Abstract

Exogenous expression of pharmacological targets in transformed cell lines has been the traditional platform for high throughput screening of small molecules. However, exogenous expression in these cells is limited by aberrant dosage, or its toxicity, the potential lack of interaction partners, and alterations to physiology due to transformation itself. Instead, primary cells or cells differentiated from precursors are more physiological, but less amenable to exogenous expression of reporter systems. To overcome this challenge, we stably expressed c-Photina, a Ca^{2+}-sensitive photoprotein, driven by a ubiquitous promoter in a mouse embryonic stem (mES) cell line. The same embryonic stem cell line was also used to generate a transgenic mouse that expresses c-Photina in most tissues. We show here that these cells and mice provide an efficient source of primary cells, cells differentiated from mES cells, including cardiomyocytes, neurons, astrocytes, macrophages, endothelial cells, pancreatic islet cells, stably and robustly expressing c-Photina, and may be exploited for miniaturized high throughput screening. Moreover, we provide evidence that the transgenic mice may be suitable for ex-vivo bioimaging studies in both cells and tissues.

Introduction

Movements of Ca^{2+} ions are fundamental for signal transduction in cells. Because the intracellular level of Ca^{2+} is highly regulated and compartmentalized, transient alterations in Ca^{2+} concentration are excellent signals, and are downstream targets of G-protein coupled receptors (GPCRs), ion channels and transporters, all important examples of therapeutic targets [1]. One effective tool to measure Ca^{2+} mobilization sensitively, and non-invasively, are photoproteins, which release photons upon binding to Ca^{2+} (in presence of a cofactor). These photoproteins are widely used in cell-based assays for high throughput screening (HTS) as reporter genes to monitor Ca^{2+} movements associated with signals [2–4]. The sensitive detection, virtually undetectable background and high signal to noise ratio favour photoproteins over Ca^{2+}-sensitive fluorescent dyes and permit small assay-volumes [5,6]. The cofactor, coelenterazine, is added to mammalian cells expressing the photoprotein and photon emission is detected as an indicator of intracellular Ca^{2+} concentration [7].

Usually, cell-based assays exploit transformed cell lines, which express both a photoprotein and a target receptor. These cell lines have been selected for limited expression of other receptors, for their easiness to be cultured, and to be expanded. However, exogenous expression of targets and the transformed environment can create artefacts of gene dosage, toxicity, or stoichiometry of the receptor target itself, when it requires assembly of multiple subunits. An alternative to transformed cell lines are primary cells, isolated from mammals. They have a more physiological environment and may express targets endogenously, but they are frequently complicated to purify and culture in sufficient numbers, and furthermore, are sometimes very difficult to transfect with (reporter) genes.

The embryonic stem cells are a possible alternative. By maintaining the self-renewal property of the undifferentiated state, they can be cultured and expanded in vitro for long periods, and they are quite easily transfected [8]. Moreover, embryonic stem cells can differentiate into virtually any cell type, resembling primary cells [9,10]. Accordingly, they offer a natural environment for the receptor targets, and they can form stochiometrically
appropriate complex targets (like multi-subunit ion channels), that are regulated natively [11].

Therefore, we generated clones of mouse embryonic stem cells expressing a photoprotein as a Ca\textsuperscript{2+} reporter system under the control of a ubiquitous promoter. We show that multiple types of cells differentiated from one of these clones report Ca\textsuperscript{2+} signals in response to physiological stimuli. Furthermore, we exploited these undifferentiated photoprotein mES cells to produce a transgenic mouse, which may be useful for \textit{ex vivo} imaging studies and as a source of differentiated primary cells expressing a Ca\textsuperscript{2+} reporter gene.

Results

1. c-Photina Photoprotein

To identify a photoprotein which combines sensitive detection of intracellular mobilized Ca\textsuperscript{2+} and stable expression, we performed random mutagenesis of Clytin, a natural photoprotein isolated from Clytia gregaria jellyfish (syn. with Phialidin) [12,13]. One of those modified photoproteins displayed the desired characteristics and was called c-Photina\textsuperscript{B}. To improve the transduction efficiency in mammalian cells, the c-Photina gene was optimized for mammalian codon usage (as described for Photina\textsuperscript{B} in [6], and then fused to a mitochondrial tag (human Cytochrome C Oxidase, subunit VIII) [14], in an expression vector that contained no antibiotic resistance gene.

To investigate the function and stability of mito c-Photina expression, we transfected CHO-K1 cells, in order to create a stable clone [6]. The cells were kept in culture for more than 7 months and 58 passages, in absence of antibiotic selective pressure, and we confirmed function regularly by stimulating with agonists for endogenous GqPCRs (Gq Protein Coupled Receptors), whose activation induce a Ca\textsuperscript{2+} release from internal stores through the Gq/q/phospholipase C pathway. The kinetics of the bioluminescent response indicated high affinity for Ca\textsuperscript{2+}, and stable expression over time, as specified by the consistent EC\textsubscript{50} for ATP (Figure 1). This was an important feature since several mammalian cells transfected with other natural or recombinant photoproteins, tended to loose function over time in the absence of selective pressure (unpublished). The stability of mito c-Photina function made this mutant the first choice for transfection into mES cells.

2. Generation of a mES Cell Line Expressing a Photoprotein

We electroporated mouse ES cells with the \textit{mito c-Photina} gene. After neomycin selection, 114 drug-resistant colonies were picked, and expanded on mouse embryonic fibroblasts (MEFs), and screened for the ability to emit light after functional stimulation with histamine, which is known to activate the endogenously expressed GqPCR histamine-1 receptor in mES cells [15] and consequently to raise transiently the cytoplasmic Ca\textsuperscript{2+} concentration. A typical GqPCR-mediated response curve [6] was obtained after injection of 100 \textmu M histamine from almost all the clones (Figure 2A).

To verify that the response amplitude correlated with amount of photoprotein, cells, after primary measurement, were lysed to expose all coelenterazine-c-Photina complexes to Ca\textsuperscript{2+}. This response was much higher than that of all clones (data not shown) and was not always correlated to the differences in amplitude observed after GqPCR stimulation, suggesting that total coelenterazine-c-Photina reacting complex was not limiting.

The final mES mito c-Photina clone was selected from 12 high responders on the basis of different parameters: primarily the ability to respond to histamine in a dose-responsive manner normalized for cell number, secondly the total photoprotein content after cell lysis, but also the number of copies of the transgene in the host genome, karyotype, cell morphology and growth rate. The number of copies in the genome was analyzed by Southern blot and quantitative PCR analysis. Clone 29 was selected on the basis of the parameters described before (see its histamine doseresponsiveness in Figure 2B) and because it has the transgene inserted into the genome in a single copy. Further confirmation of this was the impossibility to detect signals by FISH analysis (data not shown).

3. mES/mito c-Photina/29 Clone Is Pluripotent

Indirect immunofluorescence assays were performed on the mES mito c-Photina clone in order to evaluate the presence of specific markers of the undifferentiated pluripotent mouse embryonic stem cells, such as the stage specific embryonic antigen-1 (SSEA-1) [16] (Figure 3A–B) and the transcription factor oct 3/4 [17] (Figure 3C–D). As shown in Figure 3 both SSEA-1 and oct 3/4 are present selectively in stem cells and not in the surrounding feeder cells. Additionally, we could detect also alkaline phosphatase activity (Figure 3E–F), another characteristic of undifferentiated stem cells [18].

Since the “\textit{bona fide}” demonstration of stemness is the germline transmission test, clone 29 was injected into blastocysts of pregnant host female mice. Two chimeric mice, with a high degree of chimerism (almost 100%) and male phenotypes, were obtained. When these 2 mice reached sexual maturity, they were crossed with C57BL/6 female mice and gave rise to more than 95% agouti progeny (Table 1), indicating robust germline transmission.

4. In Vitro Differentiation Assays Performed with mES/mito c-Photina/29 Clone

To show that the introduction of the transgene did not influence the “\textit{in vitro}” differentiation capabilities of the mito c-Photina/29 clone, we cultured the cells under conditions to favour either cardiomyocyte or neuronal fates, employing well described protocols including suspension protocols for embryoid body (EB) formation, and adhesion protocols [19,20] all optimized for miniaturized formats.

4.1. Cardiomyocytes. Cardiomyocytes are one of the most important cell types for drug discovery projects and the hallmark of cardiomyocytes is the Ca\textsuperscript{2+} dependent contractility and its

![Figure 1. ATP dose response curves in CHO c-Photina cells at different passages.](image)
characteristic Ca2+ channel driven depolarisation curve. In addition, the heart rate is controlled by GqPCR-dependent Ca2+ release, for example mediated by the adrenergic receptors. EBs were formed in hanging drops for two days and then in suspension for another three days. The fifth day, EBs were plated on gelatin-coated tissue culture dishes. Within one day, we observed spontaneously pulsating cardiomyocytes. The percentage of EBs containing pulsating areas was about 80% (Figure 4A–B, and Video S1 and Video S2). The protocol was adapted to miniaturized formats, putting exactly a single EB per well of 96 or 384 micro titre plates (MTP). The cardiomyocyte development occurred directly in the micro titre plate format, maintaining the same proportion of pulsating areas.

To verify the presence of mature cardiomyocytes we stained for specific cardiomyocyte markers, such as the transcription factor, GATA-4 (Figure 4C), and for the cytoskeleton protein, myosin heavy chain (MHC) (Figure 4D-E). As seen in Figures 4C, D, and E all the markers were present and showed appropriate localization demonstrating proper cardiomyocyte development.

Next, functional tests were performed by disaggregating EBs and seeding 5,000 cells/well in a 384 MTP under the same differentiation conditions. 48 hours after seeding, the cells were stimulated with standard Tyrode’s buffer as control, 50 nM endothelin-1, and 100 μM norepinephrine, which are agonists for the endogenously expressed GqPCR endothelin receptors and for the α1-adrenergic receptor, respectively. The cells were also stimulated with a depolarizing solution such as 60 mM KCl able to activate the voltage-gated channels, including the Ca2+ ones. As shown in Figure 4F, all compounds led to characteristic kinetics indicating that these cells not only express markers of mature cardiomyocytes, but are also able to respond to stimuli for endogenous GPCRs and channels.

**4.2. Neurons.** Neurons are another cell type in which Ca2+ plays a fundamental role in signal transduction, particularly in the release of neurotransmitters. For neuronal differentiation, the mES cells were differentiated in monolayer. The presence of cellular processes were visible 4–5 days after plating on gelatin-coated tissue culture dishes, and their length increased over time (Figure 5A). The presence of specific markers in these cells was investigated by immunofluorescence. The nestin staining indicated the presence of neural precursors at day 13 of differentiation (Figure 5B), together with betaIII tubulin and MAP-2 labelling which detected differentiated neurons (Figure 5C–D), as well as staining for GFAP (Figure 5E) which indicated the presence of astrocytes, among the population of differentiated cells.
The functionality of these cells was explored with the Lumibox luminometer. At differentiation day 13, the cells were stimulated by injecting glutamate and NMDA plus glycine, in order to investigate the glutamate receptors, or with ATP and abmethylene ATP, for purinergic receptors, or depolarized with 60 mM KCl, for the activation of voltage-gated channels, including the Ca\(^{2+}\) ones. All stimulations produced the expected response curves (Figure 5F).

To validate the reporter characteristics of c-Photina in this context, we characterized activation of a GqPCR (group I metabotropic glutamate receptor), a Ca\(^{2+}\)-permeable, ligand-gated ion TRP (Transient Receptor Potential) channel (vanilloid receptor-1), and voltage-gated Ca\(^{2+}\) channels, in our neuronally differentiated cells (at differentiation day 13) by comparing the photoprotein-based luminescent read-out to an acetoxymethylester-coupled dye-based (Fluo4NW) fluorescent read-out on the FLIPRtetra instrument (Figure S1). The similar results further confirm the ability of our system to detect both extracellular and intracellular Ca\(^{2+}\) influxes.

Table 1. Germline transmission results for clone 29 c-Photina mouse embryonic stem cells.

| Litter | nr agouti mice | nr c-Photina positives | nr agouti mice | nr c-Photina positives |
|--------|----------------|------------------------|----------------|------------------------|
| I      | 10             | 4                      | 4              | 2                      |
| II     | 5              | 1                      | 7              | 5                      |
| III    | 12             | 6                      | 9              | 7                      |
| IV     | 10             | 4                      |               |                        |
| V      | 6              | 3                      |               |                        |

List of agouti and c-Photina mice born from the 2 chimeric males crossed with C57BL/6 female mice, indicating the germline transmission.

doi:10.1371/journal.pone.0008882.t001

Figure 3. Immunofluorescence analysis on undifferentiated mES/mito c-Photina/29 clone. The assay was performed using the following antibodies: A. B. Anti-SSEA-1 primary antibody (A.), counterstained with Hoechst 33342 dye (B.). Scale bar -50 μm. C.-D. Anti-oct 3/4 primary antibody (C.), counterstained with Hoechst 33342 dye (D.). Scale bar -50 μm. E.-F. Alkaline phosphatase activity measured with the ELF\(^\circledR\) Phosphatase fluorescent staining kit (E.) and its corresponding contrast phase image (F.). Scale bar -200 μm. doi:10.1371/journal.pone.0008882.g003
compounds at various concentrations, we observed a dose-dependent response with both agonists (Figure 5G–H). By using picrotoxin, a specific GABA channel antagonist, we verified that this GABA-mediated signal was mainly due to activation of the GABA channels and not of the GABA transporters (data not shown). Note that the GABA response in Figure 5G is depolarizing. This is consistent with the previous observation that GABAergic reversal potential (EGABA) is more liable to depolarize the membrane potential in immature as compared to adult neural cells. This difference is due to a variation of the intracellular Cl\(^{-}\) concentration which changes during development, due to the presence of the K\(^{+}\)-Cl\(^{-}\) cotransporter KCC2 [21].

5. Systematic Characterization of Ca\(^{2+}\) Signals in mES As Compared to Neural Cells

Extrinsic signals from ‘niche’ play an important role in maintenance of multipotency in stem cells. mES cells are pluripotent and model the cell intrinsic response to signals that maintain multipotency. But the cellular pathways known to transduce these signals are few [22], and Ca\(^{2+}\) signalling has been only partially characterized in embryonic stem cells [23]. Therefore, we screened undifferentiated and neuronal differentiated cells with an unbiased library of pharmacologically active compounds (LOPAC\(^{1280}\)). This library contains all the major pharmacological target classes, including GPCRs, and ion channels active compounds. We asked which receptors and ions were functional in undifferentiated mES-c-Photina cells, as compared to neural differentiated c-Photina cells. The results were expressed as “percent activity” with respect to ATP (“max” signal for the undifferentiated cells) and glutamate (“max” signal for the differentiated cells). ATP and glutamate were selected as reference compounds, since these agonists show the highest response in the cell populations tested. The data were then analyzed with the two-sample unequal variance, one-tailed t-Student test. All the compounds showing a t-test value less than 0.05 and a percent activity mean value higher than the one showed by the mean of the min signals were selected as positives and included in table 2 (see also Table S1 and Figure S2).
The data revealed very few extrinsically activated Ca\textsuperscript{2+} signalling pathways in the undifferentiated mES cells. The Ca\textsuperscript{2+} related signals that were present are mainly associated with histamine and purinergic compounds, indicating that histamine and purinergic receptors are present. Interestingly, one of these receptors, the adenosine 1, in the P1 purinergic class, has not been

Figure 5. In vitro neuronal differentiation protocol on mES/mito c-Photina/29 clone. A. Morphological analysis of the cells at different day of the development (contrast phase images). Scale bar – 200 μm. B.–E. Immunofluorescence analysis with: B. Anti-nestin antibody counterstained with Hoechst 33342 dye. Scale bar – 100 μm. C. Anti-beta III tubulin antibody (green) and anti-GFAP antibody (red) counterstained with Hoechst 33342 dye. Scale bar – 100 μm. D. Anti-MAP-2 antibody counterstained with Hoechst 33342 dye. Scale bar – 100 μm. E. Anti-GFAP antibody counterstained with Hoechst 33342 dye. Scale bar – 100 μm. F. CCD camera-based functional test on in vitro differentiated neurons. Tyrode’s buffer, 60 mM KCl, 100 μM NMDA + 10 μM Gly, 100 μM Glu, 10 μM Gly, and 100 μM ATP responses were measured in 384 MTP on day 13 differentiated neurons. Lumibox luminometer conditions: high sensitivity, reading time 60 seconds. G.–H. FLIPR\textsuperscript{B84} functional test on in vitro differentiated neurons at day 13. For FLIPR\textsuperscript{B84} analysis the medium was replaced with 25 μL/well of Membrane Potential (MP) fluorescent dye. The plate was then incubated for 30 min at 37°C and for 30 min at room temperature and then 12.5 μL/well of GABA (G.) and glutamate (H.) solution at different concentration were injected (3X concentrated) and the fluorescence signal was recorded and expressed as RFU (Relative Fluorescence Units). FLIPR\textsuperscript{B84} settings: Exp. Time: 0.3 sec; injection speed: 20 μL/sec; injection height: 50 μL; reading time: 360 seconds.

doi:10.1371/journal.pone.0008882.g005
Table 2. Active LOPAC1280TM agonist compounds.

| Group                        | Name                                      | Percent Activity Mean ± Standard Deviation |
|------------------------------|-------------------------------------------|------------------------------------------|
| Undifferentiated mES cells   | 2-Chloroadenosine                         | 57±22.3                                  |
|                              | Calcimycin                                | 61±10.4                                  |
|                              | 2-Chloroadenosine triphosphate tetrasodium| 36±10.5                                  |
|                              | Histamine dihydrochloride                 | 13±6.1                                   |
|                              | Hydrochlorothiazide                       | 34±13.5                                  |
|                              | P1,P4-Di(adenosine-5')tetraphosphate triammonium | 24±13.5                              |
|                              | Spiperone hydrochloride                   | 44±18.5                                  |
|                              | D-609 potassium                           | 17±0.8                                   |
|                              | Thapsigargin                              | 41±12.1                                  |
| Differentiated mES cells, Day 13 | Chelyrythine chloride                     | 15±2.0                                   |
|                              | L(−)-Norepinephrine bitartrate            | 97±32.0                                  |
|                              | Acetyl-beta-methylcholine chloride        | 12±6.3                                   |
|                              | 6-Fluoronorepinephrine hydrochloride      | 58±23.4                                  |
|                              | (±)-Norepinephrine (+)bitartrate          | 108±21.7                                 |
|                              | Tryptamine hydrochloride                  | 9±4.0                                    |
|                              | 2-Methylthioadenosine triphosphate tetrasodium | 132±23.1                              |
|                              | Calcimycin                                | 41±24.4                                  |
|                              | (S)-3,5-Dihydroxyphenylglycine            | 17±11.5                                  |
|                              | (±)-AMPA hydrobromide                     | 21±4.8                                   |
|                              | (−)-Epinephrine bitartrate               | 390±162.8                                |
|                              | 2-Chloroadenosine triphosphate tetrasodium | 226±3.9                                  |
|                              | (−)-alpha-Methylnorepinephrine            | 57±21.7                                  |
|                              | Histamine dihydrochloride                 | 28±15.5                                  |
|                              | (±)-cis-Dioxolane iodide                  | 54±41.8                                  |
|                              | (±)-Epinephrine hydrochloride             | 113±8.6                                  |
|                              | L-Glutamic acid hydrochloride             | 16±9.1                                   |
|                              | OXA-22 iodide                             | 10±1.3                                   |
|                              | N-Methyl dopamine hydrochloride           | 21±14.0                                  |
|                              | FPL 64176                                 | 166±77.9                                 |
|                              | P1,P4-Di(adenosine-5')tetraphosphate triammonium | 16±5.6                                  |
|                              | Kainic acid                               | 24±16.2                                  |
|                              | 2-Methylthioadenosine diphosphate trisodium | 122±46.9                              |
|                              | Phenylephrine hydrochloride               | 17±9.6                                   |
|                              | Oxotremorine methiodide                   | 41±16.4                                  |
|                              | Spiperone hydrochloride                   | 47±34.9                                  |
|                              | Sanguinarine chloride                     | 45±34.0                                  |
|                              | (−)-Quisqualic acid                       | 57±38.6                                  |

List of compounds active on undifferentiated mES cells (9 compounds) and on neural differentiated mES cells, at day 13 (28 compounds), with the relative percent activity mean ± the standard deviation. doi:10.1371/journal.pone.0008882.t002

Previously noted in undifferentiated mES cells. There were a number of additional active compounds able to induce intracellular Ca<sup>2+</sup> elevations in the neural cells. Most of these substances suggested receptors compatible with the expected prevalence of receptors in neurons. These data offer additional evidence for the robust and appropriate differentiation of the c-Photina neurons.

6. Photoprotein Transgenic Mouse

In addition to providing cells differentiated in culture, the mES cells can be exploited to generate a transgenic mouse, which might serve as a direct source of primary cells that could express both the photoprotein transgene, and an endogenous pharmacological target in the native physiological context. The 2 chimeric mice obtained by germline transmission (as described above) were crossed with C57BL/6 female mice and gave rise to agouti progeny. All the litters were genotyped in order to check for the presence of the transgene. As expected, half of the mice born from these crosses were heterozygous for the c-Photina photoprotein gene (32/64) (Table 1). We named these animals PhotopTopo<sup>®</sup> mice. The heterozygous mice were crossed, in order to obtain a homozygous population. One fourth of the offspring were homozygous and phenotypically normal, demonstrating that the transgene did not disrupt any gene crucial for survival.

7. c-Photina Expression and Activity in PhotoTopo

To determine the c-Photina mRNA expression profile, we performed a TaqMan<sup>®</sup> qPCR analysis on transgenic and control samples. The results were normalized to the amount of 18S rRNA level (Figure 6A). Expression was detected in most tissues and at high levels. In order to check for functional c-Photina, 8 transgenic mice containing the c-Photina gene and 6 negative mice from the same litter were sacrificed in 3 different experiments. Several tissues were removed from mice, and all samples were incubated in an isotonic solution containing coelenterazine to form the active photoprotein complex. All the isolated tissues were tested in triplicates, injecting a 1% Triton<sup>®</sup> X-100 plus 250 mM CaCl<sub>2</sub> solution, in order to discharged all the photoprotein-coelenterazine active complexes into an enriched Ca<sup>2+</sup> environment (Figure 6B). We found a good correlation between c-Photina mRNA expression and light emission across tissues. In addition, light emission remained approximately stable in all expressing tissues of animals of 3, 6, or 10 months old (data not shown).

Furthermore, we investigated the bioavailability of coelenterazine after intravenous systemic injection via the tail vein [24]. After 3 hours, one transgenic and one non-transgenic animal were sacrificed and several tissues/organs were removed. Half of the material was tested immediately with the Lumibox luminometer after cell lysis and injection of a Ca<sup>2+</sup> solution (Figure 6C). The other half of the material was incubated for another 3 hours with a solution containing coelenterazine, and tested in the same way (Figure 6D). We found that the profile of luminescence across tissues was comparable between intravenous coelenterazine injection-in vivo formation of the photoprotein-coelenterazine complex, or after incubation ex vivo. The tissue samples with highest luminescent signals were: spleen, heart, lung, testis, kidney, and skeletal muscle (Figure 6).

8. Primary Cells Cultured from PhotoTopo

8.1. Aortic endothelial cells. Aortic endothelial cells are very important cells for cardiovascular diseases, and are difficult to obtain in primary culture. Since Ca<sup>2+</sup> has a fundamental role also in these endodermally-derived cells, we decided to isolate these cells. Seven animals (4 positives and 3 negatives) were sacrificed and aortas explanted. After plating on Matrigel<sup>®</sup> and culture for...
11 days in presence of endothelial cell growth supplement and heparin, we obtained endothelial cells [25]. The presence of these cells was demonstrated by flow cytometry and immunofluorescence analysis using von Willebrand Factor (vWF) and CD31/PECAM-1 markers. The cells were also tested functionally by seeding 50,000 cells/well in a 96 MTP and stimulated with endothelin-1 and TRAP 10 and 6 peptides, agonists for the endothelin receptor and proteinase-activated receptors, respectively, both of which are highly expressed in endothelial cells. As shown in Figure 7A–D activation of both receptors induced a Ca^{2+} mobilization from internal stores giving rise to typical kinetics of light emission.

8.2. Bone marrow-derived monocytes/macrophages.

The PhotoTopo mice are also a source of stem cells and precursors. To verify that the c-Photina was active in hematopoietic monocyte lineage, bone marrow-derived monocyte/macrophage precursors were isolated from the femurs of 4 positive and 3 negative transgenic mice and cultured for 10 days in the presence of M-CSF (Macrophage - Colony Stimulating Factor) [26]. We confirmed the presence of mature macrophages in cell culture, by staining for specific markers F4/80 and scavenger receptor type III (CD204) in flow cytometry and immunofluorescence analysis (Figure 7E-G). Functional studies were performed in these cells by injecting a solution of 100 mM UTP, UDP and ATP in order to stimulate the purinergic receptors. As reported in Figure 7H, all agonists were shown to induce appropriate Ca2+-mediated light emission.

8.3. Micro-organs: beta islets.

Next we analysed if c-Photina would trace the glucose-triggered and Ca2+-mediated secretion of insulin in islet cells, representing micro-organs. A pancreatic islet isolation and purification was performed from PhotoTopo animals and from negative controls. Islets were cultured overnight at 37°C, and, the following day transferred to 96 MTP (10 islets/well). After incubation with Krebs-Ringer’s buffer in presence of coelenterazine, they were stimulated with 11 mM glucose in order to activate the Ca2+-mediated insulin pathway. As control, mannitol which does not induce the Ca2+-mediated insulin response was injected at the same final concentration (Figure 7I). We observed waves of Ca2+-mediated luminescence, induced only after stimulation with glucose and not with mannitol. The islets were then stimulated with a depolarizing agent (60 mM KCl) which induces a massive Ca2+ influx through the voltage-gated Ca2+ channels and produced robust light emission (Figure 7J).

9. Ex Vivo Bioimaging from the PhotoTopo

To further explore the potentiality of c-Photina, we performed luminescence-based bioimaging studies on pancreatic islets. In order to detect topographical light emission in islets, we exploited a...
microscope-based device, equipped with an intensified CMOS camera (Photron Fastcam). Subsequent to a polarized injection of 60 mM KCl solution, we observed light emission representing Ca\(^{2+}\) moving across the entire beta islet (Figure 8A-B-E and Video S3). After acquisition, it was possible to retrieve either the response kinetics recorded from the whole islet (Figure 8C), or define the response kinetics from single areas (Figure 8D).

### Discussion

The generation of a pluripotent embryonic stem cell line containing a Ca\(^{2+}\)-activated photoprotein offers many opportunities to study Ca\(^{2+}\)-based signals. In fact, we demonstrated that c-Photina stem cells can be differentiated into two specific cell types and can be used as source of multiple primary-like cells for Ca\(^{2+}\) functional studies. Furthermore their pluripotency allowed the generation of transgenic mice, which can be an interesting reserve of cells, such as the adult stem cells (for example haematopoietic stem cells), committed progenitors, and also primary cells containing the photoprotein, for pharmacological or bioimaging studies. Interestingly, the animals can additionally be crossed with other animal models, in order to exploit these possibilities in the context of disease.

Monitoring Ca\(^{2+}\) signalling with a Ca\(^{2+}\)-sensitive reporter gene in mES cells, primary cells, and in a whole animal model, opens many opportunities to understand the development, function, and plasticity of many crucial Ca\(^{2+}\)-mediated pathways. Several techniques have been described for measuring intracellular Ca\(^{2+}\). Patch-clamp and Ca\(^{2+}\)-selective microelectrodes allow quantitative measurements of Ca\(^{2+}\) fluxes in single-cell analysis. These ion-selective microelectrodes (ISMs) are highly sensitive and selective, but suffer from a slow response time, and high levels of noise [27]. Furthermore this technology can be applied only to a restricted number of cells. On the other hand, large populations of cells can be investigated for intracellular Ca\(^{2+}\) dynamics with fluorescent probes [28]. In addition to fluorescent dyes there are also genetic tools, which provide fluorescent-based methods for Ca\(^{2+}\) monitoring, and are basically divided in two groups. The first category...
uses the principle of fluorescence resonance energy transfer (FRET) between two variants of the green fluorescent protein (GFP), covalently linked with Ca$^{2+}$ binding proteins like calmodulin [29,30]. The second category is composed of bioluminescent proteins such as aequorin [31] fused with a GFP molecule. This latter approach is used both in single cells and in transgenic animals [32,33]. The configuration of the construct with the fusion of the two proteins allows intramolecular chemiluminescence resonance energy transfer (CRET). In fact, after Ca$^{2+}$ binding, aequorin, in presence of coelenterazine, emits a quantum of light that is transferred to GFP, which works as an acceptor and emits green light [32]. The advantage of using CRET approach, instead of using only the aequorin bioluminescent signal, is to overcome the low light quantum yield of the photoprotein. The combination of the two proteins in CRET, in fact, permits detection of the Ca$^{2+}$-mediated signal, even with unavoidable loss of energy during the transfer.

Here we reported the development of a more direct and simpler system to measure efficiently Ca$^{2+}$ movements without any loss of energy during the transfer from the photoprotein to the GFP proteins. To do this we transfected directly only the photoprotein gene in mES cells and then we used these cells to develop a transgenic animal. The choice to use only the bioluminescent reporter gene avoids fusion proteins that could induce, even in presence of tethers, problems of folding and translation. This might be of particular relevance if we consider the larger presence of mitochondria, given the crucial role of Ca$^{2+}$ homeostasis in them. In fact, besides a central function in cell energy metabolism, mitochondria are able to modulate cytosolic Ca$^{2+}$ variations, after activation of both Ca$^{2+}$ channels, P2X purinergic channels, and TRP channels such as the vanilloid receptors.

An important additional feature of the mitochondrial tagged c-Photina photoprotein is its cellular stability, both in mammalian cells even in absence of a selective pressure, and in an embryonic stem cell line, and in a transgenic mouse model. This is not trivial since from our unpublished observation the expression of many natural and recombinant photoproteins often decreases during time, especially in the absence of selective pressure. The photoprotein was targeted to mitochondria, given the crucial role of Ca$^{2+}$ homeostasis in them. In fact, besides a central function in cell energy metabolism, mitochondria are able to modulate cytosolic Ca$^{2+}$ concentration and participate in Ca$^{2+}$ signalling. Moreover, mitochondrial Ca$^{2+}$ uptake is a phenomenon involved not only after Ca$^{2+}$ release from intracellular stores, as happens after stimulation of GqPCRs, but also after Ca$^{2+}$ influx through specific Ca$^{2+}$ channels from extracellular space [34,35], for review see [36,37]. Accordingly, our mitochondrial tagged photoprotein actually efficiently recorded cytoplasmic Ca$^{2+}$ variations, after activation of both Ca$^{2+}$ channels, such as the voltage-gated Ca$^{2+}$ channels, P2X purinergic channels, and TRP channels such as the vanilloid receptors.

We demonstrated that the presence of the photoprotein does not interfere with the pluripotency of mES cells. In fact, these cells still express stemness markers like oct 3/4 transcription factor and the SSEA-1 surface antigen and possess alkaline phosphatase activities [16–18]. Furthermore, the c-Photina mES cells, when injected into the blastocyst of a recipient surrogate mother, gave rise to germline transmission with a high efficiency (Table 1). The pluripotency of the c-Photina stem cells was also confirmed by the ability of the c-Photina mES cells to differentiate in vitro into cell types derived from different germ layers, such as cardiomyocytes and neurons.

The possibility to generate primary cells such as cardiomyocytes and neurons containing a bioluminescent system for monitoring
Ca\(^{2+}\) movements finds many applications. Since, Ca\(^{2+}\) has a crucial role, for example, in controlling cardiac rhythm, one could investigate Ca\(^{2+}\) behaviour in many models of different cardiac diseases. Also in neurons it could be interesting to visualize Ca\(^{2+}\) movements, in normal development or during neurodegeneration, or address synaptic function. We optimized the differentiation protocols also in miniaturized formats, like the 96 and the 304 MTP, sine qua non condition for the feasibility of their usage in high throughput screening. Cells differentiated in these formats showed all the characteristics of primary-like cells, such as the expression of cell-specific targets (demonstrated by immunofluorescence analysis) or the ability to spontaneously pulse or to respond to agonist for receptors highly expressed in these cells. Moreover, the differentiation processes were shown not to interfere with the expression of the reporter gene. The functional presence of the photoprotein in the differentiated cells was demonstrated by the ability of the cells to respond to different stimuli, inducing Ca\(^{2+}\) movements, confirming their utilization suitable for the development of cell-based assays.

This approach was exploited to address which Ca\(^{2+}\) receptors or channels might have a functional role in mouse embryonic stem cells. We screened an unbiased library of 1280 pharmacological active compound (LOPAC\(^{1280}\)) modulating all of the major classes of important receptors and channels. This signalomic approach allowed high throughput identification of all cell surface receptors and channels whose activation induce a variation of intracellular Ca\(^{2+}\) in undifferentiated mES cells. As a positive control for known Ca\(^{2+}\) signalling a parallel approach was performed on neurally-differentiated cells. The results indicated sparse activation of Ca\(^{2+}\) response in the undifferentiated mES cells. The only classes of receptors that were shown to be activated were the one of histamine-1 receptor (H\(_1\)) and those of purinergic receptors, both already described in literature [15,23,38]. The presence of the H\(_1\) receptor in these cells suggests a role of histamine in early mammalian development. Furthermore, histamine was shown to have a role in regulating neural stem cells proliferation and the expression of the H\(_1\) receptor was shown to favour neuronal fate [39]. Also the presence of P2 purinergic receptors was already described in mouse embryonic stem cells. In particular it was proposed a role of extracellular ATP in stimulating mouse embryonic stem cell proliferation [38]. Here the authors revealed the presence of P1 purinergic receptors too by the functional response to 2-chloroadenosine only in undifferentiated and not in neurally-differentiated cells. Its activation suggests that the role of the P1 receptor in pluripotent mouse embryonic stem cells should be further investigated.

Thanks to germline transmission of c-Photina mES cells, a transgenic animal containing the c-Photina photoprotein (Photo-Topo) was derived. The cells cultured from photoprotein transgenic animals can be used as positive controls for the “primary-like” cells obtained after differentiation of mES cells, and directly as primary cells, for a pharmacological screening process per se. As proof of principle, we isolated from PhotoTopo animals primary endothelial cells and hematopoietic precursors, which we differentiated into bone marrow-derived monocytes/macrophages. We demonstrated that these cells contain the photoprotein and that they can be used in miniaturized format for functional assays. Moreover, the organism-wide expression of the transgene in PhotoTopo mice (Figure 6A-B) suggests the availability of a larger spectrum of useful primary cells. Interestingly, the expression of the reporter gene was shown not to decrease with animal age, further confirming the stability of this photoprotein and indicating that the transgene is not inserted in a position subject to chromatin inactivation over the course of time.

The c-Photina transgenic mouse, can be used in combination with optical microscope systems, like CCD cameras, which are able to detect in real time light emission of bioluminescent reporter within the animal’s cells. This application opens the possibility to charge the photoprotein by systemic injection of coelenterazine, representing a suitable model for monitoring modulation of intracellular Ca\(^{2+}\) levels, and for the generation of in vivo bioluminescence imaging (BLI) based studies. As proof of principle we isolated PhotoTopo pancreatic islets since they are a perfect source of material for studying complex Ca\(^{2+}\) exchanges, occurring between different cell types. In fact they are multicellular structures, in which Ca\(^{2+}\) plays a fundamental role in insulin secretion. Actually, the entrance of glucose through the type 2 glucose transporters induces the activation of voltage-gated Ca\(^{2+}\) channels. The consequential entry of Ca\(^{2+}\) ions from the extracellular space induces insulin release from insulin-storing granules exocytosis. We demonstrated the feasibility of Ca\(^{2+}\) movement observation in PhotoTopo islets, after a glucose and a depolarizing stimulus, through the photoprotein activation, not only by a CCD camera-based luminometer but also by using an intensified CMOS-based camera with 512\(\times\)512 pixel resolution. The potentiality to study Ca\(^{2+}\) movements in whole islets and within single cells opens very interesting potential applications for diabetes research.

Materials and Methods

c-Photina Generation

The c-Photina gene (mutant 12, Patent Application EP06000171) was obtained by random mutagenesis from the gene Cytin (GenBank accession number Q68121) using the GeneMorph II Random Mutagenesis kit (Stratagene, La Jolla, CA, USA) following the supplier’s instructions with the following primers:

Upper: GATGACGAGCAGAAG-ATGGCCGACCACGGC-CAG
Lower: GAGGAGAACGCCGTT-TTATCAAGGACAC-GAAAT.

mES Cell Culture

TBV2 (129S2/SvPas) mouse embryonic stem cells [40] were cultured in the undifferentiated state on a monolayer of Mitomycin C treated mouse embryonic fibroblasts in the presence of leukemia-inhibiting factor (LIF) (Chemicon) [41].

Photoprotein mES Cell Clone Generation and Selection

The c-Photina gene was cloned into the pcDNA3.1+ vector (Invitrogen) downstream the mitochondrial tag (mito) of the human Cytochrome C Oxidase, subunit VIII [14].

7\(\times\)10\(^6\) mES cells were electroporated using 30 \(\mu\)g of the mito c-Photina DNA, linearized with BglIII (New England Biolabs). Positive clones were selected with 200 \(\mu\)g/mL G418 (geneticin, SIGMA) [40]. Four hours after the test the medium was replaced with 50 \(\mu\)l/well of Tyrode’s buffer (130 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM NaHCO\(_3\) and 20 mM HEPES, pH 7.4, 2 mM Ca\(^{2+}\)) and 10 \(\mu\)M coelenterazine, in the dark, and incubated at 37°C in a humidified atmosphere with 5% CO\(_2\) in order to reconstitute the active photoprotein. The number of photons emitted after injection of the different ligands for 60 seconds was measured on the Lumibox CCD camera-based luminescence detector designed and built by Bayer Technologies GmbH (Wuppertal, Germany), and expressed as RLU (Relative Luminescence Units).

DNA from mES cells plated on gelatin-coated dishes was extracted with standard methods [42]. 10 \(\mu\)g of ES genomic DNA of ES/mito c-Photina cells was digested with the restriction enzymes, HindIII, XbaI, BamHI, HindIII/XbaI (New England
Biolabs), transferred to a positively-charged nylon membrane (Roche) for Southern blot analysis. The [32P]dCTP–labelled c-Photina coding sequence [42] was used as the probe. The Southern blot analysis was performed by digesting the genomic DNA with restriction enzymes which cut only once in the transfected vector (to discriminate concatamers).

Quantitative PCR – Sybr Green®

QPCR (Quantitative Polymerase Chain Reaction) was performed using approximately 3 ng of DNA per reaction with the “Platinum® SYBR Green® QPCR SuperMix UDG” protocol (Invitrogen). The primers were used designed on the primer pair: cPH-for: CACCAAGTTGCGTGGAAGG; cPH-rev: GCCAGTCCTGGCCTGACTC;

For the comparison between luminescent and fluorescent read-outs the cells were or incubated with with 40 μL/well of Tyrode’s buffer and 10 μM coelenterazine four hours before the test or with 40 μL/well of the Fluor4NW dye (Molecular Devices) solubilised in Tyrode’s buffer for 60 min at 37°C. The signals were recorded at FLIPRtetra® reader, for 220 sec for fluorescence and 60 sec for luminescence read-out.

Immunofluorescence Analysis

The mES cells were fixed with 4% paraformaldehyde (PFA, MERCK, Whitehouse Station, NJ, USA) solution and simultaneously blocked and permeabilized with 10% normal goat serum (Chemicon)/0.2% Triton X-100 in 1X PBS. The different antibodies were incubated in 10% normal goat serum 0.1% Triton X-100 in 1X PBS.

The primary antibodies used were: mouse anti-oct 3/4 (C-10) (Santa Cruz Biotechnology), mouse anti SSEA-1 (Santa Cruz Biotechnology), rabbit anti myosin heavy chain (MHC) (H-300) (Santa Cruz Biotechnology), rabbit anti-GATA-4 (H-112) (Santa Cruz Biotechnology), mouse anti-sarcomeric alpha-actinin (EA-53) (SIGMA), rabbit anti-microtubule-associated protein 2 (MAP2) (Chemicon), rabbit anti-beta III tubulin (Chemicon), rabbit anti glial fibrillary acidic protein (GFAP) (Dako), and mouse anti-nestin (Rat-401) (Chemicon).

The secondary antibodies used were the fluoresceinated antimouse (Chemicon), alone or in combination to the anti-rabbit IgG/IgM rhodamine-conjugated (Millipore), or anti rabbit FITC-conjugated (Chemicon) secondary antibody in 10% normal goat serum/0.1% Triton X-100 in 1X PBS.

The Hoechst 33342 dye (Invitrogen) (2 μg/mL final concentration) was incubated for 5 min at room temperature.

Images were acquired by using either an Olympus IX51 microscope equipped with a F-View II camera and the dedicated software cell-F (Olympus) or an Olympus IX70 microscope coupled to a Leica digital camera with a customized acquisition system. In some cases the brightness and/or the contrast were modified in order to reduce the background signal deriving from the white wall 384 plates used for the experiments (Matrix-Thermo Scientific 384 well plates-polystyrene white/clear).

Alkaline Phosphatase Staining

The alkaline Phosphatase activity was measured with the ELIP® Phosphatase staining kit (ATCC) following manufacturer’s instructions.

Tagman® PCR

Total RNA was isolated by TRIzol® (Gibco/BRL, Gaithersburg, MD). Reverse transcription-PCR (RT-PCR) was performed with the Invitrogen Superscript II RT-PCR kit (Invitrogen), as recommended by the manufacturer.
PhotoTopo CCD Camera Functional Tests

Mice were perfused with a physiological solution and tissues were harvested and incubated in a reaction solution containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM DTT, 1 mM EDTA, 0.1% BSA, 20 μM coelenterazine plus protease inhibitor cocktails (Roche), for 3 h at room temperature. Luminescence was determined on the Lumibox, after injection of a solution of Triton X-100 and 250 mM CaCl₂.

300 μl of coelenterazine (375 μM coelenterazine, 3.3% DMSO, 990 nM glutathione in physiological solution or 2.8 mg of coelenterazine/kg), was injected via the tail vein. Tissues were explanted, triturated with a scissors, and divided into two batches. One part was put in the reaction solution (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM DTT, 1 mM EDTA, 0.1% BSA, plus protease inhibitor cocktails) without coelenterazine and tested immediately on the Lumibox; the other part was incubated with the same reaction solution in presence of 20 μM coelenterazine for 3 h at room temperature before the Lumibox luminometer test, after injection of a solution of Triton X-100 and 250 mM CaCl₂.

Flow Cytometry

Cells were resuspended in 100 μL of a blocking solution containing 4% FCS serum, 1 mM EDTA in PBS at a cell concentration of 1 × 10⁶ cells/mL for 10 min, then incubated with 5 μg/10⁶ cells of the primary antibodies for other 30 minutes.

Cells were washed twice with blocking buffer. After 30 min of incubation with the secondary antibody and another 2 washes, cells were resuspended at 10⁶/mL in blocking buffer.

Cell acquisition was performed with FACSort Becton Dickinson in a region (R1) defined gating out only debris by forward and side scatter characteristics. 40,000 gated events were analysed with CellQuest software (BD).

The list of the antibodies used is:

- Rabbit anti-human von Willebrand Factor (DAKO),
- Goat anti-mouse CD31/PECAM-1 (R&D systems),
- Rat anti-mouse CD204 (AbD Serotec),
- Rat anti-mouse F4/80 (AbD Serotech),
- Goat anti-mouse IgG & IgM, (H+L) FITC conjugated (Chemicon),
- Goat anti-rabbit IgG, (H+L) FITC conjugated (Chemicon),
- Goat anti-rat IgG, FITC conjugated (AbD, Serotech),
- Chicken anti goat IgG (H+L), FITC conjugated (Chemicon).

PhotoTopo Endothelial Cell Preparation

Endothelial cells were isolated from mouse aorta. The aorta was removed from anaesthetized and heparinised mice, and after cleaning was placed (with the intima side down) on Matrigel¹⁶ (BD)-coated plates in DMEM medium plus 10% FCS, glutamine, not essential amino acids, and 75 μg/mL endothelial cell growth supplement (EGGS, Sigma). See Suh et al., 1999 [23] for extensive details.

PhotoTopo Bone Marrow Derived Monocytes/ Macrophages Preparation

Mice were sacrificed in order to isolate the bone marrow. The haematopoietic precursors were isolated from bone marrow flushed from femurs and differentiated in vitro into macrophages as described [26].

PhotoTopo Beta Islet Isolation and Culture

Pancreatic islets were isolated from mice (nine weeks old, 20–22 g) by a collagenase digestion method [44]. Briefly, 2 mL of cold Hank’s buffer/collagenase type V solution (1 mg/mL Sigma, St Louis, MS, USA) was infused into the pancreatic duct in situ, and the removed pancreas was digested at 37°C for 15 min. Islets were purified on a discontinuous Ficoll gradient (Sigma). The islets
(250 islets/mL) were cultured free-floating (37°C, 5% CO2) in medium RPMI 1640 (Bio-Whittaker, Walkersville, MD, USA) supplemented with L-glutamine (Sigma), penicillin-streptomycin (1,000 U/mL-10 mg/mL; Sigma) and 10% FCS (HyClone, Logan, UT, USA) for 20–24 h before the utilization. Islet purity was >90%.

Ten mito c-Photina transgenic mice islets/well were put in a white 96 MTP and incubated in Krebs-Ringer’s solution (125 mM NaCl, 5 mM KCl, 1.2 mM MgSO4,1.2 mM KH2PO4, 2 mM CaCl2, 25 mM HEPES pH 7.4, 0.1% BSA and 3 mM glucose) with 10 µM coelenterazine for 4 h at 37°C. The islets Ca2+ kinetic responses were measured at the Luminoskan Ascent (Labsystems) luminometer after stimulation with a glucose stimulus (11 mM), or with mannitol (11 mM), as the negative control. The glucose concentration was then normalized to 3 mM and the islets were then stimulated with a depolarizing stimulus (60 mM KCl) on the Lumibox. The total photoprotein content in the islets was measured after cell were lysed with a Triton X-100-based buffer.

Supporting Information

Video S1 Pulsating cardiomyocytes at differentiation day 15. Embryoid body containing a spontaneous beating area of cardiomyocytes obtained 15 days after differentiation of mES c-Photina cells. Found at: doi:10.1371/journal.pone.0008882.s001 (8.51 MB AVI)

Video S2 Pulsating cardiomyocytes at differentiation day 20. Embryoid body containing a spontaneous beating area of cardiomyocytes obtained 20 days after differentiation of mES c-Photina cells. Found at: doi:10.1371/journal.pone.0008882.s002 (80.19 MB AVI)

Video S3 Luminescence-based bioimaging studies on pancreatic islets. Ca2+-mediated light release obtained after stimulation of a PhotoTopo beta islet with a depolarizing stimulus (60 mM KCl). The light was acquired with a microscope based device, equipped with an intensified CMOS camera (Photon Fastcam). Found at: doi:10.1371/journal.pone.0008882.s003 (3.92 MB AVI)

Figure S1 Comparison of c-Photina and FluorNW readout systems in differentiated neurons (day 13). A–B. Example of responses after activation of a Ca2+-permeable, ligand-gated ion TRP (Transient Receptor Potential) channel, such as the Vanilloid Receptor-1 (VR-1), measured at FLIPTO© instrument with c-Photina (C.) or FluorNW fluorescent dye (D.). C–D. Example of responses after activation of a Ca2+-permeable, metabotropic glutamate receptor, measured at FLIPTO© instrument with c-Photina (C.) or FluorNW fluorescent dye (D.). E–F. Example of responses after activation of voltage-gated Ca2+ channels, measured at FLIPTO© instrument with c-Photina (E.) or FluorNW fluorescent dye (F.). FLIPTO© settings for luminescence: Integ. time: 1 sec; exp. time: 0.90 sec; injection speed: 20 µL/sec; injection height: 35 µL; reading time: 60 seconds. FLIPTO© settings for fluorescence: Integ. time: 1 sec; exp. time: 0.53 sec; injection speed: 20 µL/sec; injection height: 35 µL; reading time: 220 seconds. Found at: doi:10.1371/journal.pone.0008882.s004 (1.55 MB TIF)

Figure S2 LOPAC1280© screening schematic representation. For differentiated (Day 13) cells, Percent Activity is computed based upon the median response value of Min Signal wells and Glutamate (as Max Signal) wells on each plate. For undifferentiated cells, Percent Activity is computed based upon the median response value of the Min Signal wells and ATP (as Max Signal) wells on each plate. The large symbols (square) indicate that the %Activity is statistically significant based upon a t-test, in comparison to the %Activity of Min Signal wells (taking all of the Min Signal wells for each Day as a large group). The t-test performed was the “Two-sample unequal variance, one-tailed”. The threshold lines indicated the percent activity mean ± the standard deviation. Found at: doi:10.1371/journal.pone.0008882.s005 (2.45 MB TIF)

Table S1 Active LOPAC1280© compound description list. List of all the active compounds retrieved after screening of undifferentiated and neuronal differentiated cells (day 13) with an unbiased library of pharmacologically active compounds (LOPAC1280©). For all the compounds is indicated their complete name, the class, the action, the selectivity and the description (information provided directly by SIGMA LOPAC). Found at: doi:10.1371/journal.pone.0008882.s006 (0.11 MB DOC)

Acknowledgments

Paola Saveri (San Raffaele Scientific Institute, DIBIT, Division of Genetics and Cell Biology, Milan, Italy), Tod Flak, Marianna Stabilini, Andrea Brambilla, Claudia Caserini and Claudio Gattuso (Axxam S.p.A.), Fabio Grohovaz (ALEMBIC), Jacopo Tessadori and Stefano Pitasii (Optotech), Raffaella Melzi, Barbara Antonioli and Elisa Cantarelli (Beta Islet Unit, DIBIT, HSR, Milan, Italy).

Author Contributions

Conceived and designed the experiments: SC CN SL LW SC. Performed the experiments: SC SF CF CN PA AM LP LW. Wrote the paper: SC LW SC.

References

1. Rink TJ (1990) Receptor mediated calcium entry. FEBS Lett 268: 381–385.
2. Kendall JM, Badminton MN (1998) Aequorea victoria bioluminescence moves into an exciting new era. Trends Biotechnology 16: 216–224.
3. Campbell AK, Hallet RA, Daw ME, Ryall RC, Hart RG, et al. (1993) Application of the photoprotein obelin to the measurement of free Ca2+ in cells. In Bioluminescence and Chemiluminescence, basic Chemistry and Analytical applications, deLuca MA and McElroy WD (ed). Academy Press, New York. pp 601–607.
4. Johnson FH, Shimomura O (1978) Introduction to the bioluminescence of medusae, with special reference to the photoprotein aequorin. Methods Enzymol 57: 271–291.
5. Matthews LC (2000) Seeing the light: calcium imaging in cells for drug discovery. Drug Discovery Today: HTS supplement 1: 10–19.
6. Bovolenta S, Foti M, Lohmer S, Corazza S (2007) Development of a Ca2+-activated photoprotein, Photina, and its application to high-throughput screening. J Biomol Screen 12: 694–704.
23. Yanagida E, Shoji S, Hirayama Y, Yoshikawa F, Otsu K, et al. (2004) Recombinant aequorin and green fluorescent protein as valuable tools in the study of cell signalling. Biochem J 355: 1–12.

22. Stewart MH, Bendall SC, Bhatia M (2008) Deconstructing human embryonic stem cells: prospects for developmental biology and cell therapy. Physiological Reviews 88: 635–678.

21. Lee K, Xu E (2008) The role and the mechanism of h+- and Ca2+-selective microelectrodes suitable for in vitro extracellular recording. J Neurophysiol 96: 919–924.

20. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca2+-indicators with greatly improved fluorescence properties. J Biol Chem 260: 3440–3450.

19. Miyawaki A, Griesbeck O, Heim R, Tsien RY (1999) Dynamic and quantitative Ca2+ measurements using improved cameleon. Proc Natl Acad Sci USA 96: 12765–12770.

18. Romoser VA, Hinkle PM, Persichini A (1997) Detection in living cells of Ca2+-dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. A new class of fluorescent indicators. J Biol Chem 272: 13270–13274.

17. Bähring SS, Gambhir SS (2002) Optical imaging of Renilla luciferase reporter gene expression in living mice. Proc Natl Acad Sci U S A 99: 377–382.

16. Solter D, Knowles BB (1978) Monoclonal antibody defining a stage specific receptor. Biochemical and Biophysical Research Communications 191: 110–125.

15. Heo JS, Han HJ (2006) ATP Stimulates Mouse Embryonic Stem Cell Proliferation via Protein Kinase C, Phosphatidylinositol 3-Kinase/Akt, and Mitogen-Activated Protein Kinase Signaling Pathways. Stem Cells 24: 2637–2648.

14. Melzi R, Sanvito F, Mercalli A, Andralojc K, Bonifacio E, et al. (2000) Intrahepatic islet transplant in the mouse: functional and morphological characterization. Cell transplantation 17: 1361–1370.

13. Kirichok Y, Krapivinsky G, Clapham DE (2004) The mitochondrial calcium uniporter is a highly selective ion channel. Nature 430: 325–328.

12. Fedirko N, Svachar N, Cheshler M (2006) Fabrication and use of high-speed, concentric h+- and Ca2+-selective microelectrodes suitable for in vitro extracellular recording. J Neurophysiol 96: 919–924.

11. McNeish J (2004) Embryonic stem cells in drug discovery. Nat Rev Drug Discov 3: 70–80.

10. Wobus AM, Boheler KR (2005) Embryonic stem cell differentiation to the cardiac lineage. Methods in Mol Biol: Basic Cell Culture Protocols, Helgason CD, Miller CL (ed). Totowa, New Jersey: Humana press. pp 91–103.

9. Boheler KR (2003) ES cell differentiation to the cardiac lineage. Methods in enzymology 365: 228–241.

8. Faco A, Manganelli G, Simone M, Guido S, Minchiotti G, et al. (2000) High-throughput screening-compatible single-step protocol to differentiate embryonic stem cells in neurons. Stem Cells Dev 7: 573–584.

7. Bähring SS, Gambhir SS (2002) Optical imaging of Renilla luciferase reporter gene expression in living mice. Proc Natl Acad Sci U S A 99: 377–382.

6. Shirouzu A, Shibuya K (1983) Halistaurin, phialidin and modified forms of aequorin as Ca2+ indicator in biological systems. Biochem J 225: 745-749.

5. Libor LD, Ward WW (1982) Isolation and characterization of a photoprotein sequence analysis of cDNA for the luminescent protein aequorin. Proc Natl Acad Sci USA 79: 77–85.

4. De Giorgi F, Brini M, Bastianutto C, Marsault R, Montero M, et al. (1996) Targeting aequorin and green fluorescent protein to intracellular organelles. Gene 175/1 Spec No: 113–117.

3. Bloemers SM, Leurs R, Smit MJ, Verheule S, Tertoolen LGJ, et al. (1993) Characterization of explanted endothelial cells from mouse aorta: electrophysiological properties with greatly improved fluorescence properties. J Biol Chem 272: 77–85.

2. Rizzuto R, Simpson AW, Brini M, Pozzan T (1992) Rapid changes of mitochondrial calcium uniporter revealed by specifically targeted recombinant aequorin. Nature 358: 325–328.

1. Scholer HR, Hatzopoulos AK, Balling R, Suzuki N, Gruss P (1989) A family of receptors. Biochemical and Biophysical Research Communications 191: 110–125.