven and due to some very small SCCs. During larval fish develop-

ment the heart is growing permanently [19]. We assume that the generated SCCs have a bigger size with increasing age of the prepared larva; this assumption is confirmed through subsequent analysis. Overall, the maximum area of the SCC (maximal aberration) is increasing with age. The generation of the SCC from rainbow trout seems to be limited on the larval age, resulting in a maximal size of the SCCs at the end of yolk sac stage. Depending on the study (e.g. studies of heart conditions), the user of this model should be aware of the different SCC sizes and should take SCCs of nearly the same size when comparing test groups.

The analysis of the CF in relation to the age of the larva also revealed dependence of the examined groups. The early embryonic heart of a rainbow trout has a frequency of 20 beats/ min. During the development to the eye point stage (~250 ATU) the frequency increased to 35 beats/ min [20]. At 378 ATU the larva has a 2.5 fold higher CF induced by a higher spontaneous electrical activity [19]. This CF analyses revealed that the CF of the SCCs is in accordance with data of larval CF in different developmental stages. The reason why the SCCs exhibit larval CFs and not CF of fully developed cardiomyocytes, which is present in the SCCs could be examined in further studies. One assumption is that the ion channel composition and the pacemaker centre are still larval and did not differentiate to an adult heart structure in the same manner as the anatomical part.

However, the aberrations of the CF of some SCCs could be induced by a very strong adhesive power of the surrounding cells resulting in lower CF. Higher CF could result in a temperature increase (up to 5°C higher; RT) of the medium during observation. Nevertheless, the differences in the frequency between the groups are not affecting the consistency of using this model in experiments. Users of this model should be aware of the age of the prepared larvae in advance.

The comparison of the CF in relation to the size of the SCC revealed no significant correlation. In mammals the CF is decreasing with increasing size of the organism. In this study we used larval rainbow trout. The heart of the prepared larva was not completely developed and the frequencies were changing. For that reason, we assume that in the SCCs different CFs were measured in the groups and this correlation of CF-SCC size could not be observed. Nevertheless, these analyses revealed the most important aspect: the mean CF of SCCs of preparations from 294 ATU to 462 ATU is similar to that of human and could possibly be used as a test system.

In the current study we observed aggregates with spontaneous contraction of up to six months. During a 30 day measurements, the CF was stable and human analogue (Figure 3f). The SCCs also show changes in their CF with temperature changes. A highly significant correlation was found between the CF and temperature in a range from 16 to 25°C. Rainbow trout is a eurythermal fish species which tolerates temperatures between ~0°C and 25°C in adult stage [21]. Aho et al. [22] showed that the heart rate correlated positively with temperature between 4 and 24°C in adult trout. Above 25°C the temperature declined with irregularity in the frequency. This effect could not be observed at the SCCs. Causation could be that optimal cell growth occurs at temperatures slightly higher than the temperature of the donor [23,24]. In that case an irregularity of the heart rate would be estimated at higher temperature than in fish. The cells of rainbow trout larvae do not grow at a temperature above 28°C (data not published), for that reason the effect of irregularity could not be measured. Also in other vertebrates especially mammals this effect of increasing heart rate with increasing temperature is commonly analyzed and is well known as temperature coefficient, Q_{10} [25].

In summary, the analysis revealed that the spontaneously contracting cell aggregates are an *in vitro* test system generated from fish larvae. In former studies we showed the opportunity of using the SCCs in regenerative biology because of the high regenerative potential after dissection and the possibility to propagate [8]. Furthermore, in [9] we showed the use of a pharmacological *in vitro* heart model system with electrophysiological properties and effects on drugs are a human analogue. In this study, the analysis of the SCC ratio and other characteristics revealed that the best preparation time is a short time before hatching. Here, *in vitro* models with human analogue characteristics can be generated in higher numbers than animals used, demonstrating the possibility as a robust system with a high-throughput as well as long-term assay in human and fish heart research which would support the efforts of reducing animal experiments at the same time.

Acknowledgements

The authors are grateful to the Reese Fischzucht in Helmstorf (Germany) providing the rainbow trout eggs for this project. This work was supported by the Ministry of science, economy and transport of Schleswig-Holstein and the European Community within the framework of the European Regional Development Fund (RDF).

References

1. Suzuki Y, Yeung AC, Ikeno F (2009) The pre-clinical animal model in the translational research of interventional cardiology. JACC Cardiovasc Interv 2: 373-383.
2. Cao F, Wagner RA, Wilson KD, Xie X, Fu JD, et al. (2008) Transcriptional and functional profiling of human embryonic stem cell-derived cardiomyocytes. PLoS One 3: e3474.
3. Leri A, Kajstura J, Anversa P (2005) Cardiac stem cells and mechanisms of myocardial regeneration. Physiol Rev 85: 1373-1416.
4. Urbanek K, Torella D, Sheikh F, De Angelis A, Nurzynska D, et al. (2005) Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci USA 102: 8692-8697.
5. Adams CP, Brantner VV (2010) Spending on new drug development1. Health Econ 19: 130-141.
6. Kohl P, Camelliti P, Burton FL, Smith GL (2005) Electrical coupling of fibroblasts and myocytes: relevance for cardiac propagation. J Electrocardiol 38: 45-50.
7. Horrobin DF (2003) Modern biomedical research: an internally self-consistent universe with little contact with medical reality? Nat Rev Drug Discov 2: 151-154.
8. Grunow B, Ciba P, Rakers S, Klinger M, Anders E, et al. (2010) *In vitro* expansion of autonomously contracting, cardiomyogenic structures from rainbow trout *Oncorhynchus mykiss*. J Fish Biol 76: 427-434.
9. Grunow B, Wenzel J, Terlau H, Langner S, Gebert M, et al. (2011) *In vitro* developed spontaneously contracting cardiomyocytes from rainbow trout as a model system for human heart research. Cell Physiol Biochem 27: 1-12.
10. Brette F, Luxan G, Cros C, Dixey H, Wilson C, et al. (2008) Characterization of isolated ventricular myocytes from adult zebrafish (*Danio rerio*). Biochem Biophys Res Commun 374: 143-146.
11. Nemtsas P, Wettwer E, Christ T, Weidinger G, Ravens U (2010) Adult zebrafish heart as a model for human heart? An electrophysiological study. J Mol Cell Cardiol 48: 161-171.
12. Shiels H, Calaghan SC, White E (2006) The cellular basis for enhanced volume-modulated cardiac output in fish hearts. J Gen Physiol 128: 37-44.
13. Eschenhagen T, Didié M, Heubach J, Ravens U, Zimmermann WH (2002)

Cardiac tissue engineering. Transpl Immunol 9: 315-321.

14. Junqueira LC, Carneiro J (2005) Basic histology. McGraw-Hill Companies, Inc by Appleton & Lange. ISSN: 0891-2106.
15. Vornanen M (1998) L-type Ca2+ current in fish cardiac myocytes: effects of thermal acclimation and beta-adrenergic stimulation. J Exp Biol 201: 533-547.
16. Kim CS, Coyne MD, Gwathmey JK (2000) Voltage-dependent calcium channels in ventricular cells of rainbow trout: effect of temperature changes *in vitro*. Am J Physiol Regul Integr Comp Physiol 278: 1524-1534.
17. Riccioppo Neto F, Mesquita Júnior O, Olivera GB (1997) Antiarrhythmic and electrophysiological effects of the novel KATP channel opener, rilmakalim, in rabbit cardiac cells. Gen Pharmacol 29: 201-205.
18. Russel WM, Burgh RL (1959) The principles of humane experimental technique. London: Methuen & Co. Special edition published by Universities Federation for Animal Welfare (UFAW), 1992.
19. Burggren W, Keller BB (1997) Development of cardiovascular systems. Cambridge University Press: 145-465.
20. Rönna KC (1977) Myogenesis and contraction in the early embryonic heart of the rainbow trout. An electron microscopic study. Cell Tissue Res 180: 123-132.
21. Threader RW, Houston AH (1983) Heat tolerance and resistance in juvenile rainbow trout acclimated to diurnally cycling temperatures. Comp Biochem Physiol 75: 153-155.
22. Aho E, Vornanen M (2001) Cold acclimation increases basal heart rate but decreases its thermal tolerance in rainbow trout (*Oncorhynchus mykiss*). J Comp Physiol B 171: 173-179.
23. Ott T (2004) Tissue Culture of Fish Cell Lines. NWFHS Laboratory Procedures Manual - Second Edition Chapter 10: 1-16.
24. Bols NC, Dayeh VR, Lee LE, Schirmer K (2005) Use of fish cell lines in the toxicology and ecotoxicology of fish. Piscine cell lines in environmental toxicology. T.P. Mommsen and T.W. Moon (Eds) Elsevier B.V. Biochemistry and Molecular Biology of Fishes 6: 43-84.
25. Swoap SJ, Gutilla MJ (2009) Cardiovascular changes during daily torpor in the laboratory mouse. Am J Physiol Regul Integr Comp Physiol 297: R769-R774.