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Review Article

OPTOGENETICS: A NOVEL APPROACH IN PACING HAERT TISSUE AND ENGENDER PROPAGATING CARDIAC IMPULSES

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ABSTRACT
The cardiac pacemaker controls the rhythmicity of heart contractions and these can be substituted by battery-operated devices as last resource. Optogenetics involves insertion of light-sensitive proteins into human embryonic stem cell to encode DNA making mammalian tissues light-sensitive. The first discovered protein of this type is Channelrhodopsin2 (ChR2), which is widely used in neuroscience. The limitation of electrical stimulation of heart, a standard technique can be overcome by using ChR2.The various methods involved in optogenetics and energy needs were discussed in this section. Initially, optogenetics is confined only to neuronal system, later on extended to heart and other organs. This method involves precise localized stimulation and constant prolonged depolarization of cardiomyocytes and cardiac tissue resulting in alterations of pacemaking, Ca^{2+} homeostasis, electrical coupling and arrhythmogenic spontaneous extra beats.
Keywords: Optogenetics, Light-sensitive proteins, Channel rhodopsin2 (ChR2), Opsins.

INTRODUCTION

In mammals, a specialised group cells in the Sino atrial (SA) which is a primary pacemaker controls the heart rate. Other pacemakers the atrioventricular (AV) node, specialized conducting system, and muscle may initiate electrical activation if the SA node is dysfunctional or suppressed. These pacemakers discharge at a slower rate and, in the absence of an appropriate increase in stroke volume, may result in tissue hypoperfusion. Spontaneous activation and contraction of the heart are the consequence of the specialized pacemaking tissue found within these anatomic locales. Action potentials (AP) in the heart are regionally heterogeneous. The AP's in cells isolated from nodal tissue are distinct from those recorded from atrial and ventricular myocytes. In dysfunction of these pacemakers there occur fluctuations in the generation of AP's which create abnormalities in the cardiac rhythm or arrhythmias or heart rhythm disorders. To bring back the rhythm of heart from many years electrical pacemakers are widely in use with technical limitations and potential complications. For this electrical stimulation an external electrical field is applied that locally induces action potentials that are propagated to electrically couple neighbouring cells[1]. This approach, however, leads to irreversible reactions resulting in production of the toxic gases H_2, O_2or $Cl_2$and alterations of pH[2]. In addition, electrical stimulation produces inhomogeneous areas of de- and hyperpolarization using unipolar point stimulation or global field stimulation of cardiomyocytes and whole hearts[3,4]. To overcome these limitations many researchers have done which finally lead to the stimulation of mammalian cells with light using o advanced technology known as Optogenetics. In this we use of a light-activated non selective cation ChR-2 which is widely used in neuroscience field[5-7]. Illumination of ChR2-expressing cells with blue light (475 nm) induces a sustained inward current and can be performed for extended periods of time[5]. The area of stimulation can be precisely determined by confined illumination. This method enabled precise localized stimulation and constant prolonged depolarization of cardiomyocytes and cardiac tissue resulting in alterations of pacemaking, Ca^{2+} homeostasis, electrical coupling and arrhythmogenic spontaneous extra beats.

Optogenetics

Optogenetics refers to the precise interrogation, stimulation, and control of excitable tissues by light (genetically altered to become light sensitive)[8].Since 1990's,it was known that photo taxis and photophobic responses in green alga Chlamydomonas reinhardtii are mediated by rhodopsins[9,10]. Microorganisms that produce proteins which directly regulate the flow of charged ions across plasma membrane in response to light have been studied. Of these the first identified protein was light ligated ion pump, bacteriorhodopsin[11].

The discovery of these channels and ion pumps in green algae and bacteria began a new era in neuroscience. Light-gated cation channels, usually variants of famous ChR2 are now routinely used by many laboratories to trigger action potentials in neurons with millisecond precision[12].

Initially applications of optogenetics have been restricted exclusively to neuronal system which was now advanced to other cells and organs[13].Optoelectric transducers in nature are a class of light –sensitive transmembrane proteins best represented by bacteriorhodopsin, converting photons into transmembrane voltage through proton pumping.

Microbial Opsins

Species from multiple branches of the animal kingdom have evolved mechanisms to sense electromagnetic radiation in their environments. Likewise many microbes, in the absence of complex eye structures employed by metazoans, have developed light-activated proteins for a variety of purposes. For some, this serves as a mechanism of homeostasis to remain at a certain depth in the ocean[14]; for others, this helps maintain osmotic balance in a highly saline environment[15]. These and other diverse roles are, in many cases, fulfilled by a family of seven-transmembrane, light-responsive proteins encoded by opsin genes. Opsin genes are divided into two distinct super families: microbial opsins (type I) and animal opsins (type II). Opsin proteins from both families require retinal, a vitamin A–related organic cofactor that serves as

the antenna for photons; when retinal is bound, the functional opsin proteins are termed rhodopsins. type I opsin genes are found inprokaryotes, algae, and fungi[16] type II opsin genes are present only in highereukaryotes and are responsible mainly forvision[17,18]. Type II opsingenes encode G protein–coupled receptors (GPCRs) and, in the dark, bind retinal in the11-*cis* configuration. Upon illumination, retinalisomerizes to the all-*trans* configuration andinitiates the reactions that underlie the visual photo transduction second messenger cascade. After photo isomerization, , the retinal-protein linkage is hydrolyzed; free all-*trans* retinal then diffuses out of the protein and is replaced by afresh 11-*cis* retinal protein for another round of signalling[19]. In contrast, type I opsins more typically encode proteins that utilise retinal in the all-*trans* configuration, which photo isomerizes upon photon absorption to the 13-*cis* configuration. Unlike the situation with type II rhodopsin, the activated retinal molecule in type I rhodopsins does not dissociate from its opsin protein but thermally reverts to the all *trans* state while maintaining a covalent bond to its protein partner[20].

Production of light-sensitive cells

Optogenetics allows light sensitive proteins such as ChR2 to be encoded with DNA to produce mammalian tissues sensitive to light. For the production of light sensitive heart cells, ChR2 which encodes DNA was inserted into human embryonic stem cell which controls flow of electrically charged ions into the cells. The primary ion for heart cell is sodium which causes the cell to contract by initiating electrochemical cascade. Then these are later transferred into light sensitive cardiomyocytes.

METHODS

Generation of the *CAG*-driven ChR2-EYFP expression vector

The mutant ChR2 (H134R) has been used because of its higher current amplitudes. The *CMV* promoter in the pcDNA3.1/hChR2 (H134R)-EYFP vector was removed by MunI-SacI excision and replaced by the *CAG* promoter (chicken β-actin promoter with *CMV* enhancer; 1.7 kb SpeI-SacI fragment) from the pCIG2 plasmid. Cloning was verified by DNA sequencing. Before transfection the plasmid was linearized by ScaI digestion.

Histology and immunofluorescence staining

Cells and hearts were fixed with 5% PFA, and cryopreserved. These hearts were sectioned with a cryotome into 10-μm-thick slices. Fixed cells and tissue slices were stained in 0.2% Triton X in PBS, supplemented with 5% donkey serum for 2 hr at room temperature using antibodies to the proteins α-actinin and Oct 3/4 as primary antibodies and Cy3-conjugated secondary antibodies. Nuclear staining was performed with Hoechst 33342[21].

Optical Stimulation

Optical stimulation was delivered to hESC-CMs via a Lambda DG-4 300W Xenon light source or with a 470 nm LED at 7mW/mm2. For multielectrode array (MEA) electrophysiology, optical stimulation consisted of a monophasic waveform with peak amplitude of 0, 33, 75, or 100% of maximum power (10 mW/mm2 for 40_ objective), pulse width of 100 ms, and frequency of 0.5, 1.0, or 1.5 Hz. For whole-cell patch-clamp electrophysiology, optical stimulation consisted of a monophasic waveform with peak amplitude of 0, 12.5, 25, 50, or 100% of maximum power (10 mW/mm2 for 40_ objective) and a pulse width of 1000Ms. Optical power delivered to cells at each microscope objective was measured with a digital power meter at the focal plane of the objective[22].

Stem Cell Culture and Differentiation

Channel rhodopsin-expressing human embryonic stem cells (hESCChR2þ) were grown as monolayers on hESC-qualified Matrigel and maintained in the pluripotent state through daily feeding with mTeSR1 media[23] supplemented with 1_ penicillin/streptomycin (Invitrogen). Cardiomyocyte differentiation was usually begun 2–5 days after initially seeding hESCChR2þ on Matrigel. At this time, the cells were transferred to RPMI-1640 media supplemented with B27, 1_ nonessential amino acids, 1_ penicillin/streptomycin, and 0.1 mM b-mercaptoethanol (all Invitrogen) and differentiation method begins using aspects of other methods. On the first day of differentiation, Day 0, RPMI media with 50 ng/mL of Activin A was added to each well. On the subsequent day, Day 1, 5 ng/mL of BMP-4 was added to each well. On Day 3, fresh RPMI media was added to each well and was replaced every 48 h until Day 11, when the cells were transferred to Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen), 1_ nonessential amino acids, 1_ penicillin/streptomycin, and 0.1 mM b-mercaptoethanol. This DMEM was then replaced approximately every 48 h. Cardiomyocytes generally began spontaneously beating between Days 9 and 2.

Opsin Sources and Lentiviral Vector

The ChR2 variant was optimized for mammalian expression by truncating the native sequence from 2241 base pair (bp) to 933 bp, by changing the native Histidine (H) codon (CAC) to the arginine (R) codon (CGC) coding for protein residue 134 of ChR2 (H134R), and by changing its gene's codon usage to conform to human codon usage distribution[24,25]. The lentiviral vector pLenti-EF1a- ChR2-eYFP-WPRE (pLECYT) was constructed as previously described[25,26]. The pLET plasmid contains the ubiquitously expressed elongation factor 1-alpha (EF1a), to obtain high levels of ChR2-eYFP expression in a mammalian system. All constructs have been fully sequenced previously for accuracy of cloning[24,26]. High-titer lentivirus was produced using a second-generation lentiviral system by cotransfection of 293FT cells (Invitrogen, Carlsbad, CA), the pLECYT viral vector described above, pCMVRD8.74 (containing GAG and POL), pMD2.G (containing VSVg), and calcium phosphate.

ESC Culture and Transfection

R1 and αPIG[27] ESCs were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 15% FCS (Promega), 0.1 mM nonessential amino acids (Invitrogen), 50 μg ml−1 penicillin and streptomycin (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma) and 500 U ml−1 leukemia inhibitory factor (Chemicon). ESCs were cultured on irradiated mouse embryonic fibroblasts derived from neomycin resistant mice. For generation of transgenic ESCs 5 × 106 cells were mixed with 30–40 μg of the linearized plasmid in PBS, electroporated with one pulse at 250 V and 500–750 μF (Bio-Rad Gene Pulser) and plated on two 100 mm plates. Selection for neomycin-resistant colonies started two days after electroporation by adding 300 μg ml−1 G418 to the medium. Resistant colonies were picked and transferred on to mouse embryonic fibroblast-coated 24-well plated. Propagated and analysed for EYEP expression. For the transfection of the αPIG9 ESC line that harbors already a neomycin resistance gene we used cotransfection with 10 μg of a pPGK-hygromycin plasmid and 40 μg of the CAG-

ChR2-EYFP plasmid and selected positive colonies with 200 μg ml−1 hygromycin on hygromycin resistant mouse embryonic fibroblasts.

ESC Differentiation and Purification of Cardiomyocytes

ESC differentiation in embryonic bodies was performed in Is cove's DMEM high-glucose medium (Invitrogen) supplemented with 20% FCS, 0.1 mM nonessential amino acids, 50 μg ml−1 penicillin and streptomycin, 0.1 mM β-mercaptoethanol in the absence of leukemia inhibitory factor using the hanging drop method as reported previously[28]. For the purification of cardiomyocytes, the αPIG/CAG-ChR2-EYFP ESC line was differentiated using a mass culture protocol. Cardiomyocytes were purified by addition of 10 μg ml−1 puromycin on day 9 of differentiation and dissociated with trypsin on day 12 or 13. Single purified cardiomyocytes were either used directly or frozen at −80 °C in 10%/50%/40% DMSO/FCS/DME.

GENERATION AND CHARACTERISATION OF CHR2-EXPRESSING CARDIOMYOCYTES

To obtain ChR2-expressing cardiomyocytes, a stable transgenic mouse embryonic stem cell (ESC) lines[27] expressing a mutant ChR2 has been generated, ChR2(H134R)7, in fusion with EYFP from the chicken β-actin promoter (*CAG*), a ubiquitous promoter that is particularly active in muscle cells[29]. Transgenic ESCs had membrane-bound EYEP signal, and patch-clamp experiment revealed that inward current could be induced by illumination. For differentiation of cardiomyocytes, embryonic bodies from transgenic ESCs were generated. Cardiomyocytes identified in embryonic bodies by staining with an antibody to the muscle-specific protein α-actinin, showed membrane-bound EYFP signal, indicating ChR2 expression. At day seven of differentiation, embryonic bodies started to beat spontaneously, and application of pulsed blue light reliably induced cellular contractions. Light intensities required for 1:1 transduction of the optical signal into contractions depended on the duration of the light pulse. Pulsed light did not induce contractions in control embryonic bodies expressing EGFP instead of ChR2-EYFP from the *CAG* promoter, indicating the specificity of the light-induced ChR2 activation. This method of easy, noncontact stimulation of cardiomyocytes opens the possibility of prolonged light stimulations without side effects. Notably, prolonged light stimulations (30 s) of beating embryonic bodies led to an increment in the spontaneous beating rate in embryonic bodies expressing ChR2-EYFP but not in control embryonic bodies expressing EGFP. This finding could be explained by light-induced continuous depolarization of the resting membrane potential, allowing the pacemaker cells that drive the spontaneous beating of the embryonic body to reach the threshold potential earlier and to spike faster. Later single cardiomyocytes derived from transgenic ESCs were investigated to determine the biophysical effects of ChR2 activation. Patch clamp analysis demonstrated that application of blue light induced inward currents at negative holding potentials. Brief pulses of light evoked free running action potentials and paired pulses were used to measure refractoriness. In addition, prolonged light stimulation induced action potentials, followed by prolonged depolarization's to 34.4 ± 10.7 mV (mean ± s.d.; n = 5) throughout the entire light pulse. To determine whether these 'prolonged depolarization's' influenced Ca2+ handling Ca2+ imaging was performed. Brief light stimulation induced typical action potential–driven Ca2+ transients, whereas longer light stimulations prolonged the elevation Ca2+ levels. Thus, light stimulation of ChR2 can be used to depolarise cardiomyocytes and to prolong the time period of systolic high Ca2+ concentration.

Immunostaining showing ESCs expressing the classical stem cell

To investigate whether local stimulation also works in a functional syncytium of cardiomyocytes, ChR2-expressing, ESC-derived cardiomyocytes were purified using a cardiac-specific resistance and plated them on multi electrode arrays. The cardiomyocytes formed a two-dimensional layer of synchronously beating cells and generated local field potentials pulsed illumination of one region evoked electrical activity in this area with subsequent spreading of the electrical signal to other regions .The pacemaker site could be shifted to other areas by moving the site of the illumination .Thus, ChR2 can be used for precise local stimulation in a two-dimensional culture *in vitro*. In addition, the effect of prolonged local illumination was examined. This led to suppression of electrical activity in the illuminated area without influencing the spontaneous activity of non-illuminated areas .This is most likely due to the ChR2-induced sustained depolarization that we observed in single cardiomyocytes leading to inactivation of Na+ channels[31] and refractoriness in the illuminated area of the monolayer.

To determine whether ChR2 can be used for stimulation of the adult heart *in vivo*, we generated transgenic mice from ChR2-expressing ESCs. The ChR2-EYFP fusion protein was located at the cell membrane of ventricular cardiomyocytes.ChR2 expression did not result in increased leak currents in cardiomyocytes or cardiac hypertrophy because resting membrane potential, membrane resistance, action potential duration and heart weight were not altered compared to controls. Light application induced typical ChR2 currents in ventricular cardiomyocytes. Time constant of decay was 19.7 ± 3.4 ms (mean ± s.d.; n = 11), and shorter than refractoriness of cardiomyocytes; therefore it is not limiting fast stimulation of cardiomyocytes. Action potentials were generated with a light intensity–dependent delay ChR2 expression and currents were also prominent in atrial cardiomyocytes.

To analyse the effect of ChR2 activation *in vivo*, the electrocardiogram was recorded from intubated and ventilated mice[32] expressing ChR2-EYFP while illuminating the beating heart with blue light in a confined area using a macroscope. Pulsed illumination of atria led to supraventricular pacing in the electrocardiogram with a delay of 12.2 ± 3.7 ms (mean ± s.d.; n = 6) from onset of the light pulse to the electrocardiogram response and with an increment of the P-wave duration (mean ± s.d.; 153 ± 28%, n = 6, P = 0.003) and of the PQ interval (delay from atrial to ventricular excitation) (mean ± s.d.; 121 ± 5%, n = 6, P = 0.001), indicating that pacing was induced in the illuminated area of the atrium. Light stimulation of the ventricle evoked ventricular extra beats (arrhythmic heart beats) with a delay of 9.3 ± 2.2 ms (mean ± s.d.; n = 6) and with longer QRS durations (time for depolarization of the ventricle) (209 ± 24% of control, mean ± s.d.; n = 6, P = 0.000) in the electrocardiogram .Also, the QRS shape changed according to the site of stimulation proving true local stimulation. The observed delay from the start of illumination to action potential generation *in vitro* or response in the

electrocardiogram *in vivo* was in a similar range (~10 ms) and reflected the time required for depolarization of illuminated cardiomyocytes. Atria needed higher light intensities for stimulation than ventricles. Higher light intensities were required when reducing the area of stimulation and that an illumination of 0.05 mm2 was sufficient to induce pacing .Assuming illumination of the first epicardial layer of longitudinal orientated ventricular myocytes this area corresponded to only ~50 myocytes.

To investigate the possibility of long-term depolarisations by ChR2 activation, longer constant illumination of ventricular areas were applied. This led to a disturbance in the regular sinus rhythm with the generation of spontaneous ventricular extra beats .It was concluded that depolarized cardiomyocytes in the illuminated area caused enhanced vulnerability for spontaneous ventricular extra beats. Uncoupling and block of activity could not be observed in these experiments because of the low penetrance of light.

Illumination of ChR2 expressing cardiomyocytes will allow new stimulation patterns *in vitro* and *in vivo*, and could be useful to investigate cellular mechanisms involved in pacemaking or a site-specific effect of prolonged depolarization's and delayed afterdepolarizations causing arrhythmias. ChR2 activation should be compatible with long-wavelength voltage-sensitive dyes[33], enabling high-resolution voltage mapping combined with patterned illumination. ChR2 activation in heart cells can be achieved using LED-based illumination with objectives from micro- and macroscopes or using light guides.

Altered Ca2+-handling is a typical consequence of cardiac hypertrophy and heart failurebut is also reported to induce such pathologies15. Use of ChR2 in ESC-derived cardiomyocytes to modify Ca2+ transients will be an *in vitro* system that allows investigating the role of excitation-transcription coupling in the development of cardiac hypertrophy and cardiac failure[34].

ENERGY NEEDS IN CARDIAC OPTOGENETICS

Earlier studies in neuroscience have reported that optical energies used to excite single neurons or brain tissue[34,35,36] in a wide range of high values (approximately 8 to 75 mW/mm2). The well-coupled spatially extended cardiac tissue was expected to present higher load for optical stimulation, thus possibly requiring even higher irradiance values. Yet, surprisingly, in Bruegmann et al's study[37], significantly lower light levels (0.5 to 7 mW/mm2) were sufficient to optically stimulate cardiac tissue in vitro or in vivo for a wide range of pulse durations. In our TCU approach, during optical pacing in the cell pairs or in the 2-dimensional cardiac syncytium, irradiance at 470 nm as low as 0.006 mW/mm2 has been measured. The strength duration curve (Figure 5) further corroborated low irradiance needed across pulse durations. Interestingly, this is much lower than reported for neuroscience applications and about 1 to 2 orders of magnitude lower than previously reported values for cardiac excitation in cells and tissue[37] for comparable pulse durations. The significantly lower optical energy needed in our study can be explained, at least partially, by the superior light sensitivity of the donor cells as seen in the higher ChR2 current densities (Figure 6). Further differences when compared with optical excitation of ventricular and atrial tissue may stem from dimensionality.

Energy needed for suprathreshold stimulation of 2-dimensional cardiac syncytium can be estimated as follows

For typical electric pacing (5 V, 0.2 A, 0.01-second pulses), 10 mJ can be obtained. For optical stimulation with an LED (5 V, 0.02 A, 0.01- to 0.05-second pulses), 1 to 5 mJ can be obtained. Considering the possibility for optimization of light focusing as well as the active development of more efficient lightemittingdiodes, it is likely that optical stimulation may be more energy efficient than its electric counterpart. Because pacemaking (unlike cardioversion) is a topologically simple problem, that is, a spatially localized (light-sensitive) cell company can be used, the TCU strategy may prove particularly valuable in pursuing potential clinical applications, given the above energy considerations. However, the challenges of achieving efficient light delivery to a densely packed tissue, such as the myocardium, should not be underestimated, and ultimately in vivo testing is needed.

CONCLUSION

The optogenetic approach offers high spatiotemporal resolution for precise interrogation and control of excitation, apparently without interfering with essential cardiac tissue properties. Therefore, it presents a new adaptable actuation tool in cardiac research for dissection of arrhythmias. Unlike traditional electrical stimulation, optogenetics allows us to precisely control the selective permeability of the plasma membrane, its conductivity with respect to different ions, its sensitivity to light of different wavelengths, and the spatiotemporal evolution of different opening and closing profiles. The concept of pacing the heart with light is believed to be widely applicable to systematically manipulate electrically active cells and, ultimately, support the design of novel therapies for various types of neuronal, musculoskeletal, pancreatic, and cardiac disorders such as depression, schizophrenia, cerebral palsy, paralysis, diabetes, pain syndromes, and cardiac arrhythmias.

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ABBREVIATIONS

ChR2: Channel rhodopsin-2
SA: Sino Atrial
AV: Atrio Ventricular
AP: Action potential
GPCRs: G-protein coupled receptors
CAG: Chicken β-actin promoter
MEA: Multi electrode array
HESCChR2þ: Human embryonic stem cells expressing channel rhodopsin 2
pLECYT: pLenti-EF1a- ChR2-eYFP-WPRE
TCU: Tandem Cell Unit
ESC: Embryonic stem cells
EF1a: Elongation factor 1-alpha
DNA: Deoxyribo Nucleic Acid

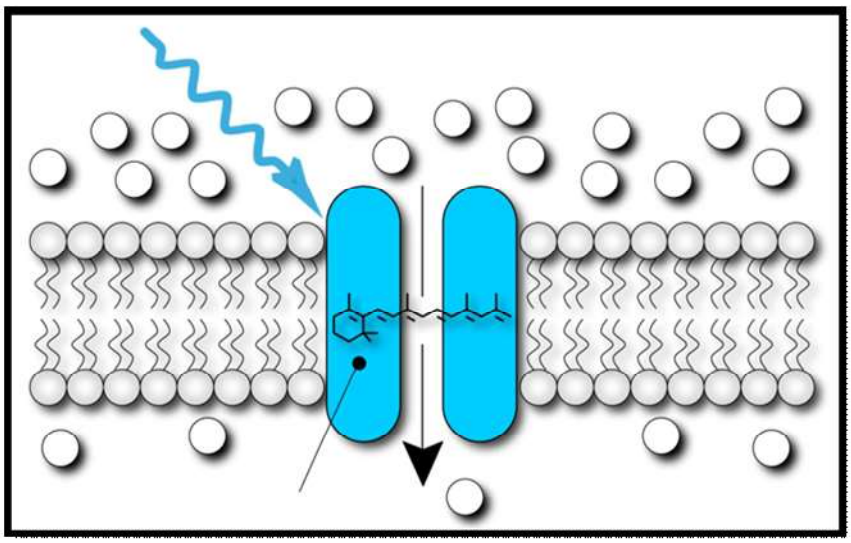

Figure 1: channelrhodopsin-2 is a light-gated cation channel consisting of seven trans membrane proteins and absorbed blue light through its interaction with retinal/ photoisomerization ofretinal opens the channel to sodium ions, which have higher concentration outside the cell than inside. to make our cells responsive to light and to allow sodium concentration to equilibrate, we induce channel rhodopsin into cardiac muscle.

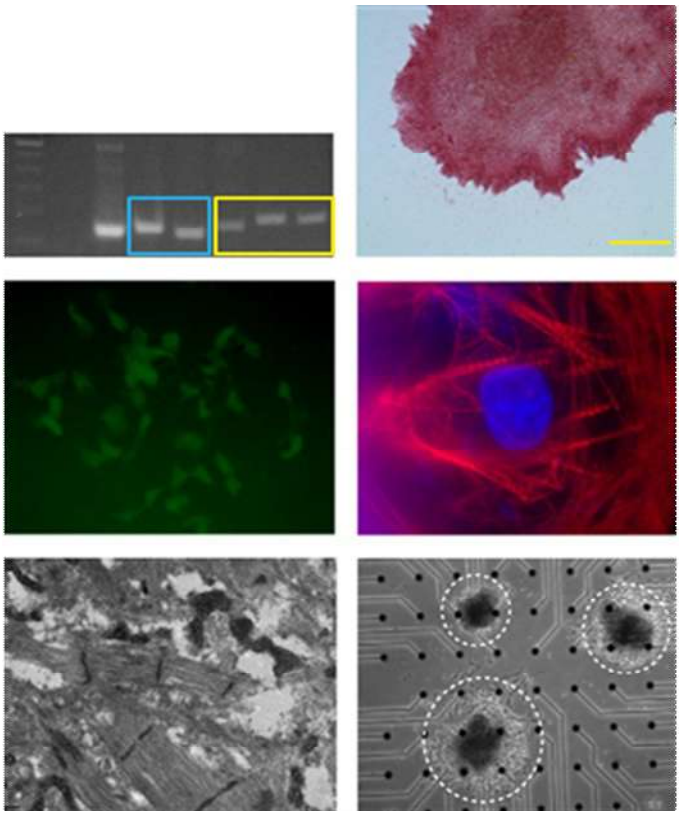

Figure 2: undifferentiated human embryonic stem cells (hesc) stably transduced with a chr2-eyfp lentiviral vector (hescchr2þ) remain pluripotent and can differentiate into cardiomyocytes (hescchr2þ-cm). (a) pcr shows that hesc chr2þ express the pluripotent oct-4 gene (169 bp, lane 4) and nanog gene (154 bp, lane 5) (blue box). in addition, amplification within the chr2 gene (174 bp, lane 6), across the chr2-eyfp gene (197 bp, lane 7), and within the eyfp gene (187 bp, lane 8), confirms stable transduction of the chr2-eyfp lentivirus in undifferentiated hescchr2þ (yellow box). a ladder (100 bp, lane 1) confirms the predicted sizes of pcr products. nontemplate control (lane 2) and gapdh (152 bp, lane 3) serve as negative and positive controls, respectively. (b) pluripotent hescchr2þ stain is positive for alkaline phosphatase (red). (c) fluorescence microscopy shows hescchr2þ has a positive eyfp signal (green). (d) hescchr2þ -cm have positive tni signals (red), consistent with a cm phenotype. dapi staining (blue) demonstrates the position of nuclei. (e) transmission electron microscopy shows sarcomeres with associated z-lines (z) and mitochondria (m) in hescchr2þ -cm. (f) light microscopy shows three hescchr2þ-cm colonies (dashed white circles) on a multielectrode array.

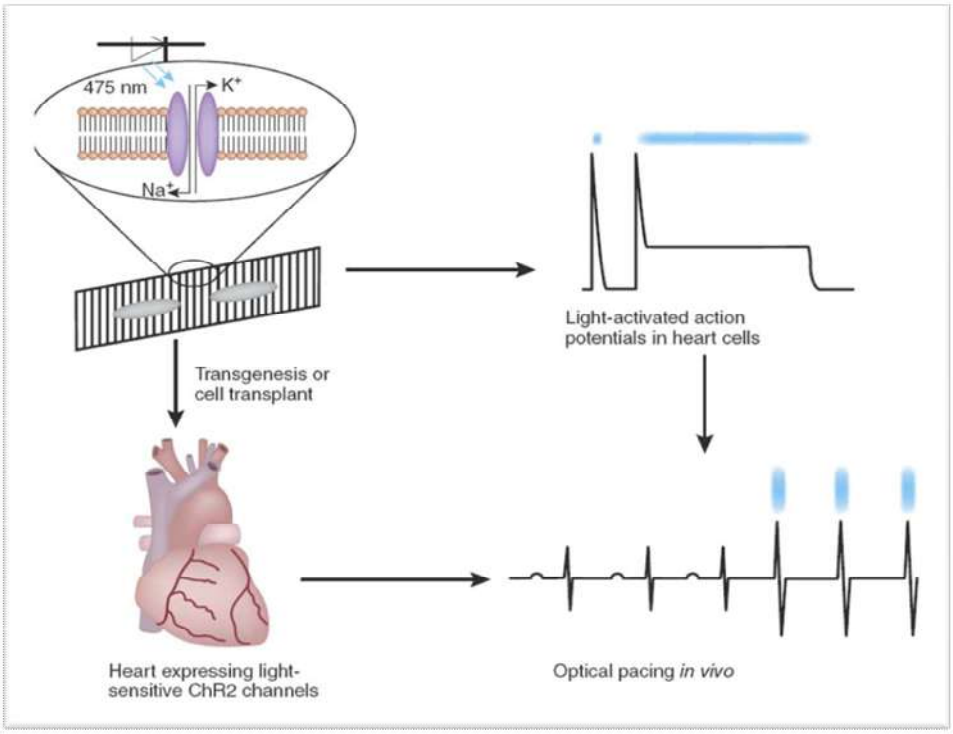

Figure 3: optical stimulation of heart

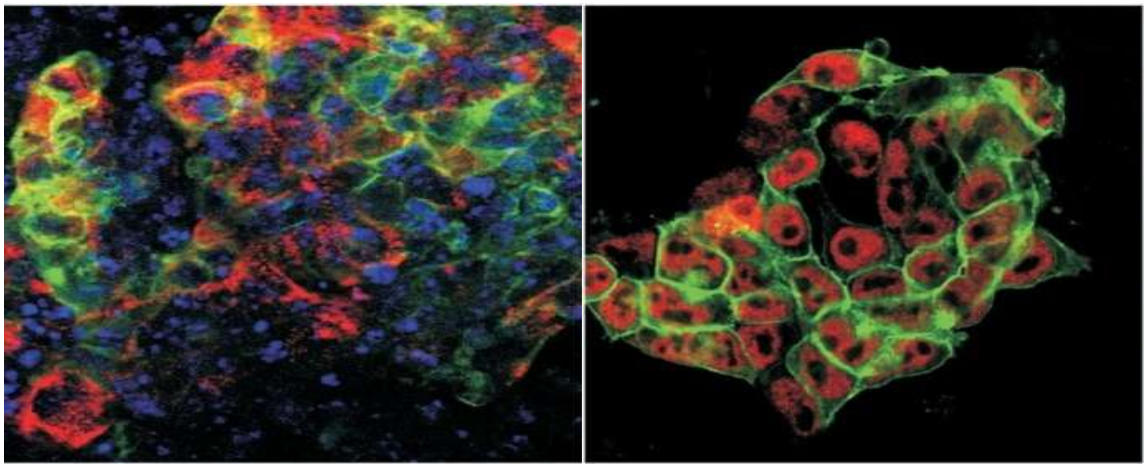

Figure 4: Immunostainings showing ESCs expressing the classical stem cell marker Oct4 in nucleus (a,red)as well as cardiomyocytes in embryoid bodies expressing alpha actinin (b,red)overlaid with fluorescence images of native EYFP signal(green:membrane bound) nuclei shown in blue

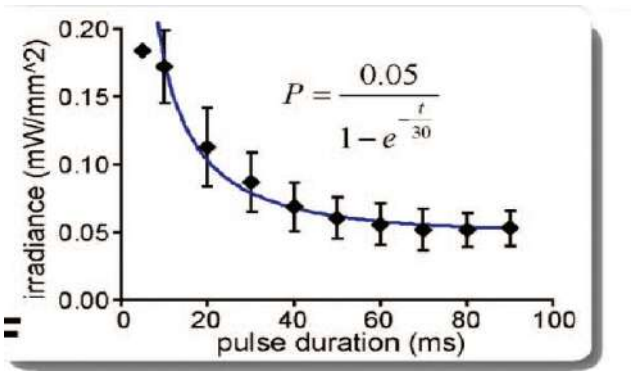

Figure 5: strength duration curve

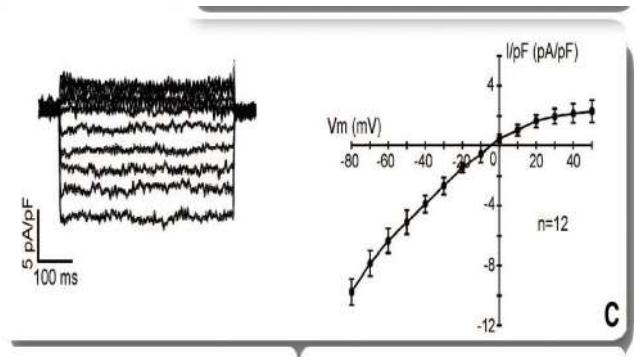

Figure 6: energy need in cardiac optogenetics