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Detection of Calcium Transients in Embryonic Stem Cells and Their Differentiated Progeny

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Abstract

A central issue in stem cell biology is the determination of function and activity of differentiated stem cells, features that define the true phenotype of mature cell types. Commonly, physiological mechanisms are used to determine the functionality of mature cell types, including those of the nervous system. Calcium imaging provides an indirect method of determining the physiological activities of a mature cell. Camgaros are variants of yellow fluorescent protein that act as intracellular calcium sensors in transfected cells. We expressed one version of the camgaros, Camgaroo-2, in mouse embryonic stem (ES) cells under the control of the CAG promoter system. Under the control of this promoter, Camgaroo-2 fluorescence was ubiquitously expressed in all cell types derived from the ES cells that were tested. In response to pharmacological stimulation, the fluorescence levels in transfected cells correlated with cellular depolarization and hyperpolarization. These changes were observed in both undifferentiated ES cells as well as ES cells that had been neurally induced, including putative neurons that were differentiated from transfected ES cells. The results presented here indicate that Camgaroo-2 may be used like traditional fluorescent proteins to track cells as well as to study the functionality of stem cells and their progeny.

Keywords

Calcium imaging; Stem cells; Camgaroo; Fluorescent indicators

Introduction

Stem cells hold tremendous promise for the study of genetic disorders and potential treatment of a variety of diseases and injuries. However, before these cells may be used for such purposes, it is vital to fully understand the differentiation of these cells and how they respond to their environment. In recent years, the functionality of differentiated cells has become increasingly important (Tsai et al. 2002; Wagers et al. 2002), as previous reports of transdifferentiation have often failed to demonstrate the functional characteristics of the newly acquired phenotype.

Stem cells of several varieties have been tested as potential therapeutic vectors for a variety of diseases and/or disorders such as Parkinson's disease (Astradsson et al. 2008; Bjorklund et al. 2002; Lee et al. 2000; Roy et al. 2006) and spinal cord injury (Coutts and Keirstead 2008; McDonald and Howard 2002; Zeng and Rao 2007), and this work provides proof of principle that stem cells are highly suitable for regenerative medicine. However, a major shortcoming of most studies has been the lack of functional data to demonstrate whether transplanted stem cells have adopted a desired phenotype, and whether these cells behave as mature, integrated cells (such as neurons) within the host. Commonly, phenotypic characterization of donor cells has been accomplished with immunohistochemical approaches. While these results are suggestive of cell type, there are other factors to consider in the characterization of a differentiated cellular population, including the transcriptional profile of these cells and their physiological characteristics. Expression of a single marker is not sufficient to assign a cellular phenotype after differentiation of a stem cell. Likewise, although expression of a variety of appropriate markers suggests a specific cell lineage, it is not definitive evidence of full phenotypic differentiation.

The ability to coax stem cells toward a neural lineage has been proposed as a means of regenerative medicine to treat disorders of the nervous system (Arenas 2002; Astradsson et al. 2008; Daley et al. 2003; Klassen et al. 2004; Park et al. 2002). Many studies have shown that a variety of stem cells are capable, under the appropriate conditions, of adopting new morphologies that are very similar to neurons, and these cells often express some neuronal markers (Black and Woodbury 2001; Brazelton et al. 2000; Kim et al. 2002; Levy et al. 2003; Woodbury et al. 2000). For instance, attempts to differentiate bone marrow-derived cells into neural cells have brought to light the importance of determining the functional capabilities of cells differentiated in vitro; this is especially important if the donor stem cells differentiate into mature cell types not normally derived from the germ layer of origin for a given tissue, such as neuron-like cells derived from bone marrow stem cells. Therefore, a variety of tests should be performed to demonstrate that a cell's original fate has been redirected along a different lineage.

Determining the functionality of many cell types including those of the nervous system is traditionally achieved with physiological approaches. These techniques offer advantages because they allow for the direct measurement of the electrical activity of cells, such as action potentials fired by neurons that are a reliable indication of the mature neuronal phenotype. A complimentary approach for studies of cellular function is the use of calcium imaging. Dynamic changes in intracellular calcium are associated with the routine functioning of many cell types. For example, calcium influx into neurons during an action potential is required for the release of neurotransmitter. Calcium imaging offers a non-invasive method to assess the functioning of a cell, and can be particularly useful when electrode access to the cell is difficult. Typically, calcium imaging is accomplished using calcium-sensitive dyes such as fura-2 (Brownlee 2000; Takahashi et al. 1999; Wang et al. 2008). However, in some cases these dyes do not provide a feasible approach, especially for

in vivo stem cell transplantation experiments in which a specific cell within the host is of interest.

Molecular studies have led to the development of a number of calcium-sensitive, fluorescent proteins, including cameleons (Miyawaki et al. 1999), pericam (Nagai et al. 2001), GcAMP (Nakai et al. 2001) and camgaroos (Griesbeck et al. 2001). Light emitted from these proteins varies with the amount of calcium present in cells. Camgaroo-1 was created by inserting the coding sequence for the calcium-binding protein, calmodulin into residue 145 in YFP (Baird et al. 1999; Yu et al. 2003). Binding of calcium to Camgaroo-1 induces a conformational change in the protein that results in a 7- to 8-fold increase in fluorescence at 514 nm. Camgaroo-1 is also pH dependent with an apparent pKa of 10.1 in the absence of Ca^{+2} and a pKa of 8.9 in the presence of Ca^{+2} . The fluorescence of Camgaroo-1 is also temperature-dependent; its fluorescence is brighter at 24°C than at 37°C.

To select a protein with better fluorescence at 37°C, Tsien and co-workers randomly mutated the Camgaroo-1 gene and screened for mutants with brighter fluorescence at 37°C (Griesbeck et al. 2001). A clone with a single nucleotide mutation in codon 70 of Camgaroo-1 was obtained. This clone, known as Camgaroo-2, possesses a mutation that converts lysine in Camgaroo-1 into a methionine. This residue is normally a glutamine in YFP. Converting amino acid 70 to a methionine reduces its dependence on Cl^- concentration, because the methionine blocks the halide-binding pocket in YFP. Because Camgaroo-1 and Camgaroo-2 both absorb light at 490 nm, a 488 nm argon laser commonly used in confocal microscopes and fluorescent-activated cell sorters excites them.

Calcium reporter genes such as the Camgaroos offer unique opportunities to visualize cells. First, the fluorescence exhibited by the cells can be used to identify donor cells upon transplantation into a host system. Furthermore, changes in levels of this fluorescence can be used to determine the changes in calcium concentration in these cells within host tissues. Previous studies have shown that stem cells can incorporate into the host retina and adopt retinal morphologies, presumably differentiating into retinal neurons (Meyer et al. 2004; Meyer et al. 2006; Takahashi et al. 1998; Van Hoffelen et al. 2003; Young et al. 2000). If these stem and/or progenitor cells incorporate into the host neural circuitry and replace cells that have been lost, they should respond to stimulation of the retina with corresponding increases or decreases in Camgaroo-2 fluorescence.

In the current study, we transfected a mouse ES cell line (GSI-1) with the Camgaroo-2 gene. Under the control of the CAG promoter, Camgaroo-2 is expressed in undifferentiated ES cells, progenitor cell types derived from differentiating ES cells, as well as mature progeny such as neurons. We evaluate the physiological responses of both undifferentiated ES cells as well as ES cell-derived, neural cells based on their Camgaroo-2 fluorescence.

Methods

Vector Design and Construction

Plasmids containing the Camgaroo-1 and Camgaroo-2 coding sequences cloned into pcDNA3 (pCam1 and pCam2) were kindly donated by Roger Tsien. As described briefly above, Camgaroo-1 and Camgaroo-2 are identical except for a single nucleotide mutation that alters codon 70 from a lysine in Camgaroo-1 to a methionine in Camgaroo-2. In these original constructs, Camgaroo-1 and Camgaroo-2 expression is driven by the enhancer and promoter from the Cytomegalovirus (CMV) major immediate-early gene. These constructs also contain a neomycin-resistance (*neo*) gene downstream of a SV40 early promoter.

Plasmid pCAG-Cam2-PAC (Fig. 1) was constructed in two steps. First, a 2,604 bp NotI to SmaI fragment from pGET015 (Tullis and Shenk 2000) containing an internal ribosomal entry site (IRES) and puromycin-*N*-acetyltransferase gene (*pac*) was ligated to a 4,842 bp NotI to SmaI fragment from pGET-GFP (Mayginnes et al. 2006) to create the plasmid pCAG-GFP-PAC. Next, the GFP gene in pCAG-GFP-PAC was replaced with an NcoI to NotI fragment from pCam2 that contained the Camgaroo-2 coding sequence to create pCAG-Cam2-PAC. Camgaroo-2 expression in pCAG-Cam2-PAC is driven by the CAG promoter, which is a composite of the CMV enhancer and the chicken β -actin promoter. Downstream of the Camgaroo-2 coding sequence is an IRES and the *pac* gene, that allows the translation of the selectable marker from the same transcript as Camgaroo-2.

Flanking the Camgaroo-2-*pac* expression cassette are two terminal repeats from adeno-associated virus type 2 (AAV2). Plasmid pCAG-Cam2-PAC can be used to generate recombinant AAV when it is transfected into mammalian cells that also express the AAV2 replication and capsid proteins as well as 5 helper genes from adenovirus (Reed et al. 2006). Although recombinant AAV was not used in these experiments, the AAV2 terminal repeats were included in pCAG-Cam2-PAC because AAV2 terminal repeats have been shown to generate more uniform and stable gene expression in vertebrate embryos (Chou et al. 2001; Fu et al. 1998; Hsiao et al. 2001).

Maintenance of Undifferentiated ES Cells

Mouse ES cells (GSI-1 cell line, kindly donated by the University of Michigan Transgenic Animal Core Facility) were maintained in the undifferentiated state as described previously (Bain et al. 1995; Meyer et al. 2004; Robertson 1997; Smith et al. 1988). Briefly, undifferentiated ES cell cultures were maintained and expanded in gelatin-coated T25 tissue culture flasks in ES cell growth medium (ESGM) in the presence of leukemia inhibitory factor (LIF; Chemicon, Cat. # ESG1106). ESGM contained DMEM, 10% newborn calf serum, 10% fetal bovine serum, nucleosides, β -mercaptoethanol (1 mM) and LIF (1,000 U/ml). Cultures were split approximately every 2 days with the addition of trypsin and reseeded into new flasks for further expansion.

Transfection of Cells

Both pCam2 and pCAG-Cam2-PAC plasmids were linearized using SmaI restriction enzyme, which cuts in the ampicillin-resistance gene, at 37°C for at least 4 h. The enzyme was inactivated at 80°C for 20 min, and then the mixture was used immediately for transfection of cells. HEK293 cells were transfected using the calcium phosphate method described previously (Reed et al. 2006). ES cells were dissociated and spun down to achieve a density of 10^7 cells/ml and were then suspended in 0.1 M PBS containing 25 μ g of linearized plasmid DNA and electroporated with a single pulse at 250 V and 500 microF. Following electroporation, cells were placed on ice for ~20 min and then plated in ESGM containing LIF and incubated at 37°C and 5% CO₂. The next day, the culture medium was changed and puromycin (4 μ g/ml) was then added to select for cells that had been transfected with pCAG-Cam2-GFP. G418 (350 μ g/ml for 2 days, followed by 150 μ g/ml for an additional 5 days) was added to the medium to select for pCam2-transfected cells. Transfected cells were expanded in culture in the presence of LIF as described above.

Neural Differentiation of ES Cells

The 4-/4+ protocol was performed as previously described (Bain et al. 1995; McDonald et al. 1999; Meyer et al. 2004) by growing ES cells as unattached embryoid bodies (EBs) for 4 days in uncoated Petri plates using ES cell induction medium (ESIM). ESIM was equivalent to ESGM except it lacked β -mercaptoethanol and LIF. The EBs were then cultured for an additional 4 days in the presence of retinoic acid (all-*trans* retinoic acid, 500 nM, Sigma Cat.

#R2625). For YFP imaging experiments, cells were then plated on culture slides coated with entactin–collagen–laminin (ECL, Upstate Biotechnology Cat. # 08–110) at a density of 50,000 cells/cm² for 4–5 days in DMEM with N2 supplement (Gibco Cat. # 17502) along with L-glutamine (2 mM), penicillin/streptomycin (100 U/ml), and nystatin supplement (10 U/ml). Cells for the immunocytochemistry experiments were plated on culture slides coated with entactin–collagen–laminin (ECL, Upstate Biotechnology Cat. # 08–110) at a density of 250,000 cells/cm² as we previously described (Pierret et al. 2007) for 2–5 days in DMEM with N2 supplement (Gibco Cat. # 17502), penicillin/streptomycin (100 U/ml) and Amphotericin B (Gibco Cat. # 041–95780) (2.5 U/ml).

Transplantation of Neuralized Camgaroo-2 ES Cells onto Organotypic Slice Cultures

Camgaroo-2 ES cells were neuralized as previously described using the 4–/4 + protocol. Mouse ex vivo organotypic brain slice cultures were prepared using established methods (Benninger et al. 2003; Stoppini et al. 1991), with the exception that the brains were sliced within a pool of ice-cold, sterile 0.1 M PBS on a Vibratome after being encased in 4% agarose. Briefly, brain hemispheres were harvested from 3-week old male mnd mice, sliced to a thickness of 400 μ m and cultured on Millicell-CM membranes (Millipore Cat. # PICM ORG 50) resting atop horse serum-containing media in 35 mm Petri dishes at 37°C and 5% CO₂. On each of the first 5 days of slice culture, the existing media was replaced by serum-free media. On the sixth day of slice culture, neuralized Camgaroo-2 cells suspended in serum-free media were applied to the slice cultures. After transplantation, serum-free media was changed once a week. Images of Camgaroo-2 cells on slice cultures were acquired eight days post-transplantation using a Leica MZFLIII stereomicroscope equipped with epi-fluorescence.

Imaging of Cells for Camgaroo-2 Fluorescence

Cells were imaged on an Olympus IX70 microscope equipped with a Photometric Sensys CCD camera. The software used with this microscope was Openlab v. 2.2.0. Both undifferentiated and neural-induced cells were exposed to the depolarizing agents KCl (60 mM) and ionomycin (10 mM), as well as solutions that prevent influx of extracellular calcium (cadmium chloride and zero-calcium/EGTA). Images were captured at various time points before, during and after exposure to these agents.

Quantification of Changes in Fluorescence Intensity

Images were acquired from at least five fields within four total experiments. The fluorescence intensity of undifferentiated ES cell colonies or differentiated neural cells, as well as the background fluorescence, was acquired by using NIS Elements software (version 3.0, Nikon). Relative fluorescence intensity was calculated by comparing the mean fluorescence intensity of cells to the background fluorescence within each field. Significance was determined with an ANOVA test.

Immunocytochemistry

Culture slides were collected 2–5 days after plating and fixed for 20 min in phosphate-buffered saline (PBS)-buffered 4% paraformaldehyde as described previously (Pierret et al. 2007). The cells were washed with 0.1 M PBS and permeabilized at room temperature for 1 hour using 0.3% Triton X-100, 0.1 M PBS, and 10% normal goat serum. Primary antibodies selective for astrocytes (rabbit polyclonal for glial fibrillary acidic protein (GFAP), 1:5,000, DakoCytomation Cat. # Z0334), neural precursors (mouse monoclonal for Nestin, 1:20, Chemicon Cat. # MAB353), radial glia (rabbit polyclonal for brain lipid binding protein (BLBP), 1:500, Chemicon Cat. # AB9558), early neurons (rabbit polyclonal for β -III Tubulin, 1:100, Chemicon Cat. # AB9324), and mature neurons (rabbit polyclonal for

Neurofilament-M, 1:200, Chemicon Cat. # AB1981) were diluted in 0.1 M PBS containing 2% normal goat serum and applied overnight at 4°C. Appropriate fluorescent-tagged goat anti-mouse (Molecular Probes Cat. #A11004) and goat anti-rabbit (Molecular Probes Cat. #A21070) secondary antibodies were diluted 1:200 in 0.1 M PBS containing 2% normal goat serum and applied for 2.5 h at room temperature. Nuclei were stained with Hoechst 33358.

Imaging of Immunocytochemistry

Cells were imaged using a Zeiss LSM 510 M-200 AXIO-VERT NLO 2-Photon confocal microscope (Molecular Cytology Core, University of Missouri). Images were processed using LSM 5 Image Examiner software version 4.0.0.157 and Adobe Photoshop CS.

Results

To compare the fluorescence of Camgaroo-1 and Camgaroo-2 in mammalian cells at 37°C, we transiently transfected HEK293 cells with pCam1 and pCam2, which expressed Camgaroo-1 and Camgaroo-2 respectively, under the control of the CMV promoter (Fig. 1). Prior to stimulation, the fluorescence of Camgaroo-1 was barely detectable above background (Fig. 2). Elevated potassium levels are known to depolarize the cell and result in the influx of calcium through voltage-gated calcium channels. When transiently transfected HEK293 cells were exposed to media containing elevated K⁺ (potassium acetate = 80 mM), Camgaroo-1 fluorescence significantly increased after 1 min and continued to increase 20 min after exposing the cells to potassium acetate.

In contrast to Camgaroo-1, the basal fluorescence of Camgaroo-2 was easily detectable at 37°C in transfected cells. This fluorescence increased dramatically when the cells were exposed to high K⁺. Although the apparent increase in fluorescence of Camgaroo-1 was greater than that of Camgaroo-2, the low basal fluorescence made it unsuitable for initial identification of donor cells for potential *in vivo* applications. Therefore, we focused on Camgaroo-2 for the remaining experiments.

Because of potential inactivity of the CMV promoter in undifferentiated ES cells (Chung et al. 2002), we cloned the Camgaroo-2 coding sequence into a new expression vector called pCAG-Cam2-PAC (Fig. 1C). Expression of Camgaroo-2 in this construct is driven by the CAG promoter that is a combination of the strong CMV enhancer and the chicken β -actin promoter (Niwa et al. 1991). This promoter has been used to generate transgenic mice that express GFP in almost every cell-type except for erythrocytes and hair follicle cells (Okabe et al. 1997). Downstream of the Camgaroo-2 gene is an IRES and puromycin-resistance gene. This allows the puromycin-resistance gene to be translated from the same transcript as Camgaroo-2. In pCam2 (Fig. 1b), the neomycin gene is expressed from a promoter from SV40 virus. There are two AAV2 terminal repeats in pCAG-Cam2-PAC that serve two purposes. First, pCAG-Cam2-PAC can be used to produce recombinant AAV. Only the DNA between the two terminal repeats will be packaged into the recombinant AAV virus. Second, the presence of AAV2 terminal repeats flanking an expression cassette produces stronger and more uniform gene expression. When plasmid DNA is integrated into the chromosomal DNA, the expression of the transgene can vary greatly depending on where the DNA has integrated. The AAV terminal repeats act similar to matrix attachment regions in the chromatin. Promoters near these attachment points tend to be highly active. This results in stronger and more uniform gene expression from one cell to the other regardless of where the DNA integrated.

To compare Camgaroo-2 expression from pCam2 and pCAG-Cam2-PAC, we transfected plasmid DNA into ES cells by electroporation. To select for cells that acquired pCam2, we added the neomycin analog G418 (350 μ g/ml for 2 days followed by 150 μ g/ml for an

additional 5 days) to the media. To select for cells transfected with pCAG-Cam2-PAC DNA, we added puromycin (4 μ g/ml) to the media. Neomycin-resistant ES cell colonies that were transfected with pCam2 displayed little Camgaroo-2 fluorescence (Fig. 3a, b). However, Camgaroo-2 fluorescence was visible upon cellular differentiation but only in certain cell types, not including neurons (data not shown). The Camgaroo-2 gene was cloned into pCAG-Cam2-PAC under the control of a CAG promoter. Upon electroporation of pCAG-Cam2-PAC into mouse ES cells, Camgaroo-2 fluorescence was clearly visible within 24 h in culture. Puromycin selection for stably-transfected cells yielded colonies of ES cells that were positive for the Camgaroo-2 gene (Fig. 3c, d). Following differentiation of these ES cells into neurons, Camgaroo-2 fluorescence was detected in most mature cell types (Figs. 5, 6).

The neural phenotypes of differentiated cells containing the Camgaroo-2 transgene were confirmed with immunocytochemistry (Fig. 4). Cells within the cultures expressed glial fibrillary acidic protein (GFAP), the neural precursor marker Nestin, neuronal markers β -III Tubulin and Neuro-filament medium chain (NF-M) and radial glial marker brain lipid binding protein (BLBP). Protein expression following retinoic acid neural induction was consistent with the neural outcomes reported previously (Bain et al. 1995; Meyer et al. 2004, 2006; Pierret et al. 2007).

Upon differentiation to a neural lineage, these cells may prove to be suitable for in vivo applications. In addition to their potential to monitor fluctuations in intracellular calcium, the inherent basal fluorescence associated with Camgaroo-2 could potentially serve to aid in the identification of these cells in the host environment. To test the ability to track donor cells expressing Camgaroo-2 in host brain tissue, ex vivo organotypic brain slice cultures were obtained from mice and a suspension of neuralized ES cells transfected with pCAG-Cam2-PAC were added to the surface of the organotypic slice (Fig. 5a). Following application of donor cells to the slice surface and incubation for 8 days, the neuralized ES cells had integrated into the host tissue, as observed using the basal level of Camgaroo-2 fluorescence produced by the donor cells (Fig. 5b).

To determine whether the Camgaroo-2 gene could be used to measure cellular activity, ES cell cultures were exposed to high K^+ (60 mM KCl) to depolarize the cells and to produce increased levels of intracellular calcium. In colonies of undifferentiated ES cells (Fig. 6a), slow changes in fluorescence were observed in response to high K^+ over the course of 10 min, indicative of increased levels of intracellular calcium (Fig. 6b, c). These changes in fluorescence were statistically significant ($P < 0.05$) and reversible upon return to normal media (Fig. 6d). In cultures of ES cell-derived neural cells, including neuron-like cells (Fig. 6e), a basal level of Camgaroo-2 fluorescence was observed (Fig. 6f). Exposure to high K^+ increased fluorescence in the somas of these differentiated cells (Fig. 6g). These changes in relative fluorescence intensity were statistically significant ($P < 0.01$) and reversible upon removal of elevated potassium (Fig. 6h).

We also induced increased intracellular calcium with the calcium ionophore ionomycin. In colonies of undifferentiated ES cells (Fig. 7a), addition of ionomycin resulted in changes in Camgaroo-2 fluorescence (Fig. 7b, c) much more rapidly than that observed in response to elevated potassium. The increase in relative fluorescence intensity was found to be statistically significant ($P < 0.01$) and reversible with wash (Fig. 7d). In cultures of ES cell-derived neural cells (Fig. 7e), basal fluorescence was observed (Fig. 7f), and upon exposure to ionomycin, fluorescence was greatly increased in the somas of these cells as well as in some of the larger processes (Fig. 7g). These changes were observed to be significant ($P < 0.01$) when compared with both baseline levels of fluorescence intensity as well as following the removal of ionomycin (Fig. 7h). In additional experiments, we applied 0.1

mM cadmium chloride to block voltage-gated calcium channels (Fig. 7i). We also exposed these cells to a zero-calcium solution containing 0.5 mM EGTA (Fig. 7j). Both of these manipulations significantly reduced the basal fluorescence produced by the cells expressing Camgaroo-2. The latter results indicated that a basal level of calcium influx exists in these cells, and when this influx was blocked, Camgaroo-2 fluorescence was reduced.

Discussion

Stem cells provide a source of cells for therapeutic transplantation in a variety of diseases. By definition, stem cells self-renew, producing more copies of themselves. Furthermore, stem cells can differentiate into one or more mature cell types (Bauwens et al. 2008; Daley et al. 2003; Gage 2000; Hua and Sidhu 2008; McKay 2000; Orlovskaya et al. 2008; Puceat 2008). These features make stem cells excellent candidates for therapeutic applications. Embryonic stem cells provide a potentially limitless source of cells for transplants, and also retain the plasticity to differentiate into many cell types. When transplanted in vivo, embryonic and adult stem cells often take cues from the local microenvironment and differentiate into cell types appropriate for their final tissue location (Bjorklund et al. 2002; Cedervall et al. 2007; Harkany et al. 2004; Parker et al. 2007; Yan et al. 2007).

Although the therapeutic promise of stem cells is clear, it has become increasingly important to demonstrate the functionality of differentiated stem cells (Castro et al. 2002; Prockop 2003; Wagers et al. 2002). Traditionally, phenotypic differentiation of stem cells has been demonstrated by using immunocytochemical labeling for markers unique to specific cell types. These procedures, along with the cellular morphology and their location following transplantation, have often been used to conclude that a stem cell has differentiated into a mature phenotype such as a neuron. However, recent studies have shown that further evidence is often necessary to determine definitively that a specific mature cellular phenotype has been acquired (Castro et al. 2002; Neuhuber et al. 2004; Pierret et al. 2006; Prockop 2003; Wagers et al. 2002). For example, neuronal-like cells derived from bone marrow were proposed to emerge from transdifferentiation events based on morphological and immunocytochemical results. However, when these cells were subjected to physiological tests, they rarely behaved as predicted, based on their morphological and marker expression data (Anderson et al. 2001). In fact, they can revert to their original phenotype when the inducing agent is removed (Neuhuber et al. 2004).

Among the first to demonstrate that ES cells can differentiate into functional neurons was work by Gottlieb and colleagues (Bain et al. 1995). This group used a neural induction protocol that includes exposure to retinoic acid to derive neuronal populations from ES cells. They used morphologic, histologic, as well as electrophysiologic data to show that mature cells derived from this protocol were true neurons. In other studies of tissue-derived stem cells, similar results have been demonstrated by Verfaillie and colleagues (Jiang et al. 2003). They reported that a sub-population of bone marrow-derived stem cells can be manipulated to achieve phenotypic characteristics of neurons; in fact, these cells exhibited electrophysiologic properties of neurons when grown under specific conditions. This type of functional data is not limited to neurons derived from stem cells. Similar criteria have also been used to demonstrate the functionality of a variety of cell types differentiated from stem cells such as cardiomyocytes (Wobus et al. 1997) and pancreatic islet cells (Lumelsky et al. 2001; Soria et al. 2000).

In the current study, undifferentiated ES cells as well as neuron-like cells derived from ES cells exhibited changes in Camgaroo-2 fluorescence in response to elevated levels of potassium or the addition of the calcium ionophore ionomycin. These agents increase intracellular calcium levels in many cell types; thus our experiments establish the proof of

principle that Camgaroo-2 can be used to monitor changes in intracellular calcium levels in embryonic stem cells and their progeny, including those of the neural lineage. By extension, Camgaroo-2 expressing stem cells will be useful in more specific situations, such as when testing for changes in calcium concentration in stem cell-derived neurons in response to neurotransmitter application or during stimulation of these cells that elicits action potentials.

Genetically-encoded calcium indicators (GECIs) provide an excellent alternative approach to assess the physiology of stem cells and their progeny (Barth 2007; Pologruto et al. 2004). Certain GECIs have properties that make them more advantageous for particular applications, including their use in mammalian model systems. Cameleons were initially developed by Tsien and colleagues (Miyawaki et al. 1999) and are dual-emission ratiometric calcium indicators comprised of a cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). Binding of calcium results in a conformational change in the protein that enhances the fluorescence resonance energy transfer from CFP to YFP. Although cameleons offer certain advantages, they can be less desirable due to the two-photon imaging necessary for quantification.

GCaMP is another type of GECI (Nakai et al. 2001) that is a chimera comprised of an N-terminal peptide followed by a circularly-permuted EGFP and calmodulin. The GCaMP excitation and emission spectra are similar to GFP. Yet another chimeric protein called pericam (Nagai et al. 2001) is comprised of the M13 peptide fused to a circularly-permuted EYFP followed by calmodulin. Binding of calcium to GCaMP or pericam results in a 4 to 5-fold or 8-fold increase, respectively, in fluorescence intensity. However, neither GCaMP nor pericam tend to be expressed well at physiological temperatures for mammalian cells.

Camgaroo-2 is based on a single fluorescence wavelength so it is easily visualized on most standard epifluorescence microscopes. Despite the fact that the changes in fluorescence intensity for Camgaroo-2 are less dynamic than some other GECIs (Barth 2007; Garaschuk et al. 2007; Heim et al. 2007; Yu et al. 2003), in many cases Camgaroo-2 is likely to be a better choice for GECI use because amino acid 70 in Camgaroo-2 has been mutated to a methionine, which improves the folding of Camgaroo-2 at physiological 37°C, and because it is pH independent, unlike both GCaMP and pericam.

It is noted that the CMV promoter was reported to be inactive in undifferentiated ES cells (Chung et al. 2002). However, two other groups reported that the CMV promoter is active in these cells (Ward and Stern 2002; Zeng et al. 2003). The simplest explanation for these apparently contradictory results is that the CMV promoter is active in ES cells, but at a reduced activity. The contradictory conclusions are likely due to differences in the sensitivity of the assays used. Chung et al. (2002) used as a reporter, a renilla GFP following an IRES; this combination is less sensitive than the GFP reporter used by the other two groups. These fluorescent proteins are excellent for detecting transfected cells, but are less useful, due to the long half-life of GFP, when the objective is to determine promoter activity. In addition, a gradual loss of reporter expression in neurons is observed when neural cells are transduced with a recombinant AAV containing a CMV promoter upstream of the transgene (Tenenbaum et al. 2004). This is caused by silencing of the CMV promoter due to DNA hypermethylation. In contrast, when the neuron-specific enolase promoter that does not become hypermethylated is used, little or no loss of reporter expression in transduced cells is observed (Tenenbaum et al. 2004).

In the current study, we show that genetically-encoded calcium indicators, such as Camgaroo-2, can effectively be used in stem cells to track these cells by using their inherent fluorescence, and they can be used to assess changes in intracellular calcium levels of these

cells during changes in cellular activity. The fluctuations in fluorescence intensity are associated with induced changes in intracellular calcium, and thus Camgarrow-2 expressing cells provide an alternative means of determining the functionality of stem cells and mature cell types derived from stem cells.

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Fig. 1.

Maps of plasmids: Plasmids used in these experiments are pCam1 (**a**), pCam2 (**b**), and pCAG-Cam2-PAC (**c**). Key features of pCam1 and pCam2: the CMV enhancer and promoter are from the human cytomegalovirus major immediate-early gene, Camgaroo-1 (Cam1), Camgaroo-2 (Cam2), bovine growth hormone polyadenylation site (BGH polyA), early promoter and origin from SV40, neomycin-resistance (*neo*) gene, SV40 polyadenylation site (SV40 polyA), β -lactamase (*bla*) gene (ampicillin-resistance). Key features of pCAG-Cam2-PAC: CMV enhancer, β -actin promoter is from the chicken β -actin gene, intron, Camgaroo-2 (Cam2), internal ribosomal entry site (IRES), puromycin-*N*-acetyltransferase gene (*pac*), SV40 polyA, AAV2 terminal repeats (TR), *bla* gene (ampicillin-resistance)

Fig. 2. Camgaroo fluorescence in transiently-transfected HEK293 cells. HEK293 cells were transfected with either pCam1 (**a**) or pCam2 (**b**). Camgaroo fluorescence was detected using an inverted fluorescence microscope 48 h after the cells were transfected. Images of the same field of view were taken 0, 1, 5, 10, 15 and 20 min after the media was replaced with media that contained elevated potassium acetate (80 mM). The high K^+ results in increased intracellular Ca^{+2} in the cells and an increase in both Camgaroo-1 and Camgaroo-2 fluorescence

Fig. 3.

Mouse ES cell colonies transfected with the Camgaroo-2 construct. Mouse ES cells shown with phase contrast microscopy (**a**) were first electroporated with the Camgaroo-2 gene under the control of the CMV promoter. Following selection with neomycin, positively transfected colonies displayed minimal fluorescence (**b**). Following cloning of the Camgaroo-2 gene into a new vector under the control of the CAG promoter, mouse ES cell colonies viewed with phase microscopy in (**c**), expressed readily visible levels of basal fluorescence (**d**). Scale bar equals 200 μm in **a**, **c**, and **d**, and 100 μm in **b**

Fig. 4.

Camgaroo-2 ES cells express neural markers upon differentiation. After neural induction, mouse ES cells transfected with pCAG2 express the early neural marker Nestin and/or the astrocytic marker GFAP (**a**). Some cells demonstrate co-expression of the two neural markers. Expression of the early neuronal marker β -III Tubulin (**b**) was also demonstrated in vitro. Neuralized Camgaroo-2 ES cells include cells that expressed brain lipid binding protein (BLBP) (**c**), indicative of radial glia. These cultures also include cells that express the mature neuronal marker Neurofilament (NF) (**d**). The Hoechst 33528 nuclear label is shown in *blue* in all panels. Scale bar equals 50 μ m and applies to all frames. (Color figure online)

Fig. 5. Identification of Camgaroo-2 ES cells in ex vivo organotypic brain slice cultures. Upon addition of neuralized ES cells expressing Camgaroo-2 to an organotypic brain slice culture (**a** is phase contrast image of slice culture), donor cells penetrated the living brain slice and were readily identifiable based upon their basal levels of fluorescence (**b**). *Arrows in b* indicate the representative identification of transplanted camgaroo-expressing cells. Scale bar equals 1 cm in **a** and 300 μm in **b**

Fig. 6.

Camgaroo-2 ES cells respond to elevated extracellular potassium. Colonies of undifferentiated Camgaroo-2 ES cells stably-transfected with pCAG-Cam2- PAC (**a**) exhibit low levels of basal fluorescence under normal conditions (**b**). In the presence of elevated potassium (60 mM), the fluorescence intensity of these cells increases (**c**). The relative fluorescence intensity was significantly (* $P < 0.05$) increased in response to elevated levels of potassium, and this effect was reversed by the removal of the high potassium solution (**d**). In Camgaroo-2 expressing cells that have undergone neural induction (**e**), the cells produce low levels of basal fluorescence in most of their somas (**f**). In the presence of elevated potassium (60 mM), the fluorescence intensity of these cells increases dramatically (**g**). The relative fluorescence intensity was significantly (** $P < 0.01$) increased in response to elevated levels of potassium, and this effect was reversed by the removal of the high potassium condition (**h**). Scale bar equals 400 μm in **a–c** and 200 μm in **e–g**.

Fig. 7.

Undifferentiated and differentiated Camgaroo-2 ES cells respond to the calcium ionophore ionomycin and blocking influx of calcium reduces basal fluorescence. Colonies of undifferentiated Camgaroo-2 ES cells stably-transfected with pCAG-Cam2-PAC (**a**) express low levels of basal fluorescence under normal conditions (**b**). In the presence of ionomycin (10 mM), the fluorescence intensity observed in these cells increases (**c**). The relative fluorescence intensity was significantly (** $P < 0.01$) increased in response to the addition of ionomycin, and this effect was reversed by the removal of ionomycin (**d**). Camgaroo-2 cells that have been differentiated into neural cells (**e**), exhibit low levels of basal fluorescence in the somas of most cells (**f**). In the presence of ionomycin (10 mM), the fluorescence intensity of these cells increases dramatically (**g**). The relative fluorescence intensity was significantly (** $P < 0.01$) increased in response to the addition of ionomycin, and this effect was reversed by the removal of ionomycin (**h**). The application of 0.1 mM cadmium chloride (**i**) or a zero-calcium bath solution containing 0.5 mM EGTA (**j**) significantly decreased Camgaroo-associated fluorescence. Scale bar equals 400 μm in **a–c** and 200 μm in **e–g**.