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Cardiac Optogenetics*

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***Abstract*— For therapies based on human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes (CM) to be effective, arrhythmias must be avoided. Towards achieving this goal, light-activated channelrhodopsin-2 (ChR2), a cation channel activated with 480 nm light, and a first generation halorhodopsin (NpHR1.0), an anion pump activated by 580 nm light, have been introduced into hiPSC. By using *in vitro* approaches, hiPSC-CM are able to be optogenetically activated and inhibited. ChR2 and NpHR1.0 are stably transduced into undifferentiated hiPSC via a lentiviral vector. Via directed differentiation, both wildtype hiPSC-CM (hiPSC^{WT}-CM) and hiPSC$^{ChR2/NpHR}$-CM are produced and subjected to both electrical and optical stimulation. Both hiPSC^{WT}-CM and hiPSC$^{ChR2/NpHR}$-CM respond to traditional electrical stimulation and produce similar contractility features but only hiPSC$^{ChR2/NpHR}$-CM can be synchronized and inhibited by optical stimulation. Here it is shown that light sensitive proteins can enable *in vitro* optical control of hiPSC-CM. For future therapy, *in vivo* optical stimulation could allow precise and specific synchronization of implanted hiPSC-CM with patient cardiac rates and rhythms.**

I. Introduction

Human induced pluripotent stem cells (hiPSC) have emerged as a valuable source for basic and translational studies since this cell type can be derived from adult cells and, in principle, can serve as an autologous cell source requiring no immunosuppression upon implantation [1, 2]. hiPSC can give rise to every cell type in the body and have been differentiated into various electrically-active cell types, including cardiomyocytes (hiPSC-CM) [3, 4]. For regenerative medicine applications, the rhythms of hiPSC-CM will need to be precisely matched to recipient rhythms to avoid arrhythmias [5]. To date, differentiation protocols for hiPSC-CM have mainly utilized growth factors; however, the resulting CM retain an immature phenotype and are electrically heterogeneous [3]. Given that recent *in vitro* and *in vivo* evidence has shown that electrical stimulation causes alignment of cultured CM along the electrical conduction axis [6] and that electrical conduction is required to preserve cardiac morphology during development [7], electrical stimulation may be needed for inducing a mature CM fate [8]. However, traditional electrical stimulation is limited by low spatial resolution, by inhomogeneous de- and hyper-polarization, and by alterations of pH at the stimulus electrodes [9]. Optogenetics, a technology which employs light-gated ion channels such as channelrhodopsin-2 (ChR2), a cation-selective channel, and halorhodopsin (NpHR), an anion-selective channel, to control genetically targeted cells with high spatiotemporal precision, offers a powerful tool for potentially overcoming these limitations as shown in Fig. 1 [10]. Recently, optogenetic studies with mouse, zebrafish, and human CM have demonstrated the ability to apply long-term depolarizations, the ability to locate and control pacemaker development, and the ability to computationally model light-activated stimulation [11-14]. In order to create a new avenue for synchronization of human autologous electrically-active cells with recipient rhythms, a stably transduced hiPSC line expressing ChR2 and a first generation NpHR (NpHR1.0) is described, differentiation of this line into hiPSC-CM is shown, and, finally, optical activation and inhibition of these cells is demonstrated below.

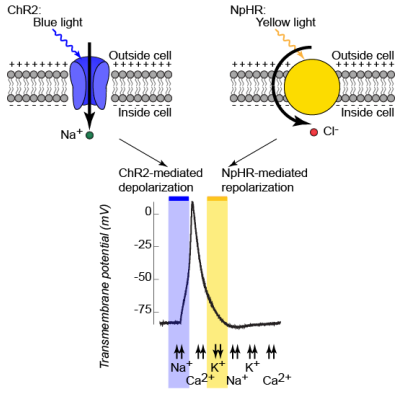

Figure 1. Activation of channelrhodopsin-2 (ChR2) and halorhodopsin (NpHR) allows depolarization and repolarization of an action potential in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM).

II. Methods

All experiments, methods, and protocols for this study were approved by the Stanford University Stem Cell Research Oversight (SCRO) committee.

Lentiviral Vector Construction. The lentiviral vector pLenti-EF1α-ChR2-eYFP-WPRE (pLECYT) and pLenti-EF1α-NpHR1.0-mCherry-WPRE (pLENMT, a first generation NpHR vector) were constructed as previously described [10]. All constructs have been previously fully sequenced for accuracy of cloning and updated maps are available online at http://www.optogenetics.org.

Lentivirus Production and Transduction. High-titer lentivirus was produced using a second generation lentiviral system by co-transfection of 293FT cells (Invitrogen, Carlsbad, CA), the pLECYT and pLENMT viral vectors described above, pCMVRΔ8.74 (containing GAG and POL), pMD2.G (containing VSVg), and calcium phosphate. The undifferentiated IMR90 hiPSC line (WiCell, Madison, WI) was transduced by adding 10-20 μl of concentrated virus per one well of a 6-well plate. ChR2-eYFP and NpHR1.0-mCherry expression was observed after approximately 3-5 days.

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Fluorescence-Activated Cell Sorting (FACS). Undifferentiated hiPSC sequentially transduced with ChR2-eYFP and NpHR1.0-mCherry lentivirus were sorted with a BD FACSAria instrument (BD Biosciences) equipped with 488 nm, 532 nm, and 635 nm lasers along with BD FACSDiva 6.0 software. Analysis of FACS data was done offline with FlowJo 7.6.1 software (Tree Star, Ashland, OR). A double-positive sorted subpopulation of hiPSC expressing eYFP and mCherry was expanded over two weeks.

Polymerase Chain Reaction (PCR). For undifferentiated hiPSC, PCR primers for GAPDH, Oct-4, Nanog, ChR2, eYFP, NpHR1.0, and mCherry were used. Non-quantitative PCR was performed by using components of the StemPro EZChek Human Tri-Lineage Multiplex PCR Kit (Invitrogen) and custom-designed PCR primers as described above. Total RNA was isolated and quantified and the total RNA was used to synthesize cDNA. For PCR amplification, AccuPrime Pfx SuperMix (Invitrogen), custom primers, and cDNA were combined and the following cycling program was used: a) initial denaturation at 95 °C for 2 min; b) 30 cycles of 95 °C, 30 sec; 60 °C, 30 sec; 68 °C, 1 min; c) final extension at 68 °C for 5 min.

Cell Culture. hiPSC (IMR90) were maintained in the pluripotent state through daily feeding with mTeSR1 media (StemCell Technologies, Vancouver, Canada) and were grown on hiPSC-qualified Matrigel (BD Biosciences, San Jose, CA).

Cardiomyocyte (CM) Differentiation. For differentiation, hiPSC were transferred to RPMI-1640 media (Invitrogen) supplemented with B27, 1x non-essential amino acids, 1x penicillin/streptomycin, and 0.1 mM β-mercaptoethanol (all Invitrogen). Our differentiation method used 50 ng/mL Activin A (Day 0), 5 ng/mL BMP-4 (Days 1-3), and 150 ng/mL Dkk-1 (Days 5-9) adapted from previously described methods [15, 16] and shown in Fig. 2. Cardiomyocytes generally began spontaneously beating sometime between days 10 and 14.

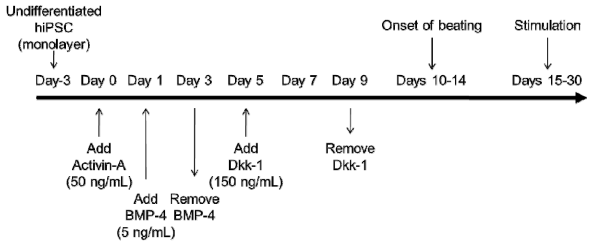

Figure 2. Differentiation protocol of hiPSC-CM

Immunocytochemistry (ICC). Using standard protocols, undifferentiated hiPSC were labeled with primary antibodies for the pluripotency markers Oct-4 (#MAB4401, Millipore), SSEA-4 (#MAB4304, Millipore), TRA-1-60 (#MAB4360, Millipore), and TRA-1-81 (#MAB4381, Millipore). hiPSC-CM were labeled with primary antibodies for the cardiac marker troponin I (TnI) (#MAB1691, Millipore). Secondary antibodies used were goat anti-mouse IgG-Alexa 633 (Invitrogen) and goat anti-mouse IgM Alexa-633 Invitrogen. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma).

Image and Video Microscopy. An AxioObserver Z1 (Carl Zeiss, Göttingen, Germany) inverted microscope was used to visualize undifferentiated hiPSC and hiPSC-CM. The Zeiss microscope was equipped with a Lambda DG-4 300 W Xenon light source (Sutter Instruments, Novato, CA), an ORCA-ER CCD camera (Hamamatsu, Bridgewater, NJ), and AxioVison 4.7 software (Zeiss). To visualize hiPSC-CM contractions, a custom edge detection algorithm in LabVIEW 2009 (National Instruments, Austin, TX) was used to detect rising and falling edge locations along a grayscale profile generated from a user-defined region of interest (ROI) based on a user-defined threshold value.

Calcium Imaging. Fluo-4 AM (Invitrogen) was incubated with hiPSC-CM for 30 min and then visualized with the Zeiss microscope and software described above.

Optical Stimulation. Optical stimulation was delivered to hiPSC-CM via a Lambda DG-4 300 W Xenon light source or with a 470 nm LED (Thorlabs, Newton, NJ) and consisted of a monophasic waveform with 100% of maximum power (10 mW/mm2 for 40x objective), pulse width of 50 ms, and frequency of 0.5 to 1.5 Hz.

III. Results

A double-positive FACS subpopulation of hiPSC expressing eYFP and mCherry was tested by PCR for expression of ChR2 and NpHR1.0. PCR confirmed the presence of pluripotency markers Oct-4 and Nanog along with the expression of ChR2, eYFP, NpHR1.0, and mCherry, as shown in Fig. 3.

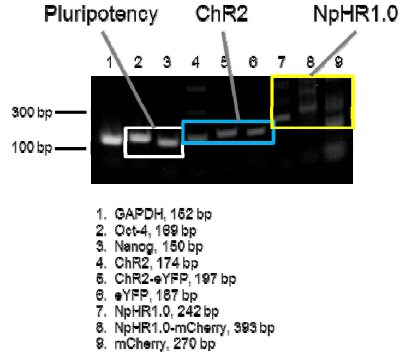

Figure 3. PCR shows expression of the pluripotency genes Oct-4 and Nanog, along with ChR2, eYFP, NpHR1.0, and mCherry in undifferentiated, double-FACS sorted hiPSC. (GAPDH, glyceraldehyde 3-phosphate dehydrogenase, serves as a positive control).

Next, this ChR2 positive, NpHR1.0 positive hiPSC line (hiPSC$^{ChR2/NpHR}$) was then differentiated into hiPSC$^{ChR2/NpHR}$-CM. As shown in Fig. 4a-d, these CM express ChR2 and NpHR1.0 as confirmed by the presence of eYFP and mCherry signals under fluorescence microscopy. In addition, these CM express the cardiac marker TnI and exhibit characteristic banding patterns indicative of sarcomere structures, as shown in Fig. 4e. Moreover, Fluo-4 fluorescence reveals that the CM have spontaneous calcium transients, as shown in Fig. 4f. Furthermore, hiPSC$^{ChR2/NpHR}$-CM show no differences from wild-type hiPSC (hiPSC^{WT}-CM) when electrically stimulated (data not shown).

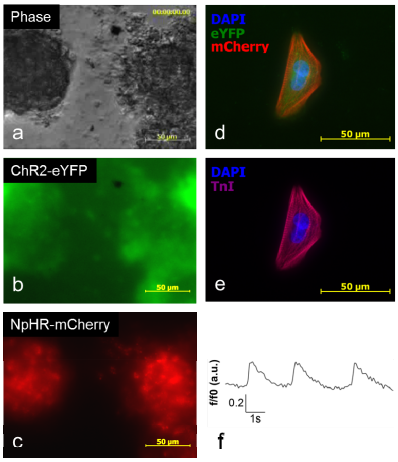

Figure 4. (a-d) hiPSC-CM express ChR2 and NpHR1.0 channels as indicated by eYFP and mCherry fluorescent protein expression, respectively. (e) hiPSC-CM express the cardiac marker troponin I (TnI). DAPI stains the nucleus. (f) hiPSC-CM exhibit spontaneous calcium transients. ((a-c) 400x total magnification; (d-e) 630x total magnification; scale bar, 50 µm; DAPI, 4', 6-diamidino-2-phenylindole).

Finally, upon stimulation with blue light at a wavelength of 480 nm, hiPSC$^{ChR2/NpHR}$-CM can be activated at frequencies ranging from 0.5 to 1.5 Hz. Fig. 5 shows the synchronization of the two colonies shown in Fig. 4a-c with blue light delivered at 1 Hz. Furthermore, hiPSC$^{ChR2/NpHR}$-CM can be inhibited with yellow light at a wavelength of 580 nm as shown in Fig. 6.

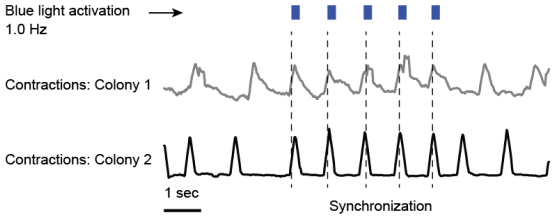

Figure 5. The two hiPSC-CM colonies shown in Fig. 4 are synchronized with blue light (480 nm) delivered at 1 Hz. Note that the colonies are asynchronous before and after the application of the light pulses.

IV. Discussion

At the gene, protein, and functional levels, the introduction of ChR2 and NpHR1.0 into undifferentiated hiPSC allows for downstream optical control of hiPSC-derived CM. Although inhibition can be achieved with this first generation of NpHR, a third generation NpHR (NpHR3.0) has been recently been described [17], which provides for more than 20-fold stronger inhibition than the initial version; use of NpHR3.0 could potentially allow for more potent inhibition as has been shown for neurons. Additionally, the use of red-shifted opsins such as the C1V1 family could increase the depth of control in tissue as longer wavelengths penetrate more and scatter less [18].

In principle, optical control should be achieved with other hiPSC-derived electrically-active cells such as neurons, smooth muscle cells, skeletal muscle cells, retinal cells, and pancreatic cells. For cell-based therapies, previous studies suggest that electrical stimulation and synchronization will be required for optimal differentiation and subsequent engraftment into host tissues. This, in turn, will be necessary to both avoid arrhythmias and restore function in damaged tissues and organs [5-7, 19]. In principle, implanted light-sensitive cardiomyocytes with a slower inherent rate than the recipient heart rate could be paced faster via ChR2, while, conversely, implanted cardiomyocytes with a faster inherent rate than the recipient heart rate could be paced slower via NpHR. In both cases, in principle, gap-junction mediated coupling would not necessarily be required for synchronization; the only requirement would be the ability to monitor the recipient heart rate in order to stimulate or inhibit the implanted light-sensitive cardiomyocytes. Such closed-loop control is already achievable in existing pacemakers.

Guided by computational predictive tools, the combined application of biochemical, spatial, mechanical, and optogenetic-mediated electrical stimuli will provide for potentially powerful tools for high spatiotemporal control and interrogation of stem cell differentiation and synchronization [20-23]. However, like traditional electrical stimulation, optical stimulation could also lead to inhomogeneous activation and inhibition; factors such as viral transduction efficiency, geometric arrangement of the cells with respect to the light source, and delivery of light through surrounding tissue and blood will need to be optimized to maximize activation and inhibition fidelity.

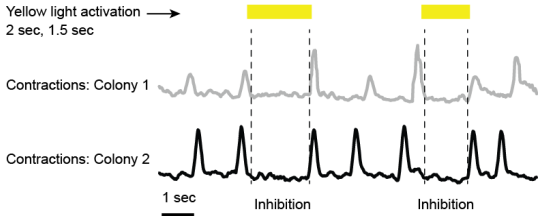

Figure 6. The two hiPSC-CM colonies shown in Fig. 4 are inhibited with yellow light (580 nm) delivered at 2 seconds then 1.5 seconds. Note that the colonies are asynchronous before and after the application of both yellow light pulses.

The next challenging step for application of optogenetic control of hiPSC-CM *in vivo* will be to establish suitable optical stimulation hardware for use in a large animal model such as swine. This hardware includes, for example, implantable optical pulse generators, fiber optic cardiac catheters, and light emitting diodes. CM implanted in zebrafish and rodents can be optically depolarized and repolarized with the same bench-top optical stimulation hardware that is currently used for activating cells *in vitro* [11, 12]. However, these small animal models, which have beating rates of ~6-10 Hz, are not suitable for electromechanical coupling with hiPSC-CM, which have beating rates of ~0.5-2 Hz [24]. Although stem cell-derived CM have been implanted as a biological pacemaker in swine [25], lack of large animal optical stimulation hardware prohibits any testing of optogenetic hiPSC-CM *in vivo* at present. It is hoped that the results presented here inspire creation of large animal and human optical stimulation technology. It is also envisioned that cardiac optogenetics will create new avenues for basic and translational studies in stem cells, developmental biology, electrophysiology, and regenerative medicine.

V. Conclusion

The light sensitive proteins ChR2 and NpHR1.0 can enable *in vitro* optical activation and inhibition of hiPSC-CM contractions. For future therapy, *in vivo* optical stimulation could allow precise and specific synchronization of implanted hiPSC-CM with patient cardiac rates and rhythms, even in the absence of connexin-mediated coupling. This, in turn, would mitigate arrhythmia generation, leading to a safe and effective autologous cell-based therapy.

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