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Multipotent progenitor cells derived from adult peripheral blood of swine have high neurogenic potential *in vitro*

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Abstract

Peripheral blood derived multipotent adult progenitor cells (PBD-MAPCs) are a novel population of stem cells, isolated from venous blood of green fluorescent protein transgenic swine, which proliferate as multicellular non-adherent spheroids. Using a simple differentiation protocol, a large proportion of these cells developed one of five distinct neural cell phenotypes, indicating that these primordial cells have high neurogenic potential. Cells exhibiting neural morphologies developed within 48 hours of exposure to differentiation conditions, increased in percentage over two weeks, and stably maintained the neural phenotype for three additional weeks in the absence of neurogenic signaling molecules. Cells exhibited dynamic neural-like behaviors including extension and retraction of processes with growth cone-like structures rich in filamentous actin, cell migration following a leading process, and various cell-cell interactions. Differentiated cells expressed neural markers, NeuN, β -tubulin III and synaptic proteins, and progenitor cells expressed the stem cell markers nestin and NANOG. Neurally differentiated PBD-MAPCs exhibited voltage-dependent inward and outward currents and expressed voltage gated sodium and potassium channels, suggestive of neural-like membrane properties. PBD-MAPCs expressed early neural markers and developed neural phenotypes when provided with an extracellular matrix of laminin without the addition of cytokines or growth factors, suggesting that these multipotent cells may be primed for neural differentiation. PBD-MAPCs provide a model for understanding the mechanisms of neural differentiation from non-neural sources of adult stem cells. A similar population of cells, from humans or xenogeneic sources, may offer the potential of an accessible, renewable and non-tumorigenic source of stem cells for treating neural disorders.

Keywords

adult stem cell; time lapse; in vitro; immunocytochemistry; blood; patch clamp; neural differentiation; extracellular matrix

Introduction

Over the past two decades, stem cell therapy has become a promising and increasingly feasible approach for repair of the central nervous system (CNS). Stem cells from various sources are being used in development of therapies for slowly progressing degenerative

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conditions such as Parkinson's disease, Huntington's disease and multiple sclerosis, and for acute damage due to stroke or spinal cord injury (reviewed in Ben-Hur et al., 2005; Duncan, 2007; Hedlund et al., 2007; Louro and Pearse, 2008; McKay and Kittappa, 2008; Melone et al., 2005; Miller, 2006; Roskams and Tetzlaff, 2005; Zietlow et al., 2008). Although much progress has been made with stem cells isolated from embryonic or fetal sources, the difficulty in obtaining large numbers of these cells and the oncogenic risk associated with their degree of pluripotency (Bjorklund et al., 2002) has resulted in increased interest in the identification of alternate renewable sources of stem cells. Adult neural stem cells can be isolated from brain tissue (reviewed in Zhu et al., 2005) and human somatic cells have been reprogrammed to regain a pluripotent stem cell phenotype through the activation of various transcription factors (Takahashi et al., 2007; Yu et al., 2007), but reprogrammed cells also exhibit tumorigenic properties. In addition, stem cells capable of differentiating into neuron-like cells have been isolated from bone marrow (Mezey et al., 2000; Song et al., 2007; Trzaska et al., 2007), skeletal muscle (Park et al., 2007), adipose tissue (Jang et al., 2010; Kingham et al., 2007), hair follicles (Amoh et al., 2009; Hunt et al., 2008), skin (Marchesi et al., 2007; Toma et al., 2001), dental pulp (Karbanova et al., 2010; Kiraly et al., 2009) and umbilical cord (Buzanska et al., 2002; Kogler et al., 2004; Yang et al., 2008). Such cells have been used in animal models to effectively alleviate the symptoms of several CNS deficiencies (Biernaskie et al., 2007; Bouchez et al., 2008; Kingham et al., 2007; Marchesi et al., 2007; Wernig et al., 2008; Yang et al., 2008).

One of the most compelling motivations for the identification of easily accessible and renewable stem cell sources from adults is the potential of such cells to be used in autologous transplants (reviewed in Passier and Mummery, 2003; Serakinci and Keith, 2006; Taupin, 2006). This approach would avoid the significant complications associated with transplant rejection and immunosuppressant therapy. We recently reported the isolation of a novel type of multipotent progenitor cell from the peripheral blood of green fluorescent protein (GFP) transgenic swine (peripheral blood derived multipotent adult progenitor cells; PBD-MAPCs)(Price et al., 2006). This regenerative population of stem cells was isolated from blood collected by venipuncture and the cells were differentiated towards myogenic, osteogenic, adipogenic, endothelial or neural lines in culture using cell-type specific media conditions. Here, we demonstrate that primordial PBD-MAPCs are primed for neural differentiation and express embryonic and neural stem cell markers, and we examine more closely the dynamics, efficiency, specificity and functionality (electrophysiology) of their neural differentiation. PBD-MAPCs, although multipotent, appear to be predisposed towards differentiation down a neural lineage and do so in response to cues from the extracellular matrix.

Materials and Methods

PBD-MAPC collection and proliferation

Peripheral blood derived multipotent adult progenitor cells (PBD-MAPCs) were isolated from transgenic green fluorescent swine as previously described (Price et al., 2006). These cells grow as spheroids and were passaged every 7–10 days by collecting them by centrifugation and washing with PBS. The spheroids were resuspended in 1ml of 0.5% (each) collagenase IV/dispase (Invitrogen) and incubated for 30min at 37°C with occasional gentle mixing. Alternatively, spheroids were mechanically dissociated using a stem cell passaging tool (Invitrogen) that cut the spheroids into small pieces. Dissociated cells were split 1:3 and seeded into 6-well plates where they regrew as spheroids.

***In vitro* neural differentiation**

Spheroids were diluted into PBS, centrifuged (365×g, 5min), and resuspended in differentiation medium consisting of Neurobasal medium (Invitrogen) plus 100U/ml penicillin, 100μg/ml streptomycin, 100μM nonessential amino acids, 430μg/ml GlutaMAX-1™, B27 (Invitrogen), N2 (Invitrogen), 2.5% Matrigel™ (vol/vol, BD Biosciences, Bedford, MA), 60ng/ml EGF, 10ng/ml bFGF, 50ng/ml SHH, 100ng/ml FGF8, and 10μM CGP55845, a GABA_B receptor antagonist. Cells were plated on poly-L-lysine (Sigma) coated cover slips (Fisher Scientific, Pittsburgh, PA) or chambered coverslips (Nunc, Rochester, NY) and incubated at 37°C, 5% CO₂ with media changes every 2 days. In some time lapse experiments, EGF, FGF8 and CGP55845 were left out of the media and replaced with 50ng/ml bFGF, 25ng/ml each of GDNF, BDNF, NT-3 and 1x ITS (Gibco); no difference in growth, phenotype, or behavior were observed between cells grown in these media. Finally, in experiments testing the stability of neural differentiation or the role of extracellular matrix (ECM) molecules, a basic medium lacking Matrigel™ and containing only Neurobasal medium, penicillin, streptomycin, nonessential amino acids, GlutaMAX-1, B27 and N2 (all concentrations as above) was used. For experiments testing ECM molecules, cover slips were coated with poly-L-lysine followed by either laminin I, fibronectin, or collagen IV (all 10μg/ml) applied overnight at 37°C.

Time lapse microscopy and immunocytochemistry

Time lapse imaging was performed on a Zeiss epifluorescent microscope equipped with a heated CO₂ incubator and motorized stage (Zeiss, Jena, Germany). This allowed imaging of large surface areas in multiple wells of chambered cover slips for 48h without disturbing the cells. Images of GFP fluorescence were collected every hour at 10x in mosaics of 20×20 images representing the same field of view (approximately 16×12mm) on each cover slip at every time point. Immediately after acquisition of the final time point, the cells were washed, fixed, and processed for immunocytochemistry.

For immunocytochemistry, cells grown on poly-L-lysine coated glass in differentiation medium for 10–20 days were washed twice with PBS (0.1M, pH7.4) and fixed with fresh 4% paraformaldehyde in PBS for 15min at RT. After washing twice again, cells were permeabilized with 0.1% Triton X in PBS (PBTX) for 10min. Cells were washed three times with PBS and blocked in 10% normal goat serum (NGS) in PBTX for 30min and then incubated with primary antibodies at a 1:500 dilution in 10% NGS/PBS overnight at 4°C. After three PBS washes, cells were incubated with secondary antibodies at a 1:500 dilution in 10% NGS/PBS for two hours. Cells were washed twice more, incubated with TO-PRO-3 iodide (1μM, Invitrogen) or DAPI (10μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS for 5min, rinsed in H₂O and mounted with Prolong Gold (Invitrogen). After curing overnight, slides were stored at 4°C until imaging. Spheroids were processed with the same protocol but in suspension and collected by centrifugation at 400xg after each step. Primary antibodies used were mouse anti-β-tubulin III, -NeuN, -tyrosine hydroxylase (TH), -glial fibrillary acidic protein (GFAP), -acetylcholine transferase, -O4, -Map2ab, -synaptophysin, rabbit anti-morphogenic bone protein (all from Millipore, Billerica, MA), mouse anti-syntaxin (Abcam, Cambridge, MA), rabbit anti-NANOG (Affinity Bioreagents, Golden, CO) and rabbit anti-nestin (Littleton, CO). Secondary antibodies were species appropriate immunoglobulins linked to Alexa Fluor 568 or Alexa Fluor 350 (Invitrogen). All immunocytochemistry experiments included control cells on cover slips processed in parallel with the omission of a primary antibody; no signal was observed in the red or blue channel in these preparations.

To visualize filamentous actin (f-actin), cells were washed, fixed and processed as above, but including 1% BSA plus 10% NGS in place of NGS alone. Cells were incubated with a

1:40 dilution of Alexa 568 conjugated phalloidin solution (200U/ml in methanol, Invitrogen) for 30min followed by three washes in PBS and mounted as above.

Neural Phenotype Classification and Quantitation

Cells were plated in differentiation medium on poly-L-lysine coated cover slips as above for a period of 35 days. In each experiment, half of the coverslips were switched to basic medium after 9 days in culture. Coverslips from each culture condition (neural differentiation vs. basic medium) were removed at timepoints of 48 hours and 7, 14, 21, 28 and 35 days after plating. Coverslips were washed and fixed with paraformaldehyde, stained with phalloidin-Alexa 568 as above, and imaged for GFP and Alexa 568 fluorescence using a 10x objective. Six morphological cell types were defined as outlined in Table 1. A cellular process was defined as a thin extension that was more than two cell body widths long (Dehmelt and Halpain, 2004). Seven random fields of view were selected from each experiment, and every cell in each field was categorized. Three separate experiments were performed and counted for a total of 7080 cells. Classification and counting of cells in each field was performed independently by two trained observers. Cell body appearance and the length, number and complexity of processes were assessed. This was correlated with the migratory and process growth dynamics of cells with the same morphology captured during time lapse acquisitions. Cell counting data for each experiment were collected in Microsoft Excel. Graphs were generated and statistics (mean, standard errors and two-way ANOVA) were calculated from the entire data set using GraphPad Prism.

Imaging

Epifluorescent and transmitted light images were collected with a Zeiss AxioObserver using 10x and 20x LD Plan Neofluar air-interface objectives. The microscope was equipped with a motorized stage and an incubation chamber that allowed maintenance of 37°C, 5% CO₂ for the duration of time lapse experiments. Image collection was performed with AxioVision 4.6.3 software and Z-projections were generated using default settings. Brightness and contrast manipulations were applied only to entire images using ImageJ (NIH, Bethesda, MD). Figures were constructed using Adobe Illustrator CS3 on a Macintosh computer.

Immunoblot

PBD-MAPC spheroids were plated on poly-lysine coated cover slips in neural differentiation medium as described above and cultured for 10 to 20 days. The medium was removed and the cells were washed twice with PBS. Cells were then collected in SDS-PAGE buffer (2% sodium dodecyl sulfate, 6M urea, 62.6mM Tris-HCl, pH6.8, 160mM dithiothreitol, 0.005% Bromophenol Blue). Primordial PBD-MAPC spheroids were washed twice in PBS, pelleted by centrifugation and resuspended in SDS-PAGE buffer. Cell solutions were heated for 15min at 72°C and stored at -20°C. Proteins were separated on a precast 4–20% gradient SDS-PAGE in MOPS-SDS running buffer (Invitrogen), transferred to Immobilon-P PVDF membrane (Millipore) in transfer buffer (0.7M glycine, 25mM Tris) and blocked with 7.5% skim milk plus 5% NGS in Tris buffered saline (50mM Tris-HCl, 0.2M NaCl, pH7.4) with 0.1% Tween (TBST). Membranes were then probed with the appropriate antibody (see above) at 1:1000 in TBST/7.5% milk/1%NGS overnight at room temperature. Membranes were washed 10 × 5min in TBST and probed with appropriate goat secondary antibodies conjugated to horseradish peroxidase at 1:5000 in TBST/7.5% milk/1%NGS and washed again 10 × 5min in TBST. Membranes were developed with chemiluminescent reagent (GE Healthcare), and exposed to film (Kodak). Membranes were then stained with Coomassie (3mM Brilliant Blue G-250 in v/v 45.4% MeOH, 9.2% Glacial Acetic Acid), destained (v/v 30% MeOH, 10% Glacial Acetic Acid) and air dried. Developed film and stained membranes were scanned at 2400dpi to generate digital images. Gel analysis software (ImageJ) was used to measure the intensity of immunoblot bands of

interest from the luminogram and the intensity of a common constitutively expressed protein band in the corresponding lane of the Coomassie stained PVDF membrane. Immunoblot band intensity was normalized to the control band and 2-tailed t-tests were used to compare expression in primordial and neurally differentiated cells for each marker. Each marker was tested on multiple samples of protein extracted from primordial or neurally differentiated cells in at least three separate experiments.

PCR

Neurally differentiated PBD-MAPCs were grown on coverslips as described for immunoblot (above). After 10–16 days they were dissociated from coverslips using 0.05% trypsin-EDTA (Gibco), washed with PBS and collected by centrifugation. Total RNA was isolated using Trizol (Invitrogen) followed by RNeasy (Qiagen) and cDNA was synthesized using Superscript III (Invitrogen). PCR products were amplified with Taq polymerase (Invitrogen) using porcine-specific primers (Table 1) designed with Primer-BLAST (NCBI) based on cDNA sequences archived at NCBI. When available, porcine gDNA sequences were consulted (ensembl.org) to ensure placement of primer sets across at least one intron-exon junction. The primers used to amplify β -actin were commercially available human primers (Stratagene) and homology between human and porcine sequence was confirmed using NCBI-BLAST, the forward primer differs in two bases between the two species while the reverse primer is a perfect match. All primer sets were tested on cDNA synthesized from fresh pig brain as a positive control. PCR products were separated on precast 8% Tris-Bis acrylamide gels (Invitrogen), stained with ethidium bromide and visualized with a digital UV imaging system (FluorChem, Alpha-Innotech).

Electrophysiology

Neurally differentiated PBD-MAPCs were grown for 11–20 days on poly-lysine coated cover slips in neural differentiation medium. Cells were visualized using an inverted microscope (Nikon Diaphot) equipped with Hoffman modulation contrast optics. Tight seal whole cell patch clamp recordings were obtained from morphologically identified cells from 8 separate experiments. Cells were bathed in medium containing 150mM NaCl, 4.5mM KCl, 2mM CaCl₂, 1mM MgCl₂, 10mM glucose, 5mM sodium HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), pH7.3. Patch electrodes (3–4 M Ω) were filled with 140mM KCl, 10mM sodium HEPES, 2mM MgCl₂, 0.1mM EGTA (Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid), 4mM Na₂ATP, and 0.2mM NaGTP, adjusted to 290mOsm, pH7.2. Recordings were performed using an Axopatch 2B (Axon Instruments), with electrode and cell capacitances compensated. For the 23 cells included in this study, series resistances averaged 11.0 ± 1.3 (mean $\pm$ one standard error of the mean) and were not compensated. Signals were low pass filtered (2 kHz), then digitized 10–50kHz (National Instruments) and stored on a personal computer using the WinWCP (Strathclyde Electrophysiology Software, John Dempster, University of Strathclyde, http://spider.science.strath.ac.uk/sipbs/software_ses.htm). Holding potentials were set to between –80 and –100mV, and were not corrected for the liquid junction potential, which was estimated to be < 1mV. Voltage-dependent inward and outward currents were elicited by stepping from the holding potential to a range of potentials up to +20mV in 10 or 20mV increments (150ms duration). Voltage-dependent inactivation was assessed by using a pre-pulse protocol where cells were stepped from the holding potential to potentials between –100 and –20mV (10mV increment, 4s duration) and then to –20mV. Depolarizing voltage steps were given at intervals of 25–30s, and leak current subtraction was done using a P/4 protocol. Initial analysis and measurement of membrane currents was done using the WinWCP program, with further analysis and curve fitting done in using the Gnumeric (<http://www.gnome.org/projects/gnumeric/>) and Origin (OriginLab) programs. Unless otherwise indicated, values are reported as mean $\pm$ one standard error of the mean.

Cells were accepted for quantitative analysis of voltage-dependent activation and inactivation only if holding current remained constant and series resistance remained stable during the stimulation protocol.

Results

Maintenance and renewal of progenitor cells

Primordial PBD-MAPCs were maintained as non-adherent spherical clusters of cells that appeared to be held tightly together by a membranous structure of extracellular matrix (Price et al., 2006). They grew by radial expansion with spheroids reaching diameters of more than 600µm before exhibiting signs of necrosis in the center of the aggregate (Figure 1A-E). Cells within the clusters were round and appeared morphologically undifferentiated. Cultures could be expanded by manual cutting or disrupting spheroids with collagenase/dispase, and have been maintained in healthy cultures through more than 200 cell doublings. PBD-MAPCs were isolated from transgenic GFP⁺ swine and all cells within a spheroid express GFP, albeit at variable levels (Figure 1F). Cells within spheroids labeled with antibodies against the embryonic stem cell marker NANOG (Figure 1G) and the neural stem cell marker nestin (Figure 1H).

Neural morphologies in differentiated PBD-MAPCs

When primordial PBD-MAPCs were plated on poly-L-lysine coated glass coverslips in neural differentiation medium, they quickly adhered and dispersed, sprouting processes exceeding 100µm in length and migrating away from the settled spheroids (Figure 2A), this process usually began within 3–4 hours. Cells developed a variety of different morphologies (Figure 2B) and could be classified into six cell types (Table 2): unipolar, bipolar, tripolar, polar, complex and undifferentiated (Figure 2B1-6, respectively). The proportion of cells within a culture acquiring a specific morphology was consistent between separate experiments (Figure 2C). Unipolar, bipolar, tripolar and polar cells exhibited a very neural morphology with long, thin neurite-like processes extending out from the cell body (Figure 2B1-4). The proportion of these four neural-like cell types in the population increased steadily between 48 hours and 2 weeks after plating and was then maintained up to 5 weeks after plating (Figure 2C). The increase in the proportion of cells exhibiting these neural-like morphotypes between 48 hours and 5 weeks reached statistical significance ($p < 0.05$, two-way ANOVA) for all but the tripolar cell group; the proportion of these followed the same trend but these were the least numerous of cell types. The total number of cells exhibiting one or more long neural-like process (ie. uni-, bi-, tri- or polar cells) was $21.5 \pm 3.04\%$ (mean $\pm$ SD) at 48 hours after plating and $77.6 \pm 13.74\%$ at 5 weeks after culture in neural differentiation medium. When the neural differentiation medium was replaced with basic medium lacking signaling molecules and Matrigel™ at 9 days after plating, the proportions of unipolar, bipolar and tripolar cells did not change significantly (Figure 2C1, $p > 0.05$ for each cell type, two-way ANOVA) but the proportion of cells exhibiting a polar morphology was somewhat reduced at 4 and 5 weeks after plating in basic medium (Figure 2C2, $p < 0.05$). Even considering this small decrease, the total proportion of cells exhibiting the four neural-like morphologies at 5 weeks in culture with basic media after day 9 was $68.0 \pm 7.27\%$. The increase in cells with neural-like morphologies came mainly at the expense of cells exhibiting a complex morphology (Figure 2C2); these had multiple short processes and appeared to be an intermediate morphotype in the process of neural differentiation (Figure 2B5). Complex cells made up $67.9 \pm 5.62\%$ of the population 48 hours after plating, but the proportion of this cell type was reduced to $17.2 \pm 6.20\%$ at 5 weeks in neural differentiation medium and $27.2 \pm 7.05\%$ at 5 weeks when cultured in basic medium after day 9. Significantly, at 48 hours after plating, only $10.5 \pm 2.99\%$ of cells remained undifferentiated (appearing as individual round cells, Figure 2B6). This number was reduced to nearly zero

after 1 week of culture and was then maintained at low levels for the duration of the experiment, regardless of whether the cells were maintained in neural differentiation or basic media (Figure 2C2). Throughout the experiment, no cells with fibroblast-like or other non-neural morphologies were observed (Figure 2A, B).

Initial neural differentiation of PBD-MAPCs depends only on extracellular matrix cues

PBD-MAPCs readily assumed neural morphologies in a basic medium without added growth factors or cytokines. When cells were plated on a thin layer of Matrigel in basic medium, they settled, migrated and extended processes just as they did in neural differentiation medium (which contains Matrigel) (Figure 3A). Similarly, cells developed neural morphologies on coverslips coated with laminin or fibronectin (Figure 3B, C) in basic medium, although these extracellular matrices resulted in a modest increase in the proportion of undifferentiated cells. In addition, processes of cells grown on laminin or fibronectin were generally shorter than those of cells grown on Matrigel. Neural-like differentiation was specific to these particular extracellular matrices, because collagen or poly-L-lysine alone were not sufficient to initiate neural differentiation of PBD-MAPCs in basic medium (data not shown).

Neural molecular phenotypes of differentiated PBD-MAPCs

The thin neural-like processes on differentiated PBD-MAPCs were rich in filamentous actin (f-actin; Figure 4A) and often exhibited growth cone-like structures (Figure 4B). Neurally differentiated PBD-MAPCs labeled for a variety of neural markers via immunocytochemistry. Most cells expressed the general neural markers β -tubulin III and NeuN (Figure 4C & D). These neuronal markers were expressed by unipolar, bipolar, tripolar, polar and complex cells with great frequency and reproducibility in neurally differentiated cultures. In addition, lower numbers of cells were observed expressing the presynaptic marker syntaxin (Figure 4E). Similarly, GFAP (astrocyte marker), acetylcholine transferase (cholinergic neurons), tyrosine hydroxylase (dopaminergic marker), synaptophysin (presynaptic marker), nestin (neural precursors) and O4 (oligodendrocytes) were expressed by small numbers of cells (data not shown and Price et al., 2006).

Neurally differentiated PBD-MAPCs co-express neural proteins in culture

The early neuronal protein β -tubulin III was expressed in a large proportion of cells after culture in neural differentiation medium (Figure 4B), as was the neuronal nuclear marker NeuN (Figure 4C). Double labeling immunocytochemistry demonstrated that these neural markers were colocalized to individual cells with neural morphologies (Figure 5A). In addition, the neural marker Map2ab also colocalized with β -tubulin III in neurally differentiated PBD-MAPCs (Figure 5B) as did the synaptic proteins syntaxin and synaptophysin (Figure 5C,D).

Immunoblot and RT-PCR analysis suggest a predisposition towards neural differentiation in primordial PBD-MAPCs

We performed immunoblot analysis of total protein isolated from primordial and neurally differentiated PBD-MAPCs. NeuN (MW 46–48kDa) and syntaxin (MW 35kDa), markers of mature neurons, were present in primordial PBD-MAPCs but increase after neural differentiation (Figure 6A,B) although this increase reached significance only for syntaxin (Figure 6D). Interestingly, β -tubulin III (MW 55kDa), a marker of immature neurons, was present at slightly higher levels in primordial PBD-MAPCs than in neurally differentiated cells (Figure 6C, D).

RT-PCR analysis of cDNA synthesized from primordial and neurally differentiated PBD-MAPC cultures (Figure 6E) showed expression of a voltage gated sodium channel ($\text{Nav} 1.2$) and the neural protein, microtubule associated protein 2 (Map2). The voltage gated potassium channel $\text{Kv}1.5$ and a modifying beta subunit of voltage gated potassium channels ($\text{Kv}1\text{b}$) were also expressed in primordial and neurally differentiated cells. Amplification of β -actin, a housekeeping gene used as a positive PCR control, from the same templates demonstrated equal input of cDNA template for PCR reactions.

Neurally differentiated PBD-MAPCs exhibit typically neural behaviors

PBD-MAPCs were plated in neural differentiation medium on poly-lysine coated coverslips and observed by time lapse microscopy as they differentiated, migrated and interacted with one another. The long neural-like processes observed in immunocytochemistry were seen to grow out from the cell body and lead migration of the cell (Figure 7A & B). The cells used these processes to investigate the substrate and neighboring cells, extending, retracting and changing direction in a series of migratory and exploratory behaviors (Figure 7C & D) interspersed with nonmotile periods. An 18 hour time lapse video of Figure 7C is available as supplemental online video 1. Each of the six morphologically defined cell types (Figure 2) exhibited unique migratory behaviors (Table 2). Unipolar, bipolar and tripolar cells appeared to be terminally differentiated; over a period of 48 hours they elongated or retracted existing processes but they did not undergo drastic morphological restructuring. Unipolar cells were often motile (14 of 18 cells), with the cell body following a leading process. Bipolar cells also traveled via a leading process (4 of 11) and sometimes reverted to unipolar morphology, while all four tripolar cells observed were stationary. Polar and complex cells, in contrast, were very plastic in their morphology. These cell types extended and retracted processes frequently, undergoing significant morphological restructuring over a 48 hour time period. Half of the 27 polar cells observed were actively migrating, either by following a leading process or via amoeboid movement while retracting and extending multiple processes. Complex cells spent most of their time extending and retracting short processes. In addition, over the course of 48 hours, all cell types often interacted with one another (Figure 7C & D). These interactions varied in duration, ranging from short investigations to established and maintained connections. Cells were observed to follow other cells, and to migrate directly along other cells' processes. Some migrating cells also appeared to follow along paths left by the retracting processes or migrating cell bodies of other cells (Figure 7D).

Voltage-dependent inward and outward currents

We made an initial qualitative assessment of the presence of voltage-dependent inward and outward currents from cells grown in neural differentiation conditions. Recordings were obtained from unipolar, bipolar, tripolar, and polar cells. Voltage-dependent outward currents (Figure 8A) were present in 22 out of 23 cells examined (Figure 8C), with no significant differences among the 4 cell types examined ($\chi^2 = 2.13$, $p > 0.35$). Voltage-dependent inward currents (Figure 8B) were observed less commonly (12/23 cells). While these currents were most common in unipolar cells and least common in bipolar cells (Figure 8D), there were no significant differences among the 4 cell types in the fraction of cells showing voltage-dependent inward currents ($\chi^2 = 1.52$, $p > 0.60$).

Voltage-dependent activation of inward and outward currents was measured from a holding potential of -100mV , and stepping to potentials between -90 and $+20\text{mV}$. Peak inward and outward currents were measured at each potential and averaged (Figure 8E). Inward currents began to activate around -70mV , peaked at -30mV , and declined at more positive potentials, consistent with reduced ion driving force. Outward currents appeared at around -60mV and increased steadily with increasing depolarization. Inward currents showed

strong voltage-dependent inactivation. Inactivation was quantified using a pre-pulse protocol (Figure 8F, inset), with inward current measured at -20mV , and normalized to maximum current obtained with no depolarizing pre-pulse. Averaged normalized inward current was fit with a Boltzmann equation: $I/I_{\text{max}} = 1/(1+\exp((V_m - V_{1/2})/k))$ (Figure 8F), yielding a value for $V_{1/2}$ (the potential at which the current is half-inactivated) of -77.3mV .

Discussion

We have identified a novel, readily accessible source of adult stem cells isolated from the peripheral blood of a large mammal (Price et al., 2006). When maintained in medium containing LIF and other factors to prevent differentiation, PBD-MAPCs continued to divide in spheroids that regenerated after mechanical or enzymatic passaging. The appearance of these structures in primordial medium and their behavior when plated in neural differentiation medium is very reminiscent of embryoid bodies formed by human embryonic stem cells (Itskovitz-Eldor et al., 2000) and neurospheres derived from neural stem cells (Ahmed, 2009; Reynolds and Weiss, 1992). The expression of the embryonic stem cell marker NANOG and the neural stem cell marker nestin is also consistent with other populations of undifferentiated adult derived stem cells (Huang et al., 2009). Significantly, neither maintenance of progenitor cells, nor neural differentiation, requires the addition of feeder cells, and progenitor cells grow unattached in non-tissue-culture treated vessels. PBD-MAPCs retain their primordial state through more than 200 cell doublings and appear to have formed stable cell lines. Although they grow slowly, these cells are ideal candidates for therapies due to their ability to be scaled up in suspension culture. In addition, they are derived from peripheral blood that can be easily collected repeatedly from the same subject with minimal discomfort. Finally, the potential for neural differentiation is not lost with expansion and passage, as has been reported for some neural progenitor cell lines (Anderson et al., 2007). These characteristics potentially allow for the isolation of a large number of stem cells from a single individual and make this cell population very attractive as a possible source for autologous cell transplants. It is also important to note that once PBD-MAPCs are seeded into an environment conducive for differentiation, they no longer appear to divide. Although they actively undergo dramatic morphological and migratory changes, they do not revert to an undifferentiated state even when neurogenic signaling molecules are removed from the media. This further supports the use of PBD-MAPCs in therapies due to the lower risk of unwanted cell growth and tumorigenesis.

A high proportion of PBD-MAPCs readily differentiate toward neural morphologies including the extension of at least one long, thin cellular process, within 48 hours. This occurs even in the absence of signaling molecules, as long as the cells are provided with an appropriate extracellular matrix such as laminin. Interestingly, cells attach to the extracellular matrix and begin to extend processes very rapidly (within hours) after plating. This suggests that the primordial cells contain all of the signal transduction machinery required for the initial phases of neural differentiation (attachment, migration, process formation). Neural differentiation occurred only on Matrigel (a laminin-based matrix), laminin, and fibronectin, suggesting that the integrin family of cell adhesion molecules is involved in signaling that leads to adhesion, migration and neurite extension. A similar role for integrin signaling has been described for neural stem cells and mature adult neurons (Campos, 2005; Harper et al., 2009; Plantman et al., 2008). Matrix composition, particularly laminin, is critical in driving neural differentiation of progenitors isolated from adult blood (Jose and Krishnan, 2010) and embryonic and neural stem cells (Zhang et al., 2010). For PBD-MAPCs, matrigel was superior to laminin or fibronectin alone in inducing neural morphologies, indicating that a combination of extracellular matrix molecules and/or trace levels of cytokines enhance differentiation efficiency. Because primordial PBD-MAPCs also express nestin and neural markers, this suggests that undifferentiated PBD-MAPCs may be

predisposed towards a neural lineage, and that interactions with the extracellular matrix provide sufficient signal for initial differentiation.

Using time lapse microscopy in combination with histological techniques, it became evident that the processes of neurally differentiated PBD-MAPCs fulfill all four criteria defining a neurite (Dehmelt and Halpain, 2004). First, the processes are thin protrusions, at least as long as the cell body, which grow out from the cell body in a series of starts and stops, changing direction and reversing as though searching for appropriate chemoattractant cues. Second, the processes contained microtubulin (β -tubulin III) and, third, ended in growth cones that were rich in f-actin. Finally, these cellular behaviors were consistent with active processes of dynamic neurite outgrowth, not cytoskeleton collapse and cytoplasm shrinkage that has been implicated in the artifactual formation of cells with a neuron-like morphology from bone marrow derived stem cells (Croft and Przyborski, 2006; Lu et al., 2004; Neuhuber et al., 2004). Interestingly, development of neural morphology by PBD-MAPCs appears to achieve a steady state after 2 weeks in culture, as there was little change in the proportion of cell types represented or their behavior over three additional weeks in culture. Cells also did not appreciably increase in number following plating. We suggest that the extracellular matrix is sufficient to initiate the genesis of processes, but that additional factors are required to specify and guide axonogenesis, dendritogenesis and development of specialized neural identities. Significantly, while these signals are required to initiate neural differentiation, they do not appear to be important for maintaining the differentiated state as the proportion of cell types does not change if signaling molecules are removed on the ninth day of culture.

Approximately 50% of PBD-MAPCs achieve the terminally differentiated morphology and behavior characterized by unipolar, bipolar and tripolar cell shapes after 5 weeks in culture. The remaining cells develop polar or complex morphologies reminiscent of neural progenitors. Based on their strikingly neuronal behaviors, their complex morphologies and their expression of neuronal markers such as β -tubulin and NeuN, in addition to synaptic proteins and active membrane properties, these cell types appear to have differentiated towards a neural lineage. It is likely that the polar and complex morphotypes are primed for a chemical, electrical or structural signal to complete their differentiation program. A similar state of incomplete differentiation has been described in neurally induced bone marrow derived mesenchymal stem cells which expressed neural markers but were not physiologically active (Trzaska et al., 2009; Trzaska et al., 2007). Future identification of these terminal differentiation signals may allow directed differentiation of a large proportion of PBD-MAPCs to a specific phenotype.

We suggest that our efficient neural differentiation protocol induces an initial step in neural differentiation and results in cells waiting for additional signals to finalize their phenotype. This idea is supported by our finding that a large proportion of the cells in neurally differentiated cultures label reproducibly for general neural markers such as β -tubulin III and NeuN. In contrast, the proportion of cells labeling for synaptic proteins, indicating functional neurons, are similar to what has been reported in the literature, a few percent, with the exception of complex and lengthy protocols designed to develop a specific cell type. Significantly, individual cells co-express multiple neural markers, suggesting a coordinated development of neural phenotype. Neurally differentiated PBD-MAPCs label very infrequently for markers of astrocytes or oligodendrocytes, indicating that the cells are bound for neural and not glial fates. Since glial expansion is a common unfavorable side effect of stem cell transplantation, the lack of any appreciable astrocytic lineage in PBD-MAPCs, especially in pre-terminally differentiated cells of polar or complex morphologies, bodes well for their therapeutic use.

Neurally differentiated PBD-MAPCs exhibit remarkably dynamic migrations and cell-cell interactions. In fact, their process extensions, migration, exploration, and cell-cell interrogations are indistinguishable from that behavior observed *ex vivo* in stem cells from the subventricular zone (Kim et al., 2009). This provides strong evidence supporting the identification of these cells as neural precursors; they exhibit chain migration like developing neurons and, in many instances, cells are observed migrating along another cell's process, identical to mechanisms seen in the rostral migratory stream (Doetsch and Alvarez-Buylla, 1996; Wichterle et al., 1997). In addition, cells often appear to follow paths left by other migrating cells suggesting that PBD-MAPCs, like neuronal precursors in the rostral migratory stream (Bovetti et al., 2007), modify the extracellular matrix, perhaps with matrix metalloproteases, as they migrate.

The signature of a true neuron is demonstration of active membrane properties (reviewed in Lu and Tuszynski, 2005), and we performed whole-cell patch clamp analysis of neurally differentiated PBD-MAPCs to begin investigation of their functional properties. We observed voltage dependent outward currents in almost all cells, regardless of morphology. The outward current was slowly-inactivating, consistent with potassium currents required for fast repolarization of action potentials (Schaarschmidt et al., 2009b). Neuronal excitability is regulated by differential expression of potassium channels with specific kinetics; immature proliferating human neural progenitor cells express fast-inactivating A-type voltage-gated potassium channels (K_V) and upregulate delayed-rectifying channels, such as those that could mediate the outward currents observed in our cells, upon neural differentiation (Schaarschmidt et al., 2009b). Accordingly, RT-PCR analysis indicated that neurally differentiated PBD-MAPCs express $K_V1.5$, a delayed-rectifying channel responsible for maintaining membrane potential and modulating excitability in CNS neurons (Gutman et al., 2005). The outward currents we recorded from PBD-MAPCs had variable slow inactivation properties suggesting that $K_V1.5$ is one of a mixture of potassium channels expressed by these neurally differentiated cells. K_V1b is a beta subunit that associates with, and modulates, members of K_V1 and K_V2 families (Gutman et al., 2005), including $K_V1.5$ (Nakahira et al., 1996). This modulator is specifically expressed in the nervous system (Rettig et al., 1994) and functions to accelerate the inactivation rate of potassium currents (Nakahira et al., 1996). K_V1b is expressed in PBD-MAPCs and could contribute to slowing the inactivation kinetics of the outward current. The current-voltage dependency (IV curve) and amplitude of the outward current recorded from neurally differentiated PBD-MAPCs is comparable to pharmacologically identified delayed rectifier potassium currents recorded from neurally differentiated human neural progenitor cells (Schaarschmidt et al., 2009a) and human adipose tissue derived stem cells (Jang et al., 2010).

Approximately half of the neurally differentiated cells we recorded from exhibited fast voltage-dependent inward currents with activation and inactivation kinetics characteristic of sodium currents. Using RT-PCR we detected expression of the $Na_V1.2$ voltage gated sodium channel normally localized to the CNS (Goldin et al., 2000). Neurally differentiated adult mesenchymal stem cells similarly expressed genes for various voltage gated ion channels but, upon further examination, were found to be unable to fire action potentials and judged immature (Trzaska et al., 2007). The IV curve of these mesenchymal stem cells, however, was shifted very far to the right compared to the inward current we recorded, and had a lower amplitude. In addition, raw current traces from the differentiated mesenchymal cells did not show a prominent fast activating inward current like the ones we recorded from PBD-MAPCs. In contrast, the IV curve and amplitude of the inward currents we recorded are very comparable to those recorded from neurally differentiated stem cells derived from adipose tissue (Jang et al., 2010). These currents were identified as sodium currents and the cells were capable of firing tetrodotoxin-sensitive action potentials. Further electrophysiological studies incorporating tetrodotoxin, TEA, and other pharmacological

agents will help identify the individual channel subtypes expressed by PBD-MAPCs and direct further RT-PCR studies. In addition, neurally differentiated PBD-MAPCs vary greatly in cell size which may result in large variations in current densities between cells. Normalization to cell size and more detailed physiological studies of each morphological cell type will shed further light on the differentiation status and program of these cells. Data from these experiments may present additional modulatory targets to drive differentiation towards specific terminal neural phenotypes.

Future studies will focus on the translation of the findings reported here to *in vivo* applications in the context of PBD-MAPC grafts to the nervous system. Several stem cell populations from non-neural sources survive and differentiate in models of neural degeneration or injury (Biernaskie et al., 2007; Bouchez et al., 2008; Marchesi et al., 2007; Wernig et al., 2008; Yang et al., 2008). Given their high apparent neurogenic potential, PBD-MAPC transplants may prove effective in transplant therapies for neurodegenerative conditions such as Parkinson's disease or for spinal cord or peripheral nerve injury. In addition, the finding that only exposure to extracellular matrix molecules common to neural tissue is required to initiate neural differentiation of PBD-MAPCs suggests that primordial cells may potentially be successfully transplanted without pre-differentiation treatments. Our results indicate that transplanted primordial PBD-MAPCs could differentiate towards neural phenotypes in response to cues from endogenous extracellular matrix molecules.

Swine are considered to be physiologically quite similar to humans (Halperin, 2001) suggesting that a population of stem cells homologous to PBD-MAPCs might be isolated from human peripheral blood. Such a resource would be ideal for development in autologous transplant therapy as blood can be easily and repetitively collected from the same donor with little discomfort. Alternatively, the genetic engineering of human immunocompatible swine is currently of great interest due to the limited availability of human organs for transplant operations (Halperin, 2001; Petersen et al., 2008). Were such swine developed, the PBD-MAPCs isolated from their blood could provide stem cells for xenotransplant therapies.

Summary

Stem cells isolated from adult animals have the potential to function in autologous cell therapies of neural disorders without the complications associated with immunorejection and immunosuppression. Here, we show that a population of regenerating multipotent progenitor cells isolated from the peripheral blood of a large mammal have high neurogenic potential and may be predisposed towards differentiation down a neural lineage. These cells potentially represent an easily accessible, renewable source of cells that can be driven towards neural fates easily and with high fidelity in preparation for use in cell therapies of the nervous system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DAPI	4',6-diamidino-2-phenylindole
GFP	green fluorescent protein
NeuN	Neuronal Nuclei specific protein
PBD-MAPCs	peripheral blood derived multipotent adult progenitor cells

Figure 1.

Progenitor PBD-MAPCs grew as non-adherent spheroids in primordial media and expressed stem cell markers. (A-E): Transmission light micrographs of spheroids of varied sizes. (F): Epifluorescent micrograph superimposed on transmission light image of spheroid showing GFP expression in individual cells. (G): GFP⁺ (green) spheroid immunolabeled for NANOG (red) and nuclei (DAPI, blue). (H): GFP⁺ (green) spheroid immunolabeled for nestin (red) and nuclei (DAPI, blue). Scale bar 100µm.

Figure 2.

PBD-MAPCs irreversibly developed neural morphologies in vitro. (A): When plated in neural differentiation conditions, spheroids (asterisks) settled and cells actively migrate in a radial fashion, developing long processes. (B): All cells in such cultures could be categorized into one of six morphological classes: unipolar (B1), bipolar (B2), tripolar (B3), polar (B4), complex (B5) and undifferentiated (B6). (C): Cells were plated in neural differentiation medium and either maintained in this medium or switched to basic medium containing no matrigel or signaling molecules after the first 9 days of culture (vertical arrow on each panel). (C1): The proportion of cells acquiring unipolar, bipolar and tripolar morphologies increased until a steady state was attained at approximately two weeks after

plating in neural differentiation medium (filled symbols, solid lines). This pattern of differentiation was unchanged in populations that received only basic medium after 9 days in culture (open symbols, dashed lines). (C2): The proportion of cells developing a polar morphology increased for approximately two weeks and then held steady until 5 weeks after plating. Conversely, the proportion of cells with complex morphology decreased dramatically for the first two weeks and was then maintained. Approximately 10% of cells exhibited an undifferentiated morphology at 48 hours after plating and this number dropped to, and was maintained at, less than 5% until five weeks after plating. The patterns of differentiation were the same when neural differentiation conditions were maintained (filled symbols, solid lines) and when basic medium replaced neural differentiation medium after 9 days in culture (open symbols, dashed lines). A total of 7080 cells were counted in duplicate by two blind observers in three separate experiments. Scale bars A, 500 μ m; B, 50 μ m.

Figure 3. PBD-MAPCs developed neural morphologies when provided only with extracellular matrix and basic medium containing no growth factors. (A): GFP⁺ PBD-MAPCs adhered, migrated, and extended lengthy processes on Matrigel in basic medium. (B&C): PBD-MAPCs developed neural morphologies on laminin (B) and fibronectin (C). Green: GFP, red: f-actin, blue: nuclei. Scale bars, 100 μ m.

Figure 4.

PBD-MAPCs developed neural phenotypes *in vitro*. (A): GFP⁺ (green) cells extended thin processes rich in f-actin as visualized with phalloidin-Alexa 568 (red). (B): Higher magnification of box in A. Neurites often ended in growth cone-like structures. (C&D): Many GFP⁺ cells expressed the general neuronal markers β -tubulin III and NeuN, though some cells in the same field did not (arrowheads). (E): Subsets of cells labeled for syntaxin, a marker of more specialized cells. For C-E, left panel: GFP (green), middle panel: immunolabel for indicated neural markers (red), right panel: merged images. Blue in C is the nuclear marker, TO-PRO-3 iodide. Scale bars A, 100 μ m; B, 20 μ m, inset 5 μ m, C-E 50 μ m.

Figure 5.

Individual neurally differentiated PBD-MAPCs were immunopositive for multiple neuronal markers. After plating in neural medium, PBD-MAPCs co-expressed the neural marker protein, β -tubulin III (blue) and other neural markers (red) including (A) NeuN, (B) Map2ab, (C) syntaxin or (D) synaptophysin. Green is GFP. Merged images (right column) show co-localization of both markers in individual cells. Scale bars 50 μ m.

Figure 6.

Primordial and neurally differentiated PBD-MAPCs contained protein and mRNA for neural markers. (A-C): Immunoblot analysis demonstrated that PBD-MAPCs grown in neural differentiation conditions (N) contained higher levels of the neural proteins NeuN (A) and syntaxin (B) than primordial PBD-MAPC spheroids (P). However, primordial and neurally differentiated PBD-MAPCs expressed comparable levels of the early neural marker β-tubulin III (C). Coomassie staining (bottom) of membranes after visualization of immunopositive signals confirmed equal loading of lanes. (D): Quantitation of immunoblot data. The intensity of each immunopositive band was determined using ImageJ and normalized to the intensity of a constitutively expressed control band in the corresponding lane of the Coomassie stained membrane (double arrowhead in A-C). T-tests were used to determine if expression of a marker significantly differed between primordial and neurally differentiated cell samples (* $p < 0.05$). The normalized intensity of the band obtained from primordial cells was set to equal 1 for each marker for graphing purposes. The number of bands analyzed for each sample is indicated at the base of each bar. (E): Primordial (P) and neurally differentiated (N) PBD-MAPCs expressed voltage gated sodium and potassium channels, Na_v1.2 and of K_v1.5, in addition to the neural markers Map2 and K_v1b, as determined by RT-PCR. Amplification of β-actin, a housekeeping gene, confirmed equal amount of template input to PCR reactions.

Figure 7.

Neurally differentiated PBD-MAPCs exhibited distinctively neuronal behaviors *in vitro*.

(A): Neurite-like projections (arrow) grew out from the cell body of a polar cell and retracted as the cell investigated its surroundings. (B): A unipolar/bipolar cell (arrow) extended a projection (arrowhead) and then engaged in leading process migration. (C): A bipolar cell (filled arrow) migrated until it made contact with another cell (arrowhead) at which time it retracted its processes. A complex cell (open arrow) was highly active, extending and retracting numerous short processes while moving in an apparently random trajectory. A small number of cells (~5%) apparently underwent apoptosis (white asterisk). (D): One cell followed another (filled arrows) until they were contacted by a third cell (open arrow). The following cell of the pair extended a process towards the newcomer and after an interaction lasting several hours, all three cells traveled in different directions. Numbers in top right of panels indicate hours elapsed after the first image in the series was taken. Scale bars, 50µm.

Figure 8.

Neurally differentiated PBD-MAPCs exhibited voltage-dependent currents. (A): Typical recording illustrating a cell with voltage-dependent outward currents, but no apparent inward current. The cell was held at -80mV and stepped to -60 , -40 , -20 , 0 , and $+20\text{mV}$ for 150ms . The cell in this example was classified as a bipolar cell. (B): Typical recording illustrating a cell with voltage-dependent inward and outward currents. The voltage protocol used is the same as in A; in this example, the recording was from a polar cell. A total of 23 cells were included in this analysis: 4 unipolar, 8 bipolar, 5 tripolar, and 6 polar. Cells were assessed for the presence of inward and outward current by visual inspection of current recordings, as indicated in A and B. (C): With the exception of one polar cell, all cells showed voltage-dependent outward currents. (D): Voltage-dependent inward currents were observed less frequently, with unipolar cells most likely to possess this current, and bipolar cells least likely, however, these differences were not statistically significant. All cells with voltage-dependent inward currents also possessed voltage-dependent outward currents. No cells with only inward currents were observed. (E): The voltage-dependence of activation was assessed by holding cells at -100mV , and stepping to potentials between -90 and $+20\text{mV}$, as illustrated in the inset. Peak inward (open circles) and peak outward (filled squares) currents were measured and averaged across cells ($n=9$; 2 unipolar, 1 bipolar, 3 tripolar, 3 polar), then plotted against command potential. (F): Voltage-dependence of inactivation of inward currents was quantified by holding cells at -100mV and measuring peak inward current at -20 following a 4s pre-pulse to potentials between -100 and -20mV (see inset). Currents were normalized relative to maximal inward current at the pre-pulse potential of -100mV , and averaged across cells ($n=3$, one each of bipolar, tripolar, and polar). The smoothed curve represents the Boltzmann equation obtained by fitting to the averaged, normalized currents (see text).

Table 1
Porcine primers for reverse transcription-polymerase chain reaction

Porcine-specific primer sets used in RT-PCR. The gene name, gene symbol for ion channels, NCBI accession number, primer sequences, annealing temperature, number of PCR cycles and amplicon size are given. Each primer set was tested on cDNA synthesized from fresh pig brain as a positive control.

Gene	Accession number	5' DNA sequence 3'	Temp # cycle	Product size (bp)
NaV 1.2 (SCN2A)	XM_003133444.1	F: TGG AGA CAG GAT CCA CTG CCT CG R: TCA TAG GAA GGT GGA GAT GTG GTG G	52° 35 cyc	384
Map2	XM_003133628.1	F: GGT GGG GCA GGG GAA GGA CT R: TCC GAG GCT GGT GAT GGG GG	59° 40 cyc	329
KV 1.5 (KCNA5)	AB042525.1	F: CCC ACA GTG GCG CCT CTC CT R: CCG GAG GAT GGC CAG CGA CA	66° 35 cyc	276
KV 1b (KCNA1)	NM_001105294.1	F: CTG GCG GCT CAT CTT GG GT R: GCG ACC GTG CGG AAT TTG GC	61° 35 cyc	596
GFAP	XM_003131329.1	F: GGC TGG ATC TGG AGA GGA AGA TCG A R: AGC TGG CGC CGG TAG TCG TT	53° 35 cyc	319
β- actin	Human (Stratagene)	F:TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA R: CTA GAA GCA TTT GCG CTG GAC GAT GGA GGG	60° 35 cyc	661

Table 2
PBD-MAPCs differentiate to specific neural phenotypes *in vitro*

Neurally differentiated PBD-MAPCs can be classified into six morphological cell types. Cells of each type exhibited typical differentiation and motility behaviors as captured by time lapse microscopy. The number of cells observed for behavior through a 48 hour time course are indicated in parentheses.

Cell Type	Morphology	Behavior
Unipolar	one long process; smooth cell body	elongation of process; leading process migration (18)
Bipolar	two long processes on opposite sides of a smooth cell body	elongation of processes; leading process migration (11)
Tripolar	three long processes on a triangular cell body	fixed morphology; immobile (4)
Polar	one or more long processes; irregular cell body	extension and retraction of multiple processes; highly motile (27)
Complex	multiple short processes with hairy appearance	extension and retraction of multiple processes (25)
Undifferentiated	round without distinct morphology	differentiation into another cell type (above); motile (2)