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Real-time and non-invasive monitoring of embryonic stem cell survival during the development of embryoid bodies with smart nanosensor

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ABSTRACT

Embryonic stem cells (ESCs)-derived embryoid body (EB) is a powerful model for the study of early embryonic development and the discovery of therapeutics for tissue regeneration. This article reports a smart nanosensor platform for labeling and tracking the survival and distribution of ESCs during the EB development in a real-time and non-invasive way. Compared with the cell tracker (i.e. DiO) and the green fluorescent protein (GFP), nanosensors provide the homogenous and highly-efficient ESC labeling. Following the internalization, intracellular nanosensors gradually release the non-fluorescent molecules that become fluorescent only in viable cells. This allows a continuous monitoring of ESC survival and distribution during the process of EB formation. Finally, we confirm that nanosensor labeling does not cause the significant influences to biological properties of the ESCs and EBs.

Statement of Significance

The distribution pattern of viable embryonic stem cells (ESCs) within embryoid body (EB) is closely related with the maturation of EBs. Noninvasive and real-time monitoring of viable ESC distribution in EBs would allow researchers to optimize the culturing condition in time during the EB development and to select the suitable EBs for subsequent applications.

1. Introduction

Embryonic stem cells (ESCs) are a population of self-renewing, pluripotent cells that are able to differentiate into cell types representing all three germ layers [1,2]. ESCs-derived embryoid bodies (EBs, spherical aggregates of ESCs in suspension) recapitulate critical events during early embryo development, which offers a perfect *in vitro* platform to study cell localization, distribution, viability and lineage commitment during embryonic development [3,4].

The distribution pattern of viable ESCs within EBs is closely related with the maturation of EBs. With the progress of ESC differentiation, programmed cell death (apoptosis) gradually occurs in the core of EBs [5,6]. Additionally, several growth factors commonly used to promote ESC differentiation, such as bone morphogenetic protein (BMP) [7–9] and fibroblast growth factor (FGF) [10,11], are also reported to be involved in inducing cell apoptosis in EBs. Therefore, noninvasive and real-time monitoring of ESC viability distribution in EBs would allow researchers to optimize the culturing condition in time during EB development and to select the suitable EBs for subsequent applications.

To monitor the *in vitro* cell viability continuously and non-invasively, fluorescent labels are always preferred due to the low-cost and convenience. For example, lipophilic carbocyanine dyes (e.g. DiO) can selectively label the plasma membrane [12] and has been utilized for the visualization of cell migration and proliferation [13–16]. Unfortunately, carbocyanine dyes cannot provide any information about the cell properties like viability. Another approach is to introduce fluorescent reporter genes (e.g. sequences coding green fluorescent protein (GFP)) into cells of interest [17]. Only if the cells are viable, there are the fluorescent proteins that can be used as the markers for tracking the cell migration and survival [18,19]. For example, transplantation of GFP-expressing tumor cells into nude mice allowed tumor cell invasion and metastasis to be visualized in vivo for the first time [20,21]. The only problem about the reporter gene-based strategy

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is the low non-viral transfection efficiency in primary cells and stem cells (usually <10%) [22,23]. In addition, there might be mutations in genetically modified cells as a result of exogenous gene insertion [24,25].

Previously, we have designed a nanoparticle-based sensor platform to track real-time expression of specific biomarkers that correlate with cell status and functions [26,27]. Based on this platform, we developed a viability nanosensor by incorporating non-fluorescent calcein acetomethoxy (CAM) into poly (lactic-co-glycolic acid) (PLGA) nanoparticles [28]. When internalized by cells, PLGA nanoparticles gradually degrade within the cytoplasm and continuously release encapsulated CAM, which are then converted by esterases in living cells into fluorescent calcein molecules. As a result, CAM nanosensors can be used not only for cell labeling but also for monitoring cell viability.

Here, we report the use of viability nanosensors for highly-efficient ESC labeling and non-invasive monitoring of ESC survival and distribution during EB development (Fig. 1). As comparisons, lipophilic carbocyanine dye (i.e. DiO) and GFP reporter gene are used in parallel. Following the homogenous and highly-efficient internalization, intracellular nanosensors gradually release the non-fluorescent molecules that become fluorescent only in viable cells. While signals from these nanosensors show a steady decrease from day 1 to day 7 due to the rapid cell division within EBs, the unique cell viability monitoring function of nanosensors allows a clear presentation of living cell distribution within the EBs. The analysis of ESC proliferation rate, pluripotency maintenance and differentiation capacity are used for proving the biosafety of the nanosensors.

2. Materials and methods

All chemicals except mentioned specifically were all purchased from Sigma-Aldrich and used without further purification. All cell culture reagents and supplements were obtained from Life Technologies (USA), unless otherwise stated.

2.1. Viability nanosensor synthesis

100 mg of PLGA (lactide:glycolide = 50:50)) was dissolved in 2 mL chloroform at 4 °C and then mixed with 250 μg of CAM (Life technologies) in 250 μL dimethyl sulfoxide (DMSO). The mixture was added dropwise into 3% (w/v) poly (vinyl alcohol) (PVA) solution followed by homogenization (Tissue Master 125, Omni International) at 24,000 rpm for 1 min. The resulted emulsion was then placed in chemical hood overnight for the evaporation of chloroform. Finally, nanosensors were collected by centrifuging at 6000 rpm for 5 min, washed thrice with double-distilled water, and freeze-dried (−80 °C) before being stored in −20 °C prior to usage.

2.2. Murine embryonic stem cell (mESC) culture

mESC (E14TG2a, American Type Culture Collection (ATCC)) were maintained on tissue culture flasks (Falcon) pre-coated with 0.1% (w/v) gelatin. The culture medium was Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 15% fetal bovine serum (FBS, PAA Laboratories), 0.1 mM nonessential amino acids (NEAA, Gibco), 0.1 mM 2-Mercaptoethanol (2-ME) and 1000 units/mL leukemia inhibitory factor (LIF, Millipore). Upon reaching 80% confluence, the cells were trypsinized and used for subsequent experiment. Only cells with passage number of 6–10 were used for experiments in this study.

2.3. Cell labeling with nanosnesors

1 mg nanosensors were placed in 100 μL of 0.1% (w/v) poly-L-lysine (PLL) aqueous solution for approximately 20 min. Then, PLL-coated nanoparticles were purified through centrifugation and re-dispersed in 1 ml mESC culture medium. mESCs were plated on the gelatin-coated flask overnight before being incubated with nanosensor-containing medium. 12 h later, the nanosensor-containing medium was then replaced with normal mESC culture medium.

2.4. Cell labeling with DiO

2 μL DiO cell-labeling solution was added to 1 ml mESC culture medium to prepare the 0.2% (v/v) working solution. Then mESCs were plated on the gelatin-coated flask overnight before being incubated with the working solution for 4 h. The DiO-containing medium was then replaced with normal mESC culture medium.

2.5. Cell labeling with enhanced GFP (EGFP) plasmid

400 ng pEAK12-EGFP plasmid (promoter: cytomegalovirus, Clontech, USA) was mixed with 1 μL Lipofectamine 2000 (Invitrogen) and incubated at room temperature for 20 min before being added to 500 μL mESC culture medium to prepare the working solution. mESCs were plated on the gelatin-coated flask overnight before being incubated with the working solution. 6 h later, the Lipofectamine-containing medium was replaced with normal mESC culture medium.

2.6. Fluorescence imaging

All fluorescent images were captured with Inverted Fluorescence Microscope (IX71, Olympus) equipped with DP71 camera (Olympus). Then fluorescent positive population percentage and average cellular fluorescent intensity were calculated. First, fluorescent area and average fluorescent intensity in one fluorescent image were quantified with ImageJ. Cell-covered area in according phase contrast image was also calculated with ImageJ. Then fluorescent positive population percentage and average cellular fluorescent intensity were achieved by normalizing the fluorescent area and average fluorescent intensity to the cell-covered area, respectively. For each group, at least 6 images were captured and analyzed.

2.7. Flow cytometry analysis

Cells were dissociated from culture plate using 0.25% trypsin (Gibco) and fixed in 4% (w/v) paraformaldehyde at room temperature for 20 min. After being washed with PBS thrice, 1 million cells from each group were re-suspended in 0.5% (w/v) bovine serum albumin (BSA) solution. Flow cytometry analysis was performed on LSRFortessa X-20 (BD Biosciences) and analyzed with the BD FACSDiva Software (BD Biosciences) and FlowJo (Tree Star).

2.8. Cell proliferation assay

mESCs were seeded at a density of 5.0×10^4 cells/well onto a 24-well tissue culture plate and labeled as above mentioned. Following labeling, the cells were continuously cultured for 7 days. At various time points (day 1, 3 and 7), the culture medium was removed, and the cells were incubated with 500 μL culture medium supplemented with 50 μL of WST-1 reagent (Roche) for 3 h. The supernatant was then collected and examined (absorbance: 450 nm; reference: 620 nm) with Multiskan Spectrum Microplate Photometers (ThermoScientific, Finland). To obtain proliferation