Human Pluripotent Stem Cell Tools for Cardiac Optogenetics*

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***Abstract*— It is likely that arrhythmias should be avoided for therapies based on human pluripotent stem cell (hPSC)-derived cardiomyocytes (CM) to be effective. Towards achieving this goal, we introduced light-activated channelrhodopsin-2 (ChR2), a cation channel activated with 480 nm light, into human embryonic stem cells (hESC). By using *in vitro* approaches, hESC-CM are able to be activated with light. ChR2 is stably transduced into undifferentiated hESC via a lentiviral vector. Via directed differentiation, $hESC^{ChR2}$-CM are produced and subjected to optical stimulation. $hESC^{ChR2}$-CM respond to traditional electrical stimulation and produce similar contractility features as their wild-type counterparts but only $hESC^{ChR2}$-CM can be activated by optical stimulation. Here it is shown that a light sensitive protein can enable *in vitro* optical control of hESC-CM and that this activation occurs optimally above specific light stimulation intensity and pulse width thresholds. For future therapy, *in vivo* optical stimulation along with optical inhibition could allow for acute synchronization of implanted hPSC-CM with patient cardiac rhythms.**

I. Introduction

Human pluripotent stem cells (hPSC), including human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC) can give rise to every cell type in the body and have been differentiated into various electrically-active cell types, including cardiomyocytes (hPSC-CM) [1-3]. hESC and hiPSC have emerged as a valuable source for basic and translational studies; hiPSC in particular, whose creation was fostered from the knowledge of hESC biology, can be derived from adult cells and, in principle, can serve as an autologous cell source requiring no immunosuppression upon implantation [4, 5]. For regenerative medicine applications, it seems likely that the rhythms of hPSC-CM will need to be acutely and chronically matched to recipient rhythms to avoid arrhythmias [6].

Optogenetics is a technology which employs the targeted genetic introduction of light-sensitive channels, such as channelrhodopsin-2 (ChR2), and pumps, such as halorhodopsin (NpHR), into cells that enables their high spatiotemporal activation and inhibition, respectively, by optical actuation [7-9]. Optogenetic-mediated electrical actuation overcomes the limitation of low spatial control by traditional electrode stimulation, which is due in part by electrode size and spacing. This limitation hinders single cell control within a population of cells [10, 11].

To date, optogenetics has mainly been demonstrated in neurons [7-9], various non-cardiac cellular types, [12, 13], and pluripotent stem cells (PSC) [14-16]. In the cardiac field, optogenetics has been used in murine stem cell-derived cardiomyocytes [14], zebrafish pacemaker cells [17], and to control light-activated behavior of human pluripotent stem cell-derived cardiomyocytes (hPSC-CM) [16, 18, 19].

We previously have described optogenetic stimulation of hESC-CM by ChR2 and both stimulation and inhibition of hiPSC-CM by ChR2 and NpHR1.0, a first generation halorhodopsin inhibitor [16, 19]. Furthermore, we have described a technology that employs an optogenetic six (6)-LED array for chronically perturbing cardiac electrophysiology [20].

As shown in Fig. 1, for stimulation, ChR2, a seven transmembrane protein channel derived from algae, is actuated by blue light of around 480 nm. This actuation leads to non-specific cation flow from outside a cell to inside; for cardiomyocytes, this depolarization, mainly due to sodium cationic flow, activates inherent voltage-gated sodium channels, which then further leads to activation of inherent calcium and potassium channels. This cascade of events leads to a cardiac action potential. Below, we briefly describe creation of ChR2-controlled hESC-CM and the optimal optical parameters, such as intensity, pulse width, and frequency that are required to control these cells.

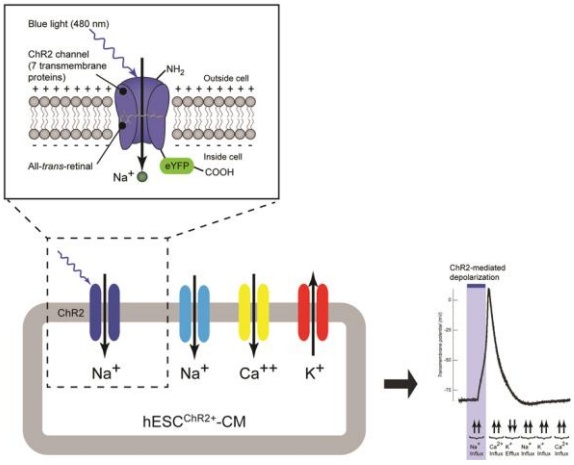

Figure 1. Activation of channelrhodopsin-2 (ChR2) allows depolarization of an action potential in human embryonic stem cell-derived cardiomyocytes (hESC-CM)

II. Methods

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

Lentiviral Construction. As shown in Fig. 2, a lentiviral vector for channelrhodopsin-2 (ChR2), pLenti-EF1α-ChR2-

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eYFP-WPRE (pLECYT) was constructed as previously described [16, 19]. All constructs have been fully sequenced and vector maps are available at http://www.optogenetics.org.

Lentivirus Production and Transduction. By co-transfection of 293FT cells (Invitrogen, Carlsbad, CA), high-titer lentivirus was produced using the pLECYT viral vector described above, pCMVRΔ8.74 containing GAG and POL, pMD2.G containing VSVg, and calcium phosphate.

Cell Line Production. The undifferentiated stable H9 (WA09) human embryonic stem cell (hESC) line (WiCell, Madison, WI) carrying ChR2 was created as previously described [16, 19]. Briefly, hESC were transduced by adding concentrated virus to the cells. ChR2-eYFP expression was observed by 5 days. ChR2-eYFP expressing cells were isolated by fluorescence-activated cell sorting (FACS). These lines are further referred to as $hESC^{ChR2}$ for simplicity while there non-transduced counterparts are referred to as $hESC^{WT}$.

Fluorescence-Activated Cell Sorting (FACS). Undifferentiated hESC transduced with ChR2-eYFP were sorted with a BD FACSAria instrument (BD Biosciences) equipped with BD FACSDiva software. Analysis of FACS data was done offline with FlowJo software (Tree Star, Ashland, OR).

Polymerase Chain Reaction (PCR). For undifferentiated hESC, PCR primers for GAPDH, Oct-4, Nanog, ChR2, and eYFP were used. Total RNA was isolated and quantified and was then used to create cDNA. For PCR amplification, AccuPrime Pfx SuperMix (Invitrogen), custom primers, and cDNA were combined.

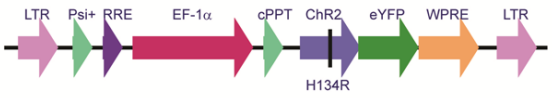

Figure 2. Lentiviral vector containing channelrhodopsin-2 (ChR2) and enhanced yellow fluorescent protein (eYFP) expressed under the control of the constitutive promoter EF-1α.

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

Cardiomyocyte (CM) Differentiation. To differentiate hESC to CM, hESC were grown in tissue culture plates containing RPMI-1640 media with B27, 1x non-essential amino acids, 1x penicillin/streptomycin, and 0.1 mM β-mercaptoethanol (all Invitrogen, Carlsbad, CA). The differentiation method utilized 50 ng/mL Activin A, 5 ng/mL BMP-4, and 37.5 ng/mL concentrated Dkk-1 as from our previously described methods [16, 19]. Cardiomyocytes generally began beating by day 14.

Image and Video Microscopy. To visualize undifferentiated hESC and hESC-CM, an AxioObserver Z1 (Carl Zeiss, Göttingen, Germany) inverted microscope was used. The microscope was equipped with a Lambda DG-4 300 Watt Xenon light source (Sutter Instruments, Novato, CA), an ORCA-ER CCD camera (Hamamatsu, Bridgewater, NJ), and AxioVison software (Zeiss).

Optical Stimulation. hESC-CM optical stimulation was achieved via a Lambda DG-4 300 Watt Xenon light source and consisted of a monophasic waveform with 100% of maximum power (10 mW/mm^2 for 40x objective), pulse width of 5-100 ms, and frequency of 0.5 to 2.0 Hz.

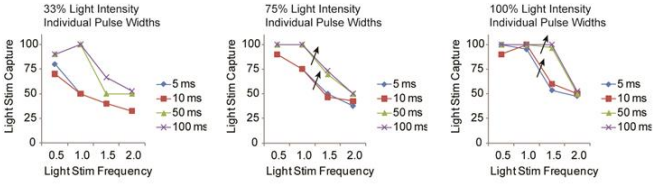

Figure 3. Percentage of light stimulation capture of $hESC^{ChR2}$-CM as a function of light intensity, pulse width, and stimulation frequency.

III. Results

Lentivirus containing ChR2 in-frame with eYFP was introduced into pluripotent hESC. These cells were FACS sorted for a positive subpopulation expressing eYFP. This single-positive subpopulation was then expanded over three weeks, when it was then tested by PCR for expression of the pluripotency markers Oct-4 and Nanog along with the expression of ChR2 and eYFP. There were no detectable phenotypic differences between $hESC^{WT}$ and $hESC^{ChR2}$ lines.

Next, the $hESC^{WT}$ and $hESC^{ChR2}$ lines were differentiated into CM. There was no difference in the time required for CM differentiation between both lines. $hESC^{ChR2}$-CM spontaneously contracted and showed no differences from $hESC^{WT}$-CM (data not shown). In addition, $hESC^{ChR2}$-CM expressed ChR2 as confirmed by the presence of eYFP signals under standard fluorescence microscopy.

Upon stimulation with a Lambda DG-4 300 Watt Xenon light source filtered for 480 nm blue light, it was confirmed that $hPSC^{ChR2}$-CM were responsive to this wavelength and could be variably activated at frequencies ranging from 0.5 to 2.0 Hz

Fig. 3 shows the percentage of light stimulation capture of $hESC^{ChR2}$-CM as a function of light intensity, pulse width, and stimulation frequency. A light intensity of 100% (corresponding to 10 mW/mm^2 for 40x objective) gave near 100% capture at frequencies up to 1.5 Hz. In addition, pulse widths of 50 and 100 ms gave the highest light stimulation capture for increasing stimulation frequencies. Finally, light

stimulation capture generally decreased as light stimulation frequency approached 2.0 Hz.

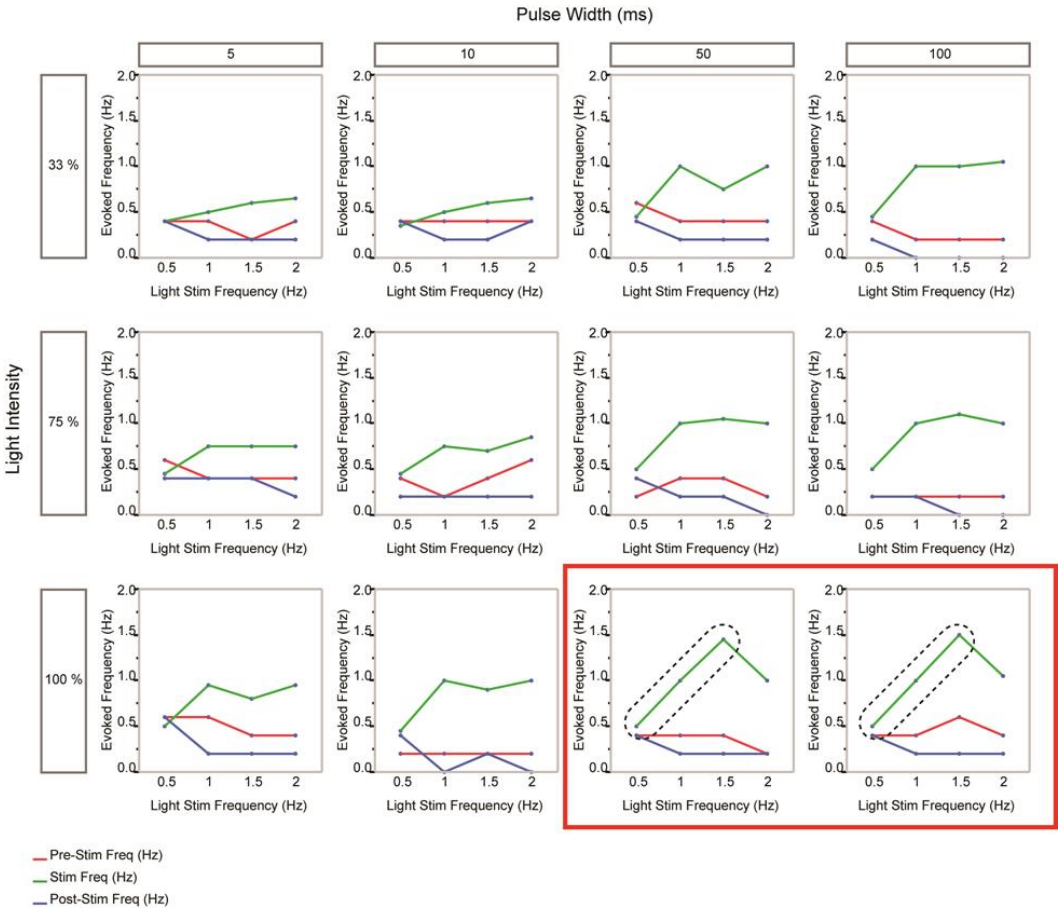

Figure 4. Evoked pre-stimulation, stimulation, and post-stimulation contraction frequencies of hESC^{ChR2}-CM as a function of input light stimulation intensity, pulse width, and frequency.

Finally, Fig. 4 shows light-evoked pre-stimulation, stimulation, and post-stimulation contraction frequencies of hESC^{ChR2}-CM as a function of input light stimulation intensity, pulse width, and frequency. Again, a light intensity of 100% (corresponding to 10 mW/mm^2 for 40x objective) resulted in evoked stimulation contraction frequencies matching input light stimulation frequencies up to 1.5 Hz at the higher pulse widths of 50 and 100 ms. Finally, light-evoked stimulation contraction frequencies matched input light stimulation at a ratio of 1:1 at frequencies up to 1.5 Hz. At 2.0 Hz, contractions occurred 1:2 compared to the input light stimulation frequency. The red rectangle identifies the optimal light stimulation intensity and pulse widths.

IV. Discussion

The introduction of ChR2 into undifferentiated hESC allows for downstream optical control of hESC-derived CM as demonstrated at the genetic, protein expression, and electrophysiological levels. As others have shown, optical control can also be achieved with other human and murine PSC-derived cells such as central and peripheral neurons [15, 21]. For cellular-based therapies, previous studies suggest that electromechanical synchronization and coupling will be required for subsequent functional engraftment into host tissues in order to both avoid arrhythmias and restore function [6, 22, 23].

As we previously outlined [20] the next steps for application of optogenetic control of hPSC-CM (including hESC-CM and hiPSC-CM) *in vivo* will be to establish suitable optical stimulation hardware for use in small, medium, and large animal models such as mice, rats, guinea pigs, and swine. This hardware includes, for example, implantable optical pulse generators, fiber optic-based leads, and light emitting diodes (LEDs). The same bench-top optical stimulation hardware that is currently used for activating cells *in vitro* can be used to optically depolarize and repolarize CM implanted in rodents [14, 17].

For human cardiomyocytes, medium-sized animal models such as guinea pigs [23] have been used to demonstrate electromechanical coupling of hPSC-CM with the recipient heart. However, it is not known whether acute and immediate synchronization will lead to faster and/or more efficient gap junction-mediated coupling. Our hope is that our results presented here for light stimulation parameters of intensity, pulse width, and frequency enable creation of large animal stimulation tools. We envision a complete optically controlled hPSC-CM line that can be activated and inhibited

in a feedback system that will allow acute and immediate synchronization of implanted CM with recipient heart rhythms. This synchronization would not require physical gap junction coupling, which will likely take days to weeks to occur. We also envision that cardiac optogenetics will create new avenues for basic and translational studies in stem cell biology and electrophysiology.

V. Conclusion

The light-sensitive protein ChR2 can enable *in vitro* optical activation of hESC-CM contractions. For blue light stimulation of these stem cell-derived cardiomyocytes, we have demonstrated optimal light stimulation intensity and pulse widths. For future therapy, *in vivo* optical stimulation combined with optical inhibition could allow immediate synchronization of implanted hESC-CM with patient cardiac rates, even in the absence of gap junction-mediated coupling. This, in turn, would mitigate arrhythmia generation, possibly augment chronic electromechanical coupling, and lead to safe and effective cell-based cardiac therapy.

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References

[1] J. Zhang, G.F. Wilson, A.G. Soerens, C.H. Koonce, J. Yu, S.P. Palecek, J.A. Thomson, and T.J. Kamp, "Functional cardiomyocytes derived from human induced pluripotent stem cells," *Circulation Research*, vol. 104, (no. 4), pp. e30-41, Feb 2009.

[2] N. Sun, M. Yazawa, J. Liu, L. Han, V. Sanchez-Freire, O.J. Abilez, E.G. Navarrete, S. Hu, L. Wang, A. Lee, A. Pavlovic, S. Lin, R. Chen, R.J. Hajjar, M.P. Snyder, R.E. Dolmetsch, M.J. Butte, E.A. Ashley, M.T. Longaker, R.C. Robbins, and J.C. Wu, "Patient-specific induced pluripotent stem cells as a model for familial dilated cardiomyopathy," *Science translational medicine*, vol. 4, (no. 130), pp. 130ra47, Apr 2012.

[3] F. Lan, A.S. Lee, P. Liang, V. Sanchez-Freire, P.K. Nguyen, L. Wang, L. Han, M. Yen, Y. Wang, N. Sun, O.J. Abilez, S. Hu, A.D. Ebert, E.G. Navarrete, C.S. Simmons, M. Wheeler, B. Pruitt, R. Lewis, Y. Yamaguchi, E.A. Ashley, D.M. Bers, R.C. Robbins, M.T. Longaker, and J.C. Wu, "Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in patient-specific induced pluripotent stem cells," *Cell stem cell*, vol. 12, (no. 1), pp. 101-13, Jan 2013.

[4] J. Yu, M.A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J.L. Frane, S. Tian, J. Nie, G.A. Jonsdottir, V. Ruotti, R. Stewart, Slukvin, II, and J.A. Thomson, "Induced pluripotent stem cell lines derived from human somatic cells," *Science*, vol. 318, (no. 5858), pp. 1917-20, 2007.

[5] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka, "Induction of pluripotent stem cells from adult human fibroblasts by defined factors," *Cell*, vol. 131, pp. 861 - 872, 2007.

[6] W. Roell, T. Lewalter, P. Sasse, Y.N. Tallini, B.-R. Choi, M. Breitbach, R. Doran, U.M. Becher, S.-M. Hwang, T. Bostani, J. von Maltzahn, A. Hofmann, S. Reining, B. Eiberger, B. Gabris, A. Pfeifer, A. Welz, K. Willecke, G. Salama, J.W. Schrickel, M.I. Kotlikoff, and B.K. Fleischmann, "Engraftment of connexin 43-expressing cells prevents post-infarct arrhythmia," *Nature*, vol. 450, (no. 7171), pp. 819-824, 2007.

[7] E.S. Boyden, F. Zhang, E. Bamberg, G. Nagel, and K. Deisseroth, "Millisecond-timescale, genetically targeted optical control of neural activity," *Nat Neurosci*, vol. 8, (no. 9), pp. 1263-1268, 2005.

[8] F. Zhang, A.M. Aravanis, A. Adamantidis, L. de Lecea, and K. Deisseroth, "Circuit-breakers: optical technologies for probing neural signals and systems," *Nat Rev Neurosci*, vol. 8, (no. 8), pp. 577-581, 2007.

[9] V. Gradinaru, F. Zhang, C. Ramakrishnan, J. Mattis, R. Prakash, I. Diester, I. Goshen, K.R. Thompson, and K. Deisseroth, "Molecular and cellular approaches for diversifying and extending optogenetics," *Cell*, vol. 141, (no. 1), pp. 154-165, 2010.

[10] R. Balint, N.J. Cassidy, and S.H. Cartmell, "Electrical stimulation: a novel tool for tissue engineering," *Tissue engineering. Part B, Reviews*, vol. 19, (no. 1), pp. 48-57, Feb 2013.

[11] T.J. Herron, P. Lee, and J. Jalife, "Optical imaging of voltage and calcium in cardiac cells and tissues," *Circulation Research*, vol. 110, (no. 4), pp. 609-623, Feb 2012.

[12] M.E. Llewellyn, K.R. Thompson, K. Deisseroth, and S.L. Delp, "Orderly recruitment of motor units under optical control in vivo," *Nat Med*, vol. 16, (no. 10), pp. 1161-5, Oct 2010.

[13] M.S. Sakar, D. Neal, T. Boudou, M.A. Borochin, Y. Li, R. Weiss, R.D. Kamm, C.S. Chen, and H.H. Asada, "Formation and optogenetic control of engineered 3D skeletal muscle bioactuators," *Lab Chip*, vol. 12, (no. 23), pp. 4976-85, Dec 2012.

[14] T. Bruegmann, D. Malan, M. Hesse, T. Beiert, C.J. Fuegemann, B.K. Fleischmann, and P. Sasse, "Optogenetic control of heart muscle in vitro and in vivo," *Nat Meth*, vol. 7, (no. 11), pp. 897-900, 2010.

[15] J.P. Weick, M.A. Johnson, S.P. Skroch, J.C. Williams, K. Deisseroth, and S.C. Zhang, "Functional control of transplantable human ESC-derived neurons via optogenetic targeting," *Stem Cells*, vol. 28, (no. 11), pp. 2008-16, 2010.

[16] O.J. Abilez, J. Wong, R. Prakash, K. Deisseroth, C.K. Zarins, and E. Kuhl, "Multiscale computational models for optogenetic control of cardiac function," *Biophysical Journal*, vol. 101, (no. 6), pp. 1326-1334, 2011.

[17] A.B. Arrenberg, D.Y.R. Stainier, H. Baier, and J. Huisken, "Optogenetic control of cardiac function," *Science*, vol. 330, (no. 6006), pp. 971-974, 2010.

[18] J. Wong, O.J. Abilez, and E. Kuhl, "Computational optogenetics: a novel continuum framework for the photoelectrochemistry of living systems," *J Mech Physics Solids*, vol. 60, (no. 6), pp. 1158-1178, Jun 2012.

[19] O.J. Abilez, "Cardiac optogenetics," *IEEE Eng Med Biol Soc*, pp. 1386-9, Aug 2012.

[20] O.J. Abilez, "Optogenetic LED array for perturbing cardiac electrophysiology," *IEEE Engineering in medicine and biology society conference proceedings*, vol. 2013, pp. 1619-1622, 2013.

[21] J.B. Bryson, C.B. Machado, M. Crossley, D. Stevenson, V. Bros-Facer, J. Burrone, L. Greensmith, and I. Lieberam, "Optical Control of Muscle Function by Transplantation of Stem Cell–Derived Motor Neurons in Mice," *Science*, vol. 344, (no. 6179), pp. 94-97, Apr 2014.

[22] K.R. Chien, "Regenerative medicine and human models of human disease," *Nature*, vol. 453, (no. 7193), pp. 302-305, 2008.

[23] Y. Shiba, S. Fernandes, W.-Z. Zhu, D. Filice, V. Muskheli, J. Kim, N.J. Palpant, J. Gantz, K.W. Moyes, H. Reinecke, B. Van Biber, T. Dardas, J.L. Mignone, A. Izawa, R. Hanna, M. Viswanathan, J.D. Gold, M.I. Kotlikoff, N. Sarvazyan, M.W. Kay, C.E. Murry, and M.A. Laflamme, "Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts," *Nature*, vol. 489, (no. 7415), pp. 322-325, 2012.