Tissue and Cell 52 (2018) 129–134

Contents lists available at ScienceDirect

Tissue and Cell

journal homepage: www.elsevier.com/locate/tice

Development of optically sensitive liver cells

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ARTICLE INFO

Keywords:
Optogenetics
Channelrhodopsin-2
Transfection
HepG2
Plasmid

ABSTRACT

Optogenetics is a new and emerging field that involves techniques of optics and genetic engineering to influence cellular functionality. In this work, we have successfully incorporated a non-selective cationic channel channelrhodopsin-2 (ChR2) into human hepatocellular carcinoma (HepG2) cells. A plasmid construct AAV-CAG-ChR2-GFP was used for liposomal transfection into the cells. ChR2 is a light sensitive membrane channel that opens upon illumination with blue light. Plasmid DNA isolation from *E. coli* XL 10 gold bacteria by alkaline lysis method resulted in a DNA concentration of 1150 µg/mL. A significant difference ($p < 0.05$) was observed between the fluorescent intensities of transfected cells and the control. The percentage of transfected cells was estimated to be 41.26%. Overall, the study delivers an optimized methodology to produce the transfected HepG2 cells that can be controlled with the light stimulation. Although ChR2 has mostly been associated with excitable cells, we anticipate that its presence into HepG2 cells may also result changes in biological functionalities by modulating the concentration of cations inside the cell. Furthermore, the transfected HepG2 cells can be co-cultured with fibroblasts such as NIH 3T3 to form liver spheroids that can serve as models for toxicological and pharmacological studies.

1. Introduction

Over the past few years, the field of optogenetics has gained widespread attention particularly in developing optically sensitive cells. Although this emerging research opportunity has been exploited first in the field of neuroscience, such combination of optics and genetic engineering has spread its wings into other domains as well, mainly because of the fact that it enables understanding and studying of the underlying biological functionality. The technique is considered to be non-invasive and non-contact, and importantly facilitates wireless control with high spatial resolution and temporal precision. Optogenetics involves development of light sensitive proteins, insertion of such proteins into the host cells, stimulation of the optically sensitive cells and finally observation of the biological responses to derive the underlying mechanism (Deisseroth, 2011; Pastrana, 2011). Light gated ion channels such as channelrhodopsin (ChR) are amongst the most exploited ion channels since its discovery in the last decade. Because of their extensive distribution over cell membrane and their association in plethora of biological processes, ion channels can be exploited as potential therapeutic targets (Bagal et al., 2013). Research findings have indicated a key involvement of ion channels in generation of reactive oxygen species (ROS) (Simon et al., 2013), inducing apoptosis, cell proliferation and cancer (Bortner and Cidlowski, 2014; Lang et al., 2005). Ion channels have been critically involved in several human diseases such as neurological disorders (Kumar et al., 2016), skeletal muscle disorders (Barchi, 1993), lung diseases (Salomon et al., 2016), liver diseases (Ramírez et al., 2016) and many more (Niemeyer et al., 2001). Ion channels have been of vital importance in human physiology and toxicology (Restrepo-Angulo et al., 2010). The study of toxicology is concerned with the harmful effects and treatment of toxins and poisons (Parasuraman, 2011). The interdisciplinary field of toxicology can be divided into two major categories namely, systemic toxicology and mechanistic toxicology. Systemic toxicology refers to the toxic effects of a toxicant on the whole body rather than a local area whereas the term mechanistic toxicology deals with the identification and understanding of the molecular and cellular events exerted by the toxins (Frazier, 1994). Mechanistic validation i.e., the understanding of cellular/molecular events that eventually lead to the visible effects of toxins has become an indispensable affair for the researchers in the field of toxicology.

Ion channels are located in the cell membrane, comprising of a diverse group of proteins that can be stimulated by a variety of stimuli such as voltage, temperature, ligands and even light (Alberts et al., 2002). Ion channels have been long known for their role in the

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https://doi.org/10.1016/j.tice.2018.05.004
Received 25 February 2018; Received in revised form 3 May 2018; Accepted 7 May 2018
Available online 08 May 2018
0040-8166/

excitability of neurons and muscles, whereas it is now well recognized for its participation in the physiology of non-excitable cells (epithelial cells) as well (Ambrosi et al., 2014; Pinto et al., 2009). It helps in maintaining the optimum concentration of salt and water in the cytoplasm, it can modulate cellular volume and adjust pH of a cell (Subramanyam and Colecraft, 2015). Such ion channels can prove very impactful in gaining an insight into the structure-function mechanism of some potentially fatal degenerative diseases. Engineered light-gated ion channels can be utilized as biosensors that further supports its application in reducing the harmful effects and identifying the symptoms of a disease at an early stage (Banghart et al., 2006). Moreover, induced pluripotent stem-cells (iPSCs)- derived cardiomyocytes transfected with ChR2 has been exploited in the development of a high- throughput toxicity screening platforms. Thereby, mimicking the physiological heart rates and characterization of the unfavourable effects of the known ion channel blockers and pro-arrhythmogenic drugs on Na^{2+}, Ca^{2+} and K^{+} channels, is being performed. The results from such experiments provide insights in developing strategies in commencement of ion channel drug discoveries (Rizzetto et al., 2018).

In this work, we demonstrate transfection of liver hepatocellular carcinoma cells (HepG2) with a plasmid construct AAV-CAG-ChR2-GFP in order to develop optically controllable HepG2 cells. In parallel, mouse embryonic fibroblast cells (NIH 3T3) were used a model cell type for establishment of the transfection procedures. The plasmid DNA contains ubiquitous mammalian promoter CAG, ChR2 gene and a green fluorescence protein (GFP) gene within it. Channelrhodopsin-2 (ChR2) is a light activated channel and is derived from the algae *C. Reinhardtii* (Nagel et al., 2005). The expression of the plasmid enables incorporation of a ChR2 membrane protein along with identification of transfected cells with the help of emission of green light. We anticipate that upon illumination with ~470 nm wavelength, the retinal chromophore inside the channel would isomerize from *cis* to *trans* configuration and vice versa and therefore open up the channel (Hegemann and Möglich, 2011) as shown in the hypothetical schematic in Fig. 1(i). This opening and closing of the ChR2 has mostly been associated with non-specific influx of cations like H^{+}, Na^{+}, K^{+} and Ca^{2+} which may have positive implications in governing cell functionality [Fig. 1(i)] (Barritt et al., 2008; Lin et al., 2009). The transfection of ChR2 channels has been previously investigated in excitable cells and to the best of our knowledge we are reporting for the first time its integration into HepG2, a non-excitable cell.

2. Materials and methods

2.1. Bacterial culture and isolation of plasmid AAV-CAG-ChR2-GFP using alkaline lysis method

The *E. Coli* XL 10 gold bacteria with the plasmid AAV-CAG-ChR2-GFP was a gift from Edward Boyden (Addgene plasmid #26929). These bacteria were grown in NZY broth with ampicillin antibiotic at 37 °C and 160 rpm for 20 h in a bacteriological incubator shaker. Further, streak method was performed on a standard Luria bertani (LB) agar plate to obtain isolated bacterial colonies. Handpicked bacterial colony was inoculated in NZY broth (under conditions of 37 °C temperature and 160 rpm for 20 h) and observed for turbidity in the broth. The bacterial culture was taken and alkaline lysis was performed to isolate the plasmid DNA. Phenol chloroform extraction method was implemented and ethanol precipitation was carried out to obtain pure plasmid DNA. After isolation of plasmid DNA, preliminary agarose gel electrophoresis was also performed to determine the presence of single intact plasmid DNA. The obtained DNA was quantified, and the concentration and purity was assessed by performing spectrophotometry (BioSpectrometer, Eppendorf) (Sambrook et al., 1989).

2.2. Culture of HepG2 and NIH 3T3 cell lines

Liver hepatocellular carcinoma cells (HepG2) and mouse embryonic fibroblast cells (NIH-3T3) cells were obtained from National Center for Cell Science (NCCS) Lab, Pune, India. They were cultured in polystyrene culture plates with a culture medium containing 89% Dulbecco's Modified Eagle Medium (DMEM, HiMedia), 10% fetal bovine serum (FBS, HiMedia) and 1% penicillin-streptomycin (100 µg/ml Penicillin, 100 µg/ml Streptomycin, HiMedia). The cells were grown at 37 °C, and 5% CO_2 in a CO_2 incubator. The cells were subcultured by trypsin-EDTA (0.25%, HiMedia) dissociation after they reached 80% confluency. Harvesting of the cells was carried out by dissociating from the culture dish using trypsin-EDTA (0.25%, HiMedia) solution for 5 min at 37 °C. The dissociated cells were then pelleted by centrifugation at 1500 rpm for 3 min. The cells were then re-suspended in cell culture media and the counting was done.

2.3. Transfection of HepG2 and NIH 3T3 cell line with the isolated plasmid DNA

Transfection was performed by using Lipofectamine™ 3000 (Thermo Fisher Scientific) reagent kit. The kit contained Lipofectamine™ 3000 (Thermo Fisher Scientific) reagent and P3000™ (Thermo Fisher Scientific) reagent. Transfection was performed on HepG2 and NIH 3T3 cell lines according to manufacturer's protocol. Initial cell density of 10,000 cells was used for plating. Cells were plated a day before the experiment in 96 well plate and transfection was performed after the cells reached a confluency of 70–80%. 0.3 µL Lipofectamine™ 3000 reagent was diluted in 5 µL of Opti-MEM™ medium. A master mix of DNA [0.2 µg] was prepared by diluting it into 10 µL of Opti-MEM™ medium and then 0.4 µL of P3000™ reagent was added to the solution. The two mixtures- diluted Lipofectamine™ 3000 and diluted DNA was added in 1:1 ratio [5 µL each] and was incubated for 15 min at room temperature. The complex was then added to the cells in a drop wise manner. The cells with transfection complex medium were top upped with Opti-MEM™ medium to make the final volume 100 µL. These cells were then incubated for 2–4 days at 37 °C in a CO_2 incubator (Galaxy 170S, Eppendorf). The cells were observed 3 days post-transfection. Transfection media were changed to cell culture media without penicillin-streptomycin. The media were changed every alternate day. Same numbers of cells were seeded for the experiments control as well. Only Opti-MEM™ medium was added to the control wells.

2.4. Microscopy

An inverted DIC/fluorescence microscope (Nikon TiU, Nikon, Japan) was used to observe the cell morphology under bright field and fluorescence images were collected using the appropriate filter. The stimulation of ChR2 was also performed using the same blue band pass filter. The expression of GFP suggests successful transfection of the cell lines with the plasmid AAV-CAG-ChR2-GFP. All images were acquired at 20× magnification.

2.5. Methodology for statistical analysis

The images were converted to grey scale using ImageJ software and background was subtracted by 50 pixels. To clearly visualize the transfected cells, the subtracted image was added to itself by image calculator module. Using multi point tool in the software the cells were marked and the mean intensity values of the fluorescence was measured using analyse module. The graphs were prepared using Origin software. The significance value was calculated by performing one-way ANOVA.