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Non-invasive label-free monitoring the cardiac differentiation of human embryonic stem cells in-vitro by Raman spectroscopy

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ABSTRACT

Background: Online label-free monitoring of in-vitro differentiation of stem cells remains a major challenge in stem cell research. In this paper we report the use of Raman micro-spectroscopy (RMS) to measure time- and spatially-resolved molecular changes in intact embryoid bodies (EBs) during in-vitro cardiogenic differentiation.
Methods: EBs formed by aggregation of human embryonic stem cells (hESCs) were cultured in defined medium to induce differentiation towards cardiac phenotype and maintained in purpose-built micro-bioreactors on the Raman microscope for 5 days (between days 5 and 9 of differentiation) and spatially-resolved spectra were recorded at 24 h intervals.
Results: The Raman spectra showed that the onset of spontaneous beating of EBs at day 7 coincided with an increase in the intensity of the Raman bands at 1340 cm^{-1}, 1083 cm^{-1}, 937 cm^{-1}, 858 cm^{-1}, 577 cm^{-1} and 482 cm^{-1}. The spectral maps corresponding to these bands had a high positive correlation with the expression of the cardiac-specific α-actinin obtained by immuno-fluorescence imaging of the same EBs. The spectral markers obtained here are also in agreement with previous studies performed on individual live hESC-derived CMs.
Conclusions: The intensity profile of these Raman bands can be used for label-free in-situ monitoring of EBs to estimate the efficacy of cardiogenic differentiation.
General significance: As the acquisition of the time-course Raman spectra did not affect the viability or the differentiation potential of the hESCs, this study demonstrates the feasibility of using RMS for on-line non-invasive continuous monitoring of such processes inside bioreactor culture systems.

1. Introduction

Research during the last decade has demonstrated the huge potential of human embryonic stem cells (hESCs) for biomedical applications, including regenerative medicine [1], drug discovery [2] or heart development and disease modeling [3]. However, the full realization of this potential relies on robust and standardized bioprocessing technologies to ensure optimized and reproducible culture conditions for the stem cells such that sufficient quantity of differentiated cells with suitable quality can be obtained [4,5]. One of the major challenges in stem cell bioprocessing is the lack of online and real-time quantitative information regarding the conditions of the cells within the bioreactors [4,5]. Current analytical techniques for the characterization of embryonic stem cells in-vitro often employ destructive assays rendering on-line monitoring impossible, or are based on crude estimates which provide only limited insight into the molecular processes (e.g. spontaneous beating of embryoid bodies in hESC-derived cardiomyocyte bioprocessing [6]). Due to these limitations, the bioprocesses are not automated and the optimization of differentiation conditions relies on manual procedures, leading to high costs, labor, time and high cell variability [4,5]. The availability of non-invasive analytical techniques which can be used in an automated manner to monitor repeatedly the properties and the condition of the cells within the bioreactor may enable a more efficient and automated optimization of the bioprocesses as well as provide overall quality assessment of the end-point differentiated cells.

A key feature of Raman micro-spectroscopy (RMS) is that important information regarding the molecular characteristics of live cells grown in-vitro can be measured without requiring labeling or other invasive procedures [7], while maintaining the cells in their physiological conditions and growth medium. Time-resolved Raman spectral measurements (up to several hours) were used to detect molecular changes during apoptosis, such as de-localization of cytochrome c [8] and re-organization of phospholipids [9]. However, the in-vitro differentiation of hESCs involves major molecular changes over days or weeks. Such molecular changes include the early signals which establish the commitment towards a specific differentiation pathway as

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well as the significant changes in the cell chemistry which provides the foundation for the structure and function of the somatic cells. The focus of this study is the cardiomyocyte differentiation of embryoid bodies (EBs) formed by aggregated hESCs. This process involves significant changes in the cells, such as formation of myofibrils required to provide the contractile properties of heart [10], the switch from the glycolytic to the more energy efficient oxidative metabolism is characterized by an expansion of the mitochondrial network [11,12] and accumulation of glycogen in the cytoplasm of the cardiomyocytes [13]. Recent studies have also showed that a high glucose concentration in the culture is a prerequisite for in-vitro cardiomyocyte differentiation of hESCs [14].

RMS has been used for label-free investigations of stem cell differentiation, including live individual cells [15,16] and fixed cells in embryoid bodies [17], and for characterization of hESCs cultured in-vitro with the aim of assessing their differentiation status [18–20]. Recently, RMS was used for label-free characterization of individual cardiomyocytes derived from hESCs [21,22] and high accuracy classification models were reported (97% specificity and 96% sensitivity) [21]. The potential of developing label-free cell-sorting techniques for hESCs-derived CMs based on RMS was also explored [23]. In this study we investigate the use of RMS to measure non-invasively the time-dependent molecular changes during the differentiation of EBs towards cardiac phenotype, a bioprocess spanning several days. In situ spatially- and time-resolved Raman measurements were carried out on EBs grown in defined culture medium for inducing differentiation towards cardiac phenotype.

2. Materials and methods

2.1. Materials, general cell culture and immuno-staining

All tissue culture reagents were purchased from Invitrogen (Paisley, UK) and chemicals were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated. Mouse embryo fibroblasts and hESC cultures were maintained at 37 °C, 5% CO2, in a humidified atmosphere. The medium was changed daily for hESC culture and every 3–4 days during differentiation.

The hESC line HUES7 was cultured in feeder-free conditions in conditioned medium in a Matrigel-coated flask and cultured using trypsin passaging between passages 17–35, as described previously [24]. Differentiation in defined conditions was achieved by forced aggregation of defined numbers of hESCs [25]. Differentiating embryoid bodies were transferred on day 5 to custom-built micro-bioreactors for Raman spectral measurements.

For immunofluorescence and flow cytometry, EBs or dissociated cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X100, and then incubated with anti-α-actinin (1:800) and anti-cardiac troponin I (1:250) for 1 h at room temperature. The secondary antibodies were Cy3 and Alexfluor-488 (1:250; Vector Labs), with staining for 1 h at room temperature. For flow cytometry, cells were then washed in PBS two times before analyzing using the Beckman Coulter FC500 flow cytometer with FlowJo software (Treestar, OR). For immunofluorescence, cell nuclei were stained with 40,6-diamidino-2-phenylindole (DAPI, 100 ng/mL) at 1:1000 dilution in PBS for 5 min at room temperature.

2.2. Analysis of beating frequency for individual cardiomyocytes

Beating frequency analysis was carried out using videos recorded under white-light illumination and software developed in MATLAB (The MathWorks, Natick, MA). The beating frequency at each pixel of the EBs was obtained by calculation using the Fourier transform of the time-dependent intensity shift at each individual pixel as described previously [21].

2.3. Raman micro-spectroscopy measurements and data analysis

A custom-built Raman micro-spectrometer optimized for live-cell studies [21] was used to acquire the Raman spectra of the EBs. A long working distance microscope objective (50×NA 0.50, Leica Microsystems) was used to focus the laser on the samples and collect the Raman scattered light. The power of the 785 nm laser was ~170 mW after objective and the diameter of the laser spot was estimated to be ~3 μm. The spectrometer was calibrated before each experiment using 4-acetamidophenol (ASTM E1840). The spectral resolution was ~1.5 cm^{-1} and accuracy was ~0.5 cm^{-1}.

The micro-bioreactors were based on titanium chambers of 3.5 cm diameter and 1.5 cm height, which included a MgF_2 cover slip (0.15 mm thick) at the bottom to enable acquisition of Raman spectra and a top glass window to allow routine observation of the cells on the microscope. The inverted microscope (Olympus IX71) was fitted with an environmental enclosure (Solent, UK) to allow the cells to be maintained in physiological conditions (culture medium, 37 °C temperature, 95% relative humidity and 5% CO_2). During the time-course Raman measurements, the EBs were kept inside the sealed micro-bioreactors within the enclosure on the Raman microscope at all time and the culture medium was not replaced. A total of 8 independent EBs were analyzed during this study, 4 beating and 4 non-beating EBs.

The size of the EBs varied from 300 to 500 μm at day 5 of the differentiation, reaching up to 1 mm at day 9. Raman spectral maps of each individual EB were recorded as raster scans with 10 μm steps (60 by 60 or 100 by 120 grids depending on the size of the EBs) and 1 s acquisition time at each position. Although the step size was larger than the laser spot, this value was considered appropriate given that the beating amplitude of the EBs was ~10 μm.

Outlier spectra (typically less that 1% per spectral map contained cosmic rays, spurious bands or large baseline) were replaced with the average of the spectra measured at the four neighboring points. A Raman background spectrum was calculated as the mean of 3600 spectra (60 by 60 grid) measured in the immediate vicinity of each EB. For principal component analysis, the individual Raman spectra in each raster scans were normalized to zero mean and unity standard deviation.

The PCA model was built using standard functions available in MATLAB and included all individual data points (over 180,000 spectra) acquired from the 8 EBs. Raman maps were built by plotting the scores of the principal component that captured the largest amount of spectral variance in the dataset.

3. Results and discussion

3.1. Differentiation protocol

To produce a model system for Raman spectroscopy time-course analysis, we first established the efficiency of the cardiomyocyte differentiation protocol. The HUES7 hESC line was differentiated using defined culture medium to produce embryoid bodies, as previously described [25]. The time-course appearance of α-actinin and cardiac troponin I, which are required for contractile function in cardiomyocytes, was established by immuno-fluorescence for embryoid bodies between day 5 and 10 of differentiation (Fig. 1). The cardiac markers appeared on day 7, which coincided with spontaneous contractile activity. Flow cytometry and immunofluorescence analysis using the cardiomyocyte markers α-actinin and/or cardiac troponin I were carried out on individual cells dispersed from EBs at day 12 of differentiation (not single EB). This indicated that approximately 85% of the cells present in beating EBs were cardiomyocytes, whereas less than 1% were identified in non-beating EBs (Fig. 2).

Since the environmental enclosure on the Raman microscope allowed cells cultured in the bioreactors to be maintained in optimal