***In vitro* Model for Assessing Arrhythmogenic Properties of Drugs Based on High-resolution Impedance Measurements**

Filomain Nguemo¹, Tomo Šarić¹, Kurt Pfannkuche¹, Manfred Watzele², Michael Reppel^{1*} and Jürgen Hescheler¹

¹Institute of Neurophysiology, University of Cologne, Cologne, ²Roche Applied Science, Penzberg, *Current addresses: Department of Cardiology, Medical University of Lübeck, Lübeck

Keys Words

Mouse iPS cells • Cardiomyocytes • Drug screening • Impedance • Arrhythmia

Abstract

Background/Aims: Cardiac dysfunction is one of the main cause of drug candidate failures in the preclinical and/or clinical studies and responsible for the retraction of large number of drugs from the market. The prediction of arrhythmic risk based on preclinical trials during drug development remains limited despite intensive and costly investigation. Moreover, methods for analyzing beating behavior of cardiomyocytes (CMs) in culture to diagnose arrhythmias are not well developed. **Methods:** In this study, we combined two emerging technologies, induced pluripotent stem (iPS) cell-derived CMs and impedance-based real-time (xCELLigence RTCA Cardio Instrument) monitoring of CM electrical activity, to assess the effect of drugs known affect cardiac activity such as isoproterenol, carbachol, terfenadine, sotalol and doxorubicin. Cells were exposed to a drug in a single dose or repeated dose scenarios and data were analyzed using RTCA Cardio software, Poincaré plot and detrended fluctuation analysis. **Results:** The

results revealed significant changes in beating parameters of iPS-CMs induced by reference compounds. Heptanol, gap junction blocker, completely disrupted the synchronous beating pattern of iPS-CMs. Decrease of beating rate, amplitude and beat-to-beat signal variations of iPS-CMs monolayer observed in the presence of doxorubicin revealed severe abnormality detected by the system. Additionally, the irregular beating rhythms recorded in the presence of Terfenadine and Sotalol at high concentration, reflect abnormalities in cell contraction and/or relaxation which may lead to arrhythmia. **Conclusions:** All these results indicated that xCELLigence RTCA Cardio system combined with iPS cells, has the potential to be an attractive high-throughput tool for studying CMs during prolonged culture times and to screen potential drugs for cardiotoxic side effects.

Copyright © 2012 S. Karger AG, Basel

Introduction

Safety assessment remains a crucial step for drug development. A significant number of drugs with high-profile have been withdrawn from the market due to their

adverse effects (e.g. cardiotoxicity), while for many others drugs, safety labels have been revised to state adequate warning about their potential side effects [1]. Cardiac arrhythmia, a leading cause of death worldwide, is the result of any interference with the well-coordinated electrical pathways that produce the heart's rhythmic contractions [2]. The mechanisms of cardiac arrhythmias are still not fully understood, despite considerable research progress in the last decade. The passage of ions such as Ca^{2+} , K^{+} and Na^{+} into and out of the cell generated electrical charges which in turn stimulate and coordinate cardiomyocyte (CM) contractions. Any alteration of these ions pathways will lead to heart dysfunction. For example, in most of the cases, cardiotoxicity arises when the compound interacts with ion channels or transporters to increase the risk for developing arrhythmias such as life-threatening Torsade de pointes (TdP) [3]. An important determinant for development of TdP is the prolongation of the QT interval, mostly arising from the prolongation of the action potential (AP) which affects the rapid potassium current (I_{Kr}) by inhibition of Ether-a-go-go-Related Gene (hERG) channel in CMs [1, 4]. Currently, assessing the risk of drug-induced QT interval prolongation is one of the main aspects of the standard preclinical drug evaluation [5, 6]. The assessments should be preferably based on repeated-dose toxicity tests. It has now become a routine for compounds to be tested for effects on hERG channel function early in drug development. However, given their involvement in a wide range of pathologies covering all major therapeutic areas, other ions channels are also known to play critical roles in physiology and disease by modulating cellular functions such as electrical excitability, secretion, cell migration, and gene transcription [7]. Therefore it is no surprise that ion channels are the second largest class of drug targets after G-protein receptors [8]. Despite being of such importance for drug discovery, the relevance and impact of ion channels data such as hERG are sometimes misinterpreted, as there are drugs that block the hERG channel but do not cause arrhythmia, and drugs that cause arrhythmia but do not block hERG channel [9]. The Ca^{2+} channel blocker Verapamil is one well-known example [1].

The creation of induced pluripotent stem (iPS) cells from adult somatic cells opened important new approaches not only for basic research but also for regenerative medicine, disease modeling and toxicology. Several recent studies have reported the generation and differentiation of iPS cell-derived cardiomyocytes (iPS-CMs) from

different sources and species [10-14]. Studying the beating activity of spontaneously active iPS-CMs can provide considerable information on the effects of any compounds on the cardiovascular system. The use of iPS-CMs has a potential to improve the confidence in the predictive value of tests and considerably reduces the number of animal experiments for toxicological testing [15]. However, drug testing using iPS-CMs requires a sophisticated platform of sensitive and cell compatible bioanalytical tools with sufficient and accurate recording capacity for key physiological and biochemical changes in the cells. Unfortunately, many of the techniques used are technically challenging, invasive and suitable only for defined endpoint measurements.

The new xCELLigence RTCA Cardio instrument, as described previously [16, 17], offers a possibility for continuous real-time monitoring of the manufacturing process and label-free assessment of the physiological state of spontaneous beating CMs. In this study, we demonstrate that xCELLigence RTCA Cardio system is a convenient tool to characterize and discriminate effects of compounds on beating activity of iPS-CMs in a time-dependent and label-free manner and, as such, is suitable for early discovery safety assessment.

Materials and Methods

Chemicals and media

Basic culture media were purchased from Gibco/Invitrogen; supplements and chemicals were from Sigma Aldrich, unless otherwise specified.

Induced Pluripotent stem cell culture

Murine iPS cell line TiB7.4 was kindly provided by Alexander Meissner and Rudolf Jaenisch (Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts). Cells were cultured as described previously [12]. Briefly, cells were maintained in an undifferentiated state in irradiated mouse embryonic fibroblasts (MEF) in Dulbecco's modified Eagle medium (DMEM) containing 15% fetal bovine serum (FBS), 1x non-essential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol (2ME), and 1000 U/ml leukemia inhibitory factor (LIF, Chemicon). The cells were stably genetically modified by plasmid electroporation to express the puromycin resistance gene puromycin N-acetyltransferase and fluorescent reporter marker EGFP under the control of cardiospecific α -myosin heavy chain promoter to allow the generation of CMs at high purity by antibiotic selection. The generation and characterization of this cell line will be described elsewhere (Fatima A. et al., manuscript in preparation).

Generation of iPS-CMs

Cardiac differentiation of transgenic iPS cells was performed in spinner flasks as previously described [12]. Briefly, after the first appearance of embryoid bodies (EBs) exhibiting the spontaneously contractile areas, usually at day 9 of differentiation, EBs were treated with 8 µg/ml puromycin for two days. Thereafter, all surviving beating clusters were dissociated into single cardiomyocytes with 0.05% Trypsin/EDTA and cells were filtered through a 35-50 micron filter to remove remaining clumps. Further, the Percoll gradient centrifugation step was used to enrich for viable CMs. Pure CMs were subsequently preplated onto Petri dishes and incubated at 37°C in 5% CO₂ for at least 24 hours. The plating medium was then removed and cells washed in pre-warmed PBS to remove debris and dead cells. At the end, about 99% of purified CMs were readily obtained for different experiments.

Immunocytochemical staining analysis

Immunostaining assays were performed according to the protocol described before [11]. Briefly, cells were fixed for 10 minutes with 4% paraformaldehyde, permeabilized in 0.25% Triton X-100 (Sigma-Aldrich) and 0.5 M ammonium chloride in 0.25 M TBS (pH 7.4) and blocked with 0.8% bovine serum albumin (BSA) for 1 hour at room temperature. Subsequently, samples were incubated with primary antibodies against α -actinin (1:500; Sigma-Aldrich) and cTnT (1:500; Abcam) at 4°C overnight and detected by AlexaFluor 555- or AlexaFluor 647-conjugated secondary antibodies (1:1000, Invitrogen). Nuclei were stained with Hoechst 33258 (1:500, Sigma-Aldrich) and samples were examined using an Axiovert 200M fluorescence microscope (Zeiss, Göttingen, Germany).

xCELLigence Real Time Cell Analysis (RTCA) Cardio Instrument and Cell Plating on E-plate Cardio 96 wells

The xCELLigence RTCA Cardio Instrument, kindly provided by Roche Diagnostics (Penzberg, Germany), was used to monitor the beating activity of iPS-CMs as well as to record the effect of various compounds on CMs after short- and long-term exposure. As previously described [16, 18], this system consists of four main components (Fig. 1A, B). The RTCA Cardio System records impedance signals and further processes and displays the data by converting impedance value into a Cell Index (CI) value. The CI is derived to represent cell status based on the changes in impedance signals, which result from cell attachment to the bottom of the wells. Thus, the CI value mainly reflects changes in the number of attached cells, their morphology and beating activity [19] and is calculated as previously described [18].

Each well of E-plates 96 was coated with 5 µg/ml fibronectin solution and incubated for at least three hours at 37°C or overnight at +4°C. Before seeding of CMs, each well was washed with PBS, filled with 150 µl of culture media and the background impedance measurement was performed during 21 seconds. To determine the appropriate iPS-CMs density for the impedance measurements, iPS-CMs were resuspended in cell culture medium (IMDM containing 20% FBS, 10 µM 2ME and 1x non-essential amino acids) and plated at a density of 5000, 10000, 15000 and 20000 cells/well (Fig. 1C). Then, the

plate was incubated for 30 minutes at 37°C to allow for an initial cells adhesion at the bottom of each well. Thereafter, the E-plate was mounted on the RTCA Cardio Station placed at 37°C in a 5% CO₂ incubator and CM adhesion and beating activity monitored using the RTCA Cardio Instrument.

Monitoring of cardiomyocyte activity using RTCA Cardio system

After the initial adhesion of CMs seeded in E-plate 96, the changes in adhesion, spreading and beating activity of the iPS-CMs were continuously recorded for up to two weeks using the impedance measurements by xCELLigence RTCA Cardio Instrument. To assess the effects of compound on iPS-CMs in this system, drugs with well-known mechanism of action on cardiac activity, such as isoproterenol (ISO), carbachol (CCh), terfenadine (TER), sotalol (SOT) and doxorubicin (DOX) were chosen. iPS-CMs were treated with these drugs at following concentration: ISO (0.01, 0.1, 1 and 10 µM), CCh (0.1, 1, 5 and 10 µM), TER (0.01, 0.1, 5, 10 µM), SOT (0.01, 0.1, 1, 10 µM) and DOX (0.1, 1, 5 and 10 µM). In some control experiments, heptanol (0.5 µM) and blebbistatin (10 µM) were used. Drugs were administrated at time 0 or 72 hrs post-plating. All drugs were dissolved in 0.1% DMSO and stored at -20°C. Control experiments with and without 0.1% DMSO were performed simultaneously. After compound administration, impedance was measured every 10 min for the following 24 h and afterwards every 15 min until the end of the experiment. Medium was partially (up to 85%) exchanged every 2 days.

Patch clamp recordings

Action potentials (APs) in spontaneously beating iPS-CMs were recorded by the whole cell patch clamp method in current clamp mode using EPC-9 amplifier (Heka Electronics, Lambrecht, Germany) as described previously [12]. The resistances of the glass electrodes used were 2-6 MΩ when they were filled with the pipette solution containing (mmol/l): 50 KCl, 80 K-Aspartate, 1 MgCl₂, 3 MgATP, 10 EGTA, 10 HEPES (pH 7.2 adjusted with KOH). Extracellular bathing solution containing (mmol/l): 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 glucose and 10 HEPES (pH 7.4, adjusted with NaOH) was used. The glass coverslips containing the cells were placed into a temperature-controlled (37°C) recording chamber and perfused continuously with extracellular solution.

Multielectrode extracellular recordings

The iPS-CMs were cultured on microelectrode-array (MEA) chamber, precoated with 5 µg/ml fibronectin. After 3 to 4 days, syncytium formation of iPS-CMs monolayer was observed. A 60-channel amplifier (MEA1040, Multichannel Systems) was used to record electrical activity of the spontaneously contracting iPS-CMs. The data was further analyzed using software written using Matlab (The Mathworks, Natick, MA, USA).

Statistical analysis

After acquisition, data were analyzed using xCELLigence Cardio Software (Roche Diagnostics, Penzberg) as previously described [20] or exported as Excel and text files and further

Fig. 1. xCELLigence system RTCA Cardio Instrument and iPS cell-derived cardiomyocytes. (A) RTCA Cardio Station used in combination with the RTCA Cardio Analyzer and Control Unit. (B) E-Plates Cardio with 96 wells, up to 80% of the bottom area of each well are covered with interdigitated gold microelectrodes (inset figure). (C) Representative images of a monolayer of EGFP-positive cardiomyocytes plated on gold microelectrodes. (D, E) Detection of typical cardiac proteins sarcomeric α -actinin (red, D) and troponin T (green; E) in iPS cell-derived cardiomyocytes by immunofluorescence. Nuclei were counterstained with Hoechst 33342 (blue). Scale bars=50 μ m. (F) Representative traces of spontaneous beating activity of monolayer cardiomyocytes recorded with impedance system (red, upper panel) in comparison to extracellular signals recorded with MEA system (black, middle panel) and intracellular signals (action potential) of single cardiomyocyte recorded using patch-clamp technique (blue, lower panel). (G) Effect of heptanol (0.5 μ M) on the beating activity of monolayer iPS-CMs detected by impedance (upper panel) and on action potentials of single iPS-CMs recorded with standard patch clamp system (lower panel). (CI=Cell Index)

analyzed with Chart5 (AD instrument Ltd., Bella Vista, NSW, Australia) and Kubios Heart Rate Variability (HRV) (University of Kuopio, Kuopio, Finland) softwares. Data were presented as mean $\pm$ SEM. Statistical significance of differences was estimated by one way ANOVA or Student's t test. $P < 0.05$ (marked with an asterik) was considered significant.

Poincaré plots and detrended fluctuation analysis

The Poincaré plot analysis (PPA) is a geometrical and non-linear method, which provides detailed information on the heart rate variability (HRV). Visual interpretations of the shape of the plot and quantitative analysis of the dispersion of the beat-to-beat signal intervals (RRI) at different beating rate have been used in previous studies [21, 22]. In the present study, beating rate variability for each well and condition were determined as previously described [21]. Usually, three important indices are derived from PPA: the standard deviation of the short-term RR-interval variability (SD1), the standard deviation of the long-term RR-interval variability (SD2) and the axes ratio ($R=SD1/SD2$) [22].

To further characterize the beating signals recorded from iPS-CMs monolayer using impedance system, the Detrended Fluctuation Analysis (DFA) was used to quantify the fractal scaling properties of short time RR-interval signals. It quantifies the presence or absence of fractal correlation properties in non-stationary time-series data. The self-similarity occurring over a large range of time scales can be defined for a selected time scale with this method. DFA usually involves the estimation of

a short-term fractal scaling exponent α_1 and a long-term scaling exponent α_2 as previously described [21, 23]. The short-term fluctuations are characterized by the slope 1 obtained from the ($\log n$, $\log F(n)$) graph within range $4 = n = 16$. Correspondingly, the slope 2 obtained from the range $16 = n = 64$ characterizes long-term fluctuations.

Results

Validation and functional properties of iPS-CMs

To screen for the effects of compound after short- and long-term exposure, the cardiomyocytes must be sensitive to a variety of toxicants, should be stable over longer periods of time and easy to cultivate to allow the generation of reproducible results. Based on these criteria, we used mouse iPS-CMs because of their ability to form functional syncytium with stable pacemaker activity and AP propagation for extended periods

The functional, structural and molecular properties of iPS-CMs used in this study were characterized extensively and will be reported elsewhere (Fatima A. et al., manuscript in preparation). These iPS-CMs express sarcomeric α -actinin (Fig. 1D) and troponin T (Fig. 1E), which play an indispensable role in contractility of

Fig. 2. Dynamic monitoring of the correlation between cardiomyocyte density and cell index using the xCELLigence RTCA Cardio system. (A) Statistics analysis of cell index at different time points after plating. Cell index are presented as mean $\pm$ SEM of at least 8 wells from two independent experiments. (B) Effect of medium change on cell index of CMs at the density of 15000 cell/well. In control condition, medium was not changed. (C) Representative impedance traces (beating activity) recorded at different time with 5000 (blue) and 20000 (red) cells/well. Six second recording time of each trace is displayed. (D, E) Beating rate (1/minute) and signal amplitude (delta CI) of iPS-CMs recorded at indicated time points after cell plating. Values are presented as mean $\pm$ SEM of 12 wells from two independent experiments.

cardiomyocytes, thus confirming their regular organization. We used impedance measurements, whole-cell patch clamp and multi-electrode array (MEA) recording to analyze the growth and function of iPS-CMs. After plating on the fibronectin-coated surface of xCELLigence E-Plate Cardio 96 and MEA chamber for, respectively, impedance and MEA experiments, the beating iPS-CMs spread over the electrodes surface and formed confluent monolayer approximately 72 hours after plating, which then exhibited synchronous beating behavior in a cell-density dependent manner. The resulting beating rate of iPS-CM monolayer detected with impedance and MEA was comparable to the frequency of APs of single iPS-CM as determined by current clamp in side-by-side experiments (Fig. 1F). The addition of 0.5 μ M heptanol (a widely used reversible inhibitor of cell-to-cell coupling) to the iPS-CMs monolayer preparation completely inhibited the beating signals detected by impedance system. However, the same concentration of heptanol did not affect the APs frequency of single iPS-CM (Fig. 1G) recorded via the conventional current-clamp

technique. Additional experiments revealed that blebbistatin (10 μ M), the excitation-contraction uncoupler, completely abolished the contractility of monolayer iPS-CMs detected by impedance, whereas no effects on AP morphology of iPS-CMs recorded with patch clamp technique were observed (data not shown), suggesting that impedance system can detect important drug effects that could not be revealed by electrophysiological tools suited for single cell analysis.

Monitoring iPS-CMs adhesion and beating activity

To characterize the beating activity of iPS-CMs with xCELLigence system RTCA cardio Instrument, we first determined the optimal density for attachment and stable beating activity recording. In this regards, iPS-CMs were plated on fibronectin-coated wells of the E-Plate Cardio 96 at densities of 5000, 10000, 15000 and 20000 iPS-CMs/well and recorded for up to 200 hours. As shown in Fig. 2A, the impedance Cell Index (CI) increased proportionally to the cell density. The CI of low iPS-CMs

Fig. 3. Assessing the effect of β -adrenergic and muscarinic receptor agonist on the cardiomyocytes beating activity via the xCELLigence RTCA Cardio system. (A) Typical examples of spontaneously beating signal recorded after 3 days and exposure to vehicle (0.1% DMSO, control), ISO (10 μ M), and CCh (10 μ M). Note the recovery after the withdrawal of the drugs. Each trace represents 5 seconds of recording time. (B) Effects of low (0.1 μ M) and high (10 μ M) concentration of ISO and CCh on beating rate in iPS-CMs. (C, D) Average amplitude and time to peak amplitude of cardiomyocyte beating signals recorded after exposure to ISO and CCh. (E-F) Poincaré plots (E) and detrended fluctuation analysis (F) for 20 s of the beating signal recorded in control, ISO (10 μ M) and CCh (10 μ M) conditions and after 48 hrs of drug withdrawal (washout). n=6 wells from two independent experiments.

density (5000 cells/well) increased continuously during the period of the study, whereas the CI of the high density (15000 and 20000 cells/well) increased and reach its maximum approximately 24 hrs after plating, thereafter remained unchanged during 200 hours, suggesting the early optimal saturation period with high cell density culture.

We further investigated the effect of medium change on CI of iPS-CMs after plating. Media change 12 and 24 hrs after cell seeding significantly influenced the CI (Fig. 2B). This observation suggests that medium should be changed after 48 hrs to maintain iPS-CMs in optimal condition for attachment. In addition, we also monitored the beating activity of iPS-CMs based on impedance measurement. After plating of iPS-CMs at low concentration (5000 cells/well), regular and stable beating signal was not detected within 120 hrs, but the signal appeared later and consistently

increased over time (Fig. 2C, left panel). However, with high iPS-CMs seeding density (20000 cells/well), consistent and synchronized beating signal was observed already 60 hrs post-plating and remained stable thereafter (Fig. 2C, right panel). To further identify the optimal cell density, we analyzed the beating frequency (Fig. 2D) and amplitude (Fig. 2E) of iPS-CMs in high versus low confluent cultures at different time points. After seeding iPS-CMs at 15000 and 20000 cells/well the maximum beating rate and amplitude were observed approximately 72 hrs later as compared to cells seeded at low density (5000 and 10000 cells/well), which exhibited irregular and unsynchronized beating signals. These results suggest that the appearance of reproducible contraction signals greatly depends on the cell density. For all further investigation on this study, we used iPS-CMs at the seeding density of 20000 cells/well that formed continuous monolayer, produced

Fig. 4. Modulation of iPS-CMs beating activity by terfenadine (TER). (A) Representative traces of beating iPS-CMs in control and in the continuous presence of low (0.1 μ M) and high (1 μ M) concentration of TER. (B) Statistical analysis of the beating ratio as described previously [17, 20]. High concentration of TER induces prominent beating irregularity. (C) Poincaré plots of the beating rate variation of in the presence of TER. (D) Average peak amplitude of the spontaneously beating cardiomyocyte signals recorded after exposure to TER. (E) Statistic analysis of time-to-peak amplitude of beating signals recorded after exposure to TER. (n=6 wells for each condition and from two independent experiments).

consistent rhythmic activity and maintained the viability of CMs over 15 days of cultivation.

Monitoring the effect of β -adrenergic and muscarinic-receptor agonists on iPS-CMs using xCELLigence system

To further evaluate the contractile activity of mouse iPS-CMs in the xCELLigence RTCA Cardio system, the influence of different drugs was examined experimentally. As one of the important critical determinants of normal cardiac electrophysiology is the intact response to hormones and transmitters of the central nervous system, we examined not only the suitability of our cell model for signaling studies but also the capability of the impedance to detect and capture changes in parameters describing spontaneous beating of CMs after drug exposure. Thus, the effect of ISO, a nonselective agonist of β -adrenergic receptors (β -AR) that activates the Gs subtype of G proteins and adenylate cyclase, and CCh, a synthetic acetylcholine analog that activates the Gi subtype of G proteins and inhibits adenylate cyclase, were studied.

Application of the high concentration of ISO (10 μ M) led to a typical increase in beating frequency compared with basal frequency, whereas the application of CCh inhibited the spontaneous beating rate (Fig. 3A). The impedance also detected the effect on the beating frequency of iPS-CMs monolayer at different concentrations of ISO and CCh (Fig. 3B). The beating rate of iPS-CMs slightly increased (from 160 to 187 beats per minute, bpm) in the presence of low concentration (0.1 μ M) of ISO, whereas 10 μ M of ISO significantly increased the beating rate to 235 bpm (n=6, p<0.01) but did not change the amplitude of the beating signal (Fig. 3C) or the time to peak amplitude, also known as the rising time (Fig. 3D).

The addition of 0.1 and 10 μ M CCh caused suitable decrease in the beating frequency (18% and 96%, respectively, Fig. 3B). The presence of 10 μ M CCh induced a significant change in contraction amplitude (Fig. 3C) and rising time (Fig. 3D) of the monolayer of iPS-CMs in a reversible manner. Additionally in some wells, the application of CCh in the presence of ISO was able to

Fig. 5. Evaluation of a non-selective beta-blocker and class III antiarrhythmic agent Sotalol (SOT) on iPS-CMs using the xCELLigence RTCA Cardio system. (A) Representative traces of beating iPS-CMs in the absence (control) and presence of SOT. (B) Note the appearance of arrhythmogenicity (irregular beat) in the presence of SOT. (C) Average peak amplitude of the spontaneous beating signals recorded after exposure of cardiomyocytes to SOT. (D) Statistical analysis of time-to-peak amplitude of the beating signals recorded after exposure to SOT. (E) Poincaré plots of the beating rate variation of in the presence of SOT. (n=6 wells for each condition).

block the ISO effect (data not shown), indicating intact and coupled β -adrenergic and muscarinic signaling cascades and the ability of the impedance system to detect drug-induced alterations of signaling pathways in cardiomyocytes.

To further quantify the effect of drugs on beating signals and beat-to-beat variations, which could be used as an indirect read-out for arrhythmias, we generated a Poincaré plots from 20 second recordings in the presence of DMSO (control), ISO and CCh. Fig. 3E (upper panel) shows Poincaré plots of representative experiments where the standard deviation measuring the short (SD1) and long (SD2) axis of RRI, and the ratio SD1/SD2 (R) are indicated. In the presence of ISO, an increase in beating rate of iPS-CMs increased the value of SD1 (from 10.7 to 15.8 ms, $p<0.05$), SD2 (from 86.8 to 101.7 ms, $p<0.05$), whereas R was slightly increased without reaching significance (from 0.12 to 0.15). However, CCh significantly decreased ($p<0.05$) the SD1 (2.1 ms), SD2

(32.6 ms) and R (0.06) compared to baseline. In most of the cases, change of these parameters was restored to near-normal levels within 48 hrs after ISO and CCh removal.

Next, we used detrended fluctuation analysis (DFA) to assess the effect of ISO and CCh on the dynamics of beating signals of iPS-CMs recorded with impedance system. The scaling exponents obtained with this analysis can be seen as self-similarity parameters. Fig. 3F shows representative double log plots of the index $F(n)$ as a function of segment length n . In the presence of ISO ($\alpha_{1-control}=1.92$ vs. $\alpha_{1-ISO}=1.84$) or CCh ($\alpha_{1-control}=1.92$ vs. $\alpha_{1-CCh}=1.93$), the shorter scales exponents do not differ significantly, but it is worth noting the shift of the position in the crossover point (circles in Fig. 3F) after the administration of the drugs. On the contrary, the long-term fluctuation was decreased in the presence of ISO ($\alpha_{2-control}=1.07$ vs. $\alpha_{2-ISO}=0.71$, $p<0.05$) and increased with CCh ($\alpha_{2-control}=1.07$ vs. $\alpha_{2-CCh}=1.16$, $p<0.05$),

Fig. 6. Monitoring the effect of doxorubicin on iPS-CMs using the RTCA Cardio system. (A) Representative recordings showing change in cell index in the presence of increasing amounts of DOX. (B) Representative traces of 5 second recordings of beating iPS-CMs shown low (0.1 μ M) and high (50 μ M) concentration effect at the indicated time point. (C, D) Effect of DOX on the beating frequency (1/minute) and signal amplitude (Δ CI) of iPS-CMs in monolayer culture recorded at indicated time points after cell plating. Within 10 hours, post-plating cells were continuously treated with different concentrations of DOX. Medium was changed every 2 days. (E) The beat-to-beat variations. DOX was applied 72 hours post-seeding, the time at which iPS-CM monolayer display regularly beating patterns. Values are presented as mean $\pm$ SEM of at least 12 wells from two independent experiments.

reflecting the chronotropic action of the agonist and demonstrate that DFA can be used to clearly discriminate the action of drugs on cardiac contractility.

Monitoring the effect of terfenadine on iPS-CMs using xCELLigence system

Pharmacological agent TER was tested to further determine the suitability of the impedance-based system for pharmacological and toxicity studies. TER is a non-sedating antihistamine drug that was withdrawn from the market due to its potent hERG blocking activity causing QT prolongation and in some cases TdP. As depicted in

Fig. 4A, TER increased at low concentration (0.1 μ M) the beating rate of iPS-CMs up to 42% above baseline whereas 10 μ M significantly induced the beating irregularity in iPS-CMs (Fig. 4B).

To further quantify the effect of high dose (10 μ M) of TER on iPS-CMs, we first determined the beating rhythm irregularity (BRI) or irregular beat ratio (IBR) as described previously [17, 20]. TER did not induce marked differences in the distribution of the beating signal amplitudes (Fig. 4C) as indicated by the error bars of the mean values (Fig. 4C, insert) but significantly decreased the time to beat amplitude (Fig. 4D) of

spontaneously beating iPS-CMs.

The Poincaré plots of iPS-CMs in control experiments and in the presence of TER at different concentrations are depicted in Fig. 4E. Compared to control values, 0.1 μM of TER significantly reduced the values of SD1 (from 12.6 to 6.2 ms, $p<0.01$) and SD2 (from 101.5 to 49.4 ms, $p<0.01$). In contrast, higher dose of TER (10 μM) significantly increased those parameters (to 21, 2 and 172.9 ms, respectively; $p<0.01$). These observations confirm that beating signals of iPS-CMs in monolayer cultures become more irregular in the presence of high concentration of TER.

Monitoring the effect of sotalol on iPS-CMs using xCELLigence system

Next, we investigated the ability of the impedance system to detect the effect of a clinically used antiarrhythmic drug on iPS-CM repolarization. Sotalol (SOT), a class III antiarrhythmic agent, is known to prolong the AP duration (APD) of cardiac cells by blocking I_{Kr} [24]. Application of SOT at different concentrations had significant effect on the beating signal properties (Fig. 5A) of iPS-CMs monolayer as monitored with impedance system. SOT induced beating irregularities in a concentration-dependent manner (Fig. 5B) and decreased the amplitude (Fig. 5C) and the time-to-peak amplitude (Fig. 5D). Poincaré plots revealed that SOT at low concentration (0.1 μM) increased the SD1 from 10.7 to 15 ms ($p<0.05$) and to 18.1 ms ($p<0.01$) at high concentration (Fig. 5E). The long axis of Poincaré plot was also increased as a function of higher drug concentration. At 0.1 μM SOT induced an increase of SD2 from 86.8 to 112.4 ms ($p<0.05$) and even more pronounced increase to 139.5 ms was obtained at concentration of 10 μM ($p<0.001$). These results clearly show that high concentrations of TER and SOT (10 μM) substantially alter the regular rhythmic activity (e.g. split beat signal into multiple peaks) of iPS-CMs cardiomyocytes.

Effects of anticancer drug Doxorubicin on the beating behavior of iPS-CMs evaluated by the impedance system

Doxorubicin (DOX) is a highly effective drug used in cancer chemotherapy. The clinical application of DOX was restricted because of its serious side effects on the heart. To evaluate the effects of DOX on iPS-CMs in monolayer cultures different concentrations (0.1, 1, 5, 10, 50 μM) were applied 10 hours after cell seeding. Fig. 6A

shows the changes of CI as function of time for different concentrations of DOX. The impedance CI decreased evenly after the addition of different concentrations of DOX and the recovery occurred gradually after washout. Within 24 hours after plating of iPS-CMs, no beat signals could be detected neither in the absence nor in the presence of DOX (Fig. 6B). Beating signals were detectable 72 and 96 hours post-plating. Beating activity was not affected by the application of 0.1 μM DOX, whereas DOX at the high concentration (50 μM) significant changed the beating pattern with immediate response (Fig. 6B). Fig. 6C displays the beating rate as a function of concentration of DOX. For this experiment, DOX at indicated concentration, were applied 10 hours after iPS-CMs plating and monitoring for at least 200 hours. In the continuous presence of 0.1 μM of DOX, the beating rate did not significantly changed whereas 5, 10 and 50 μM of DOX considerably decreased the beating rate by 54, 71 and 94% after 72 hours, respectively (Fig. 6C). Beating rate continuously decreased as a function of time and increasing drug concentration. In addition, the amplitude of beating pattern was significantly reduced for 5, 10 and 50 μM DOX compared to control cells (Fig. 6D).

Beat-to-beat variations may hold information on the electrical stability of the heart and its susceptibility to arrhythmic mortality. To further analyze this property of cardiac cells under DOX treatment, the percentage of relative beat-to-beat variations was estimated as a function of time (Fig. 6E). The response time of the beat-to-beat variations to DOX was significantly longer than that of beating rate and amplitude and this response was dose-dependent. At lower concentration of DOX, iPS-CMs took longer time to show change in beating rate and amplitude whereas at higher concentration these parameters changed much faster. Application of 0.1 μM of DOX did not induce relevant change of the beating rate (10%) of iPS-CMs 12 hours post-application, but 50 μM of DOX applied at the same time decreased the relative frequency by 47%. However, treatment of iPS-CMs with 0.1 μM DOX for a long-term (72 hours) significantly decreased the frequency (47%), an indication for cell damage. Therefore, the continuous treatment of CMs with low concentrations of DOX could induce functional impact that could be detected by impedance-based recordings, but it could also cause desynchronous contraction of the cells. These results are in accordance with those observed by Sawyer et al. [25], which showed apoptosis at low concentrations (1 μM) and necrosis at higher concentrations (>10 μM) of daunorubicin, an

another prototype agents of the anthracyclines considered as cardiotoxic as DOX [26].

Discussion

In vitro cell observation methods have been widely used by biologists and pharmacologists not only for drug screening but also in cell-based therapies for regenerative medicine. These methods should be as non-invasive as possible. In this study, we show that an impedance-based cell analysis system can be used for dynamic live cell monitoring. As previously described [16, 17], application of a low voltage signal that induces ionic currents between the microelectrodes at the surface bottom of each well generated an impedance signal, which is precisely and rhythmically interrupted by contraction and relaxation processes of spontaneously beating CMs. This system is a label-free technology which can be used to detect cellular events such as effects on the cellular morphology (e.g. agent-induced alteration of the cytoskeleton), cell death or apoptosis and G-protein coupled receptor stimulation [27]. Moreover, it can be used to monitor electrophysiological function of CMs under various conditions (e.g. in the presence of agonist or antagonist) as well as for toxicity assays. The change in the electrical impedance of microelectrodes on the bottom wells of E-plate cardio 96 through the overgrowth of cultured living cells is an attractive method to evaluate qualitatively the state of adhesion, proliferation, development of intercellular connections and APs propagation in the biological material such as iPS-CMs.

Beating CMs derived from ES or iPS, lead to complex patterns and erratic fluctuations of signal, which derive from phase shifts that individual beat signal can induce in the depolarization or repolarization phase of neighboring cardiomyocytes [28, 29]. The contraction and relaxation processes of CMs depend on different mechanisms and on different ion channel functions [30]. In addition, the format for representing the signal of impedance analysis results, which can differentiate the contraction to relaxation phase, is convenient for monitoring the beating activity of iPS-CMs. Therefore, we used this impedance system to monitor short and long-term cardioactive drugs effects on CMs generated from mouse iPS cells.

β -adrenergic and muscarinic receptors are critically implicated in modulating cardiac function and we demonstrated that application of ISO dose-dependently induced gradual increase in the beating frequency and

decreased the beat-to-beat intervals of iPS-CMs in monolayer cultures, reflecting the chronotropic action of the β -AR agonist. The increment in beating rate (up to 70%) with maximum concentration is similar to that observed in adult murine CMs (65%) and ES cell-derived CMs (40%) [31], suggesting a functionally active β receptors in iPS-CMs detected by the impedance-based system. In fact, the stimulatory β -AR response is initiated via G protein activation of adenylyl cyclase and subsequent protein kinase A (PKA)-mediated phosphorylation of L-type Ca^{2+} channels (LTCC), resulting in increased Ca^{2+} influx and augmented contractility (inotropy) as well as increased Ca^{2+} reuptake and relaxation [32, 33]. While this increase in myocyte Ca^{2+} produces the force required to meet the excessive contractile demands, it can also activate processes that cause cardiac dysfunction, such as arrhythmia, which could also be detected by the xCELLigence RTCA Cardio system [34]. Moreover, the exposure of iPS-CMs to CCh produced a dose-dependent decrease in the beating rate, which is in agreement with a negative chronotropic effect of the compound. The application of CCh in the presence of ISO was able to block the ISO effect in monolayer of iPS-CMs, indicating intact and coupled β -adrenergic and muscarinic signaling cascades and the ability of the impedance system to detect any variations in the cells environment and signaling pathways.

As Poincaré plot is useful to illustrate short and long-term variability in beating signals and internal dynamics of beatings, some previous studies discussed the perturbations of parasympathetic and sinoatrial node affects by the Poincaré plot [35, 36]. The variation observed in the distribution of the data points of Poincaré plot would be the consequence of high parasympathetic disturbance. However, in iPS-CM monolayer, there is no controls mechanism for beatings. Nevertheless, as the plots almost revealed every beat signals as a function of the previous one, then our recordings signals mainly show a systematic behavior.

The primary goal of any cardiac risk assessment test is to detect with the highest possible sensitivity the cardiac arrhythmic potential of drug candidates. We have demonstrated that both TER and SOT, compounds shown to induce cardiotoxicity, significantly increase beating variability and affect beating rate of iPS-CMs in a dose-dependent manner. Studies involving the mechanisms underlying these cardiotoxicities revealed that TER blocks the human ether-a-go-go related gene (hERG), which is involved in expression of ion channels (e.g. K^+ , Na^+ and Ca^{2+} channels) associated with electric currents in CMs

[37, 38]. Defects in hERG lead to prolongation of the QT interval. Drugs that prolong the APD and QT interval are well-known to be highly arrhythmogenic as they tend to elicit early afterdepolarizations (EADs) and enhanced dispersion of repolarization that progress to TdP [39]. In fact, the morphology variation of iPS-CMs monolayer beating signal observed in the presence of TER and SOT could be related to EAD effect induced by blocking of the hERG current leading to premature activation of Ca^{2+} channels and consequent risk of arrhythmogenesis [17, 40].

In addition, DOX has been shown to induce arrhythmia and cardiotoxicity [17, 41]. After exposure to DOX, the variability of iPS-CMs monolayer beating parameters was significantly increased with prominently declined amplitude and frequency in a dose and time-dependent manner. It is possible that organization, connection and intracellular components of iPS-CMs were impacted under treatment of DOX at high concentration, which altered cellular function and finally resulted in arrhythmia. Recent papers described a possible mechanism by which DOX induces cardiotoxicity. The most common mechanism includes the formation of free radicals and superoxides [41] and influence on the Ca^{2+} homeostasis [42], which in combination could induce QT prolongation by blocking K^+ and Ca^{2+} channels and prolonging the APD of each CMs present in the monolayer culture. In fact, before leading to apoptosis, oxidative stress can cause alterations in mitochondrial Ca^{2+} transport, which therefore can lead to tissue injury, cell death and impaired cardiac contraction.

In summary, the xCELLigence RTCA Cardio system in combination with iPS-CMs represents a useful system for assessment of cardiotoxicity of developing drug

candidates by providing a comprehensive array of data in real time. Our current studies, together with additional, recent validation studies [16-18, 20, 43], suggest that this system provides new approach to extensive, fast response, high-throughput recording and evaluation of drug-induced cardiotoxicity. Moreover, Poincaré plots and detrended fluctuation analysis could be combined with the xCELLigence RTCA Cardio software as suitable tools to describe cardiac arrhythmia and variations in beating rate. Lastly, iPS cells isolated from a patient with particular genetic disease can be manipulated *in vitro* to generated cardiomyocytes and used in this system to develop appropriate personalized therapies.

In conclusion, we shown the utility of RTCA Cardio system for pre-clinical cardio-safety screening using iPS-CMs. This system allows for high temporal resolution of iPS-CM beating cycles and offers a comprehensive assessment of cardiotoxicity. Additionally, it also possesses some useful properties such as noninvasive, continuous monitoring of changes in beating activities during compound exposure, which are unique to its design. Therefore, this system could be easily applied to screen for potential problems with promising drug molecules at the earliest possible stage, aiding their development.

Acknowledgements

We thank Dr. Azra Fatima for providing transgene iPS cell lines, Nadin Lange and Daniel Derichsweiler for their technical assistance. We also thank Matthias Matzkies, Judith Semmler and Christoph Schäfer for fruitful discussion and Roche Applied Science for access to the xCELLigence system.

References

- 1 Braam SR, Tertoolen L, van de Stolpe A, Meyer T, Passier R, Mummery CL: Prediction of drug-induced cardiotoxicity using human embryonic stem cell-derived cardiomyocytes. *Stem Cell Res* 2010;4:107-116.
- 2 Natarajan A, Stancescu M, Dhir V, Armstrong C, Sommerhage F, Hickman JJ, Molnar P: Patterned cardiomyocytes on microelectrode arrays as a functional, high information content drug screening platform. *Biomaterials* 2011;32:4267-4274.
- 3 Anson BD, Kolaja KL, Kamp TJ: Opportunities for use of human iPS cells in predictive toxicology. *Clin Pharmacol Ther* 2011;89:754-758.
- 4 Moss AJ, Kass RS: Long QT syndrome. from channels to cardiac arrhythmias. *J Clin Invest* 2005;115:2018-2024.

- 5 Chain AS, Krudys KM, Danhof M, Della Pasqua O: Assessing the probability of drug-induced QTc-interval prolongation during clinical drug development. *Clin Pharmacol Ther* 2011;90:867-875.
- 6 Sanguinetti MC, Tristani-Firouzi M: hERG potassium channels and cardiac arrhythmia. *Nature* 2006;440:463-469.
- 7 Ackerman MJ, Clapham DE: Ion channels—basic science and clinical disease. *N Engl J Med* 1997;336:1575-1586.
- 8 Overington JP, Al-Lazikani B, Hopkins AL: How many drug targets are there? *Nat Rev Drug Discov* 2006;5:993-996.
- 9 Moller C: Keeping the rhythm: hERG and beyond in cardiovascular safety pharmacology. *Expert Rev Clin Pharmacol* 2010;3:321-329.
- 10 Fatima A, Xu G, Shao K, Papadopoulos S, Lehmann M, Arnaiz-Cot JJ, Rosa AO, Nguemo F, Matzkies M, Dittmann S, Stone SL, Linke M, Zechner U, Beyer V, Hennies HC, Rosenkranz S, Klauke B, Parwani AS, Haverkamp W, Pfitzer G, Farr M, Cleemann L, Morad M, Milting H, Hescheler J, Saric T: In vitro Modeling of Ryanodine Receptor 2 Dysfunction Using Human Induced Pluripotent Stem Cells. *Cell Physiol Biochem* 2011;28:579-592.
- 11 Mauritz C, Schwanke K, Reppel M, Neef S, Katsirintaki K, Maier LS, Nguemo F, Menke S, Haustein M, Hescheler J, Hasenfuss G, Martin U: Generation of functional murine cardiac myocytes from induced pluripotent stem cells. *Circulation* 2008;118:507-517.
- 12 Pfannkuche K, Liang H, Hannes T, Xi J, Fatima A, Nguemo F, Matzkies M, Wernig M, Jaenisch R, Pillekamp F, Halbach M, Schunkert H, Saric T, Hescheler J, Reppel M: Cardiac myocytes derived from murine reprogrammed fibroblasts: intact hormonal regulation, cardiac ion channel expression and development of contractility. *Cell Physiol Biochem* 2009;24:73-86.
- 13 Brennand KJ, Simone A, Jou J, Gelboin-Burkhart C, Tran N, Sangar S, Li Y, Mu Y, Chen G, Yu D, McCarthy S, Sebat J, Gage FH: Modelling schizophrenia using human induced pluripotent stem cells. *Nature* 2011;473:221-225.
- 14 Yazawa M, Hsueh B, Jia X, Pasca AM, Bernstein JA, Hallmayer J, Dolmetsch RE: Using induced pluripotent stem cells to investigate cardiac phenotypes in Timothy syndrome. *Nature* 2011;471:230-234.
- 15 Dick E, Rajamohan D, Ronksley J, Denning C: Evaluating the utility of cardiomyocytes from human pluripotent stem cells for drug screening. *Biochem Soc Trans* 2010;38:1037-1045.
- 16 Xi B, Wang T, Li N, Ouyang W, Zhang W, Wu J, Xu X, Wang X, Abassi YA: Functional Cardiotoxicity Profiling and Screening Using the xCELLigence RTCA Cardio System. *J Lab Autom* 2011;16:415-421.
- 17 Abassi YA, Xi B, Li N, Ouyang W, Seiler A, Watzele M, Kettenhofen R, Bohlen H, Ehlich A, Kolosov E, Wang X, Xu X: Dynamic Monitoring of Beating Periodicity of Stem Cell Derived Cardiomyocytes as a Predictive Tool for Preclinical Safety Assessment. *Br J Pharmacol* 2012;165:1424-1441.
- 18 Urcan E, Haertel U, Styllou M, Hickel R, Scherthan H, Reichl FX: Real-time xCELLigence impedance analysis of the cytotoxicity of dental composite components on human gingival fibroblasts. *Dent Mater* 2010;26:51-58.
- 19 Abassi YA, Xi B, Zhang W, Ye P, Kirstein SL, Gaylord MR, Feinstein SC, Wang X, Xu X: Kinetic cell-based morphological screening: prediction of mechanism of compound action and off-target effects. *Chem Biol* 2009;16:712-723.
- 20 Guo L, Abrams RM, Babiarz JE, Cohen JD, Kameoka S, Sanders MJ, Chiao E, Kolaja KL: Estimating the risk of drug-induced proarrhythmia using human induced pluripotent stem cell-derived cardiomyocytes. *Toxicol Sci* 2011;123:281-289.
- 21 Mandel Y, Weissman A, Schick R, Barad L, Novak A, Meiry G, Goldberg S, Lorber A, Rosen MR, Itskovitz-Eldor J, Binah O: Human Embryonic and Induced Pluripotent Stem Cells-Derived Cardiomyocytes Exhibit Beat Rate Variability and Power-Law Behavior. *Circulation* 2012;125:883-893.
- 22 Charlet A, Rodeau JL, Poisbeau P: Poincare plot descriptors of heart rate variability as markers of persistent pain expression in freely moving rats. *Physiol Behav* 2011;104:694-701.
- 23 Rodriguez E, Lerma C, Echeverria JC, Alvarez-Ramirez J: ECG scaling properties of cardiac arrhythmias using detrended fluctuation analysis. *Physiol Meas* 2008;29:1255-1266.
- 24 Caspi O, Itzhaki I, Kehat I, Gepstein A, Arbel G, Huber I, Satin J, Gepstein L: In vitro electrophysiological drug testing using human embryonic stem cell derived cardiomyocytes. *Stem Cells Dev* 2009;18:161-172.
- 25 Sawyer DB, Fukazawa R, Arstall MA, Kelly RA: Daunorubicin-induced apoptosis in rat cardiac myocytes is inhibited by dexrazoxane. *Circ Res* 1999;84:257-265.
- 26 Upadhyay KK, Bhatt AN, Mishra AK, Dwarakanath BS, Jain S, Schatz C, Le Meins JF, Farooque A, Chandraiah G, Jain AK, Misra A, Lecommandoux S: The intracellular drug delivery and anti tumor activity of doxorubicin loaded poly(gamma-benzyl L-glutamate)-b-hyaluronan polymersomes. *Biomaterials* 2010;31:2882-2892.
- 27 Schwarzenberger T, Wolf P, Brischwein M, Kleinhans R, Demmel F, Lechner A, Becker B, Wolf B: Impedance sensor technology for cell-based assays in the framework of a high-content screening system. *Physiol Meas* 2011;32:977-993.
- 28 Hayakawa T, Kunihiro T, Dowaki S, Uno H, Matsui E, Uchida M, Kobayashi S, Yasuda A, Shimizu T, Okano T: Noninvasive evaluation of contractile behavior of cardiomyocyte monolayers based on motion vector analysis. *Tissue Eng Part C Methods* 2012;18:21-32.
- 29 Fahrenbach JP, Mejia-Alvarez R, Banach K: The relevance of non-excitable cells for cardiac pacemaker function. *J Physiol* 2007;585:565-578.
- 30 Bers DM: Cardiac excitation-contraction coupling. *Nature* 2002;415:198-205.
- 31 Hakuno D, Fukuda K, Makino S, Konishi F, Tomita Y, Manabe T, Suzuki Y, Umezawa A, Ogawa S: Bone marrow-derived regenerated cardiomyocytes (CMG Cells) express functional adrenergic and muscarinic receptors. *Circulation* 2002;105:380-386.
- 32 Nguemo F, Sasse P, Fleischmann BK, Kamanyi A, Schunkert H, Hescheler J, Reppel M: Modulation of L-type Ca^{2+} channel current density and inactivation by beta-adrenergic stimulation during murine cardiac embryogenesis. *Basic Res Cardiol* 2009;104:295-306.
- 33 Wang W, Zhang H, Gao H, Kubo H, Berretta RM, Chen X, Houser SR: β_1 -Adrenergic receptor activation induces mouse cardiac myocyte death through both L-type calcium channel-dependent and -independent pathways. *Am J Physiol Heart Circ Physiol* 2010;299:H322-331.
- 34 Kanno S, Kim PK, Sallam K, Lei J, Billiar TR, Shears LL 2nd: Nitric oxide facilitates cardiomyogenesis in mouse embryonic stem cells. *Proc Natl Acad Sci USA* 2004;101:12277-12281.
- 35 Bergfeldt L, Haga Y: Power spectral and Poincare plot characteristics in sinus node dysfunction. *J Appl Physiol* 2003;94:2217-2224.
- 36 Virtanen M, Kahonen M, Nieminen T, Karjalainen P, Tarvainen M, Lehtimäki T, Lehtinen R, Nikus K, Koobi T, Niemi M, Niemela K, Turjanmaa V, Malmivuo J, Viik J: Heart rate variability derived from exercise ECG in the detection of coronary artery disease. *Physiol Meas* 2007;28:1189-1200.

- 37 Liu S, Melchert RB, Kennedy RH: Inhibition of L-type Ca^{2+} channel current in rat ventricular myocytes by terfenadine. *Circ Res* 1997;81:202-210.
- 38 Abi-Gerges N, Valentin JP, Pollard CE: Dog left ventricular midmyocardial myocytes for assessment of drug-induced delayed repolarization: short-term variability and proarrhythmic potential. *Br J Pharmacol* 2010;159:77-92.
- 39 Roden DM: Early after-depolarizations and torsade de pointes: implications for the control of cardiac arrhythmias by prolonging repolarization. *Eur Heart J* 1993;14 Suppl H:56-61.
- 40 Hove-Madsen L, Prat-Vidal C, Llach A, Ciruela F, Casado V, Lluís C, Bayes-Genis A, Cinca J, Franco R: Adenosine A2A receptors are expressed in human atrial myocytes and modulate spontaneous sarcoplasmic reticulum calcium release. *Cardiovasc Res* 2006;72:292-302.
- 41 Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L: Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev* 2004;56:185-229.
- 42 Zhou S, Starkov A, Froberg MK, Leino RL, Wallace KB: Cumulative and irreversible cardiac mitochondrial dysfunction induced by doxorubicin. *Cancer Res* 2001;61:771-777.
- 43 Jonsson MK, Wang QD, Becker B: Impedance-based detection of beating rhythm and proarrhythmic effects of compounds on stem cell-derived cardiomyocytes. *Assay Drug Dev Technol* 2011;9:589-599.