Thymidine Analogs Are Transferred from Prelabeled Donor to Host Cells in the Central Nervous System After Transplantation: A Word of Caution

TERRY C. BURNS,^{a,b,c} XILMA R. ORTIZ-GONZÁLEZ,^{b,c} MARÍA GUTIÉRREZ-PÉREZ,^d C. DIRK KEENE,^{b,c} ROHIT SHARDA,^b ZACHARY L. DEMOREST,^b YUEHUA JIANG,^a MOLLY NELSON-HOLTE,^a MARIO SORIANO,^c YASUSHI NAKAGAWA,^{a,c,f} MARÍA ROSARIO LUQUIN,^g JOSE MANUEL GARCIA-VERDUGO,^e FELIPE PRÓSPER,^d WALTER C. LOW,^{a,b,c,f} CATHERINE M. VERFAILLIE^{a,c}

^aStem Cell Institute, ^bDepartment of Neurosurgery, ^cGraduate Program in Neuroscience, University of Minnesota, Minneapolis, Minnesota, USA; ^dClínica Universitaria, Universidad de Navarra, Pamplona, Spain; ^eDepartment of Cell Biology, Instituto Cavanilles and Centro de Investigacion Principe Felipe, University of Valencia, Valencia, Spain; ^fDepartment of Neuroscience, University of Minnesota, Minneapolis, Minnesota, USA; ^gDivision of Neurosciences, Centre for Applied Medical Research, Pamplona, Spain

Key Words. Adult bone marrow stem cells • Label • Bromodeoxyuridine • Thymidine analog • Control • Transplantation
Neural differentiation • In vivo tracking

ABSTRACT

Thymidine analogs, including bromodeoxyuridine, chlorodeoxyuridine, iododeoxyuridine, and tritiated thymidine, label dividing cells by incorporating into DNA during S phase of cell division and are widely employed to identify cells transplanted into the central nervous system. However, the potential for transfer of thymidine analogs from grafted cells to dividing host cells has not been thoroughly tested. We here demonstrate that graft-derived thymidine analogs can become incorporated into host neural precursors and glia. Large numbers of labeled

neurons and glia were found 3–12 weeks after transplantation of thymidine analog-labeled live stem cells, suggesting differentiation of grafted cells. Remarkably, however, similar results were obtained after transplantation of dead cells or labeled fibroblasts. Our findings reveal for the first time that thymidine analog labeling may not be a reliable means of identifying transplanted cells, particularly in highly proliferative environments such as the developing, neurogenic, or injured brain. STEM CELLS 2006;24:1121–1127

INTRODUCTION

Thymidine analogs have been used to label dividing cells in the central nervous system (CNS) for several decades [1, 2]. Thymidine analogs have also been used extensively to label cells prior to transplantation, thus allowing the fate of cells transplanted into the CNS to be determined [1, 3, 4]. As such, thymidine analogs have been used as a label to follow the migration and differentiation of stem cells, including neural stem cells [5, 6] and embryonic stem cells [7, 8], into neurons and glia after transplantation into the developing [8] and adult [4] brain.

During the past 6 years, several studies have indicated that marrow stromal cells or mesenchymal stem cells (MSCs) grafted into the brain of mice and rats can also differentiate in a region-specific manner into neurons and/or glia, suggesting greater lineage differentiation ability than was previously

thought possible [9–12]. We have also shown that multipotent adult progenitor cells (MAPCs), another stem cell population isolated from bone marrow, can contribute to the brain of mice in a region-specific manner when introduced in the blastocyst [13, 14]. We wished to determine whether MAPCs could also engraft and differentiate in the brains of postnatal animals.

MATERIALS AND METHODS

Cell Isolation Labeling and Preparation

MAPCs [13] and fibroblasts were isolated from mice expressing enhanced green fluorescent protein (GFP) under the β -actin promoter [15], ROSA26 mice (Jackson Laboratory, Bar Harbor, ME, <http://www.jax.org>), and Sprague-Dawley rats (Harlan, Indianapolis, IN, <http://www.harlan.com>). Rat MAPCs were transfected with lentivirus-GFP, and stable expression was

Correspondence: Catherine M. Verfaillie, M.D., Stem Cell Institute, Department of Medicine, University of Minnesota, 420 Delaware Street, Minneapolis, Minnesota 55455, USA. Telephone: 612-625-0602; Fax: 612-624-2436; e-mail: verfa001@umn.edu Received September 22, 2005; accepted for publication December 5, 2005; first published in STEM CELLS EXPRESS December 22, 2005. ©AlphaMed Press 1066-5099/2006/\$20.00/0 doi: 10.1634/stemcells.2005-0463

demonstrated for >35 passages. Ten micromolar bromodeoxyuridine (BrdU) or chlorodeoxyuridine (CldU) was added to the culture media 2 days prior to transplantation. Cells were washed, trypsinized, centrifuged, and rewashed prior to transplantation or in vitro coculture.

Neonatal Transplantation

Animal studies were performed in accordance with guidelines set forth by the Institutional Animal Care and Use Committee at the University of Minnesota. Two microliters containing approximately 100,000 cells were stereotactically transplanted into the brain of P5 neonatal animals as described [16]. Briefly, animals were anesthetized by cooling under 10 cm of wet ice for 1 minute per gram, then fixed in a Kopf hypothermic neonatal frame (David Kopf Instruments, Tujunga, CA, <http://www.kopfstruments.com>). Cells were injected using a 26-gauge needle attached to a 10- μ l Hamilton syringe (Hamilton, Reno, NV, <http://www.hamiltoncompany.com>). Coordinates (in mm) were as follows: i.c.v., +1.0 anteroposterior (AP), $\pm$ 1.2 medio-lateral (ML), -2.1 dorsoventral (DV) from bregma ($n = 35$ in multiple experiments including live and dead LacZ and GFP MAPCs); cerebellum, -1.0 AP, $\pm$ 1 ML, -3.0 DV from lambda ($n = 2$ LacZ MAPCs).

Transuterine Transplantation

Two microliters containing approximately 50,000 BrdU-labeled fibroblasts were injected into the lateral brain ventricles of embryos (E14.5) of one pregnant CD-1 mouse using a Harvard programmable injector (Harvard Apparatus, Holliston, MA, <http://www.harvardapparatus.com>) and flexible 30-gauge Hamilton needle.

Transplantation into the Subventricular Zone and Rostral Migratory Stream

Dead fibroblasts (150,000 in 2 μ l) were transplanted into the subventricular zone (SVZ) (right hemisphere) and rostral migratory stream (RMS) (left hemisphere) of three adult (7–8 weeks old) CD-1 mice (six grafts total) using the following coordinates: RMS: +2.2 AP, $\pm$ 1.1 ML, -3.2 DV; SVZ: +1.3 AP, $\pm$ 1.2 ML, -3.0 DV (mm relative to bregma). The olfactory bulb ipsilateral to each graft was analyzed at 3 weeks to assess the presence of graft-derived BrdU in newly born olfactory bulb neurons.

Transplantation into Ischemic Rat Brain

Permanent middle cerebral artery occlusion was performed in two 180–220 g spontaneously hypertensive rats. BrdU-labeled GFP fibroblasts (75,000 in 3 μ l) were stereotactically transplanted into the surrounding penumbra at each of three locations. The coordinates used were as follows: 1) 1.0 AP, 2.0 ML, -1.2 DV; 2) -3.0 AP, 1.5 ML, -1.2 DV; 3) -6.0 AP, 2.0 ML, -1.2 DV [17]. Animals were sacrificed 7 days after transplantation.

Striatal Transplantation of $^3\text{H-T}$ -Labeled Cells

GFP rat multipotent adult progenitor cells (rMAPCs) were cultured in the presence of 0.20 mCi/ml of tritiated thymidine (TRA120; GE Healthcare Life Sciences—Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K., <http://www.amershambiosciences.com>) for 20 hours prior to cell preparation. Twenty-two normal adult Sprague-Dawley female rats

(250 g) were anaesthetized, and 200,000 cells in 8 μ l were injected unilaterally into striatum using coordinates -0.2 AP, +2.6 ML, and -5.4 DV from bregma.

Controls

To control for the possibility that thymidine analog from transplanted cells may be incorporated into dividing host cells, cells were prepared as described but freeze-thawed three to four times on dry ice or liquid nitrogen immediately before transplantation. Absence of cell viability was confirmed by trypan blue inclusion and by replating in cell culture. Supernatant from transplanted cells ($n = 8$) was removed following the final centrifugation during cell preparation and injected as a negative control for labeled cells. Cells and supernatant were injected in parallel using the following coordinates: i.c.v., 0.0 AP, $\pm$ 1.0 ML, -2.1 DV or +1.0 AP, $\pm$ 1.0 ML, -2.1 DV (from bregma).

In Vitro Transfer

Eight hundred thousand tritiated thymidine ($^3\text{H-T}$)-labeled rMAPCs were cocultured in 12-well cell culture inserts (0.4 μ m pore size) for 2 days to determine whether unlabeled cells could take up $^3\text{H-T}$ released from labeled cells in vitro. Three variations of $^3\text{H-T}$ -labeled rMAPCs were used for coculture: live cells, cells killed by repeated freeze-thaw (liquid nitrogen), and dead cells subsequently treated with DNase (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) for 30 minutes at room temperature before coculture. Growth medium alone and growth medium with 0.20 μ Ci/ml $^3\text{H-T}$ were used as negative and positive controls for $^3\text{H-T}$ uptake, respectively. Cells were fixed and processed for thymidine autoradiography after 2 days. Similar results were obtained using BrdU-labeled fibroblasts (data not shown).

Immunohistochemistry

Animals were transcardially perfused with phosphate-buffered saline followed by 4% formaldehyde. Brains were postfixed for 24 hours and then transferred to 30% sucrose until they sank. Forty- to fifty-micrometer floating sections were processed using BrdU pretreatment as described [4]. Primary antibodies were GFP (1:1,000; Molecular Probes Inc., Eugene, OR, <http://probes.invitrogen.com>); neuron-specific nuclear protein (NeuN) (1:100; Chemicon, Temecula, CA, <http://www.chemicon.com>); nestin 401 (1:50; Chemicon); BrdU (1:250; Accurate; Accurate Chemical and Scientific, Westbury, NY, <http://www.accuratechemical.com>); BrdU (1:50; BD Pharmingen, San Diego, <http://www.bdbiosciences.com/pharmingen>), which recognized iododeoxyuridine (IdU) for double labeling of CldU with IdU [18]; glial fibrillary acidic protein (1:500; DAKO, Glostrup, Denmark, <http://www.dako.com>); and 2',3'-cyclic nucleotide 3'-phosphodiesterase (1:500; Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>). Secondary antibodies were coupled to Cy3, Cy5, fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, PA, <http://www.jacksonimmuno.com>), Alexa 488, Alexa 555, and Alexa 647 (Molecular Probes). The Tyramide Signal Amplification Kit (Molecular Probes) was used according to the manufacturer's directions for identification of GFP in animals injected with GFP-rMAPCs. Images were obtained via fluorescence and confocal microscopy and processed

using Adobe Photoshop. Confocal single optical sections were approximately 0.4 μm .

Electron Microscopy and [Methyl- ^3H]-Thymidine Autoradiography

Sagittal 200-mm sections were cut on a Vibratome tissue slicer (Leica Microsystems Nussloch GmbH, Heidelberg, Germany, <http://www.leica-microsystems.com>). The sections were post-fixed in 2% osmium for 2 hours, rinsed, dehydrated, and embedded in Araldite (Durcupan; Fluka BioChemika, Sigma-Aldrich). Serial 1.5- μm -thick semithin sections were cut with a knife and mounted onto slides, dipped in autoradiographic emulsion (Kodak NTB2), exposed for 4 weeks at 4°C, developed in Kodak D-19, and counterstained with 1% toluidine blue. A cell was considered labeled if 10 or more silver grains overlaid the nucleus and the same cell was labeled in three adjacent sections. Seventy ^3H -T-labeled cells identified in the semithin sections were selected for electron microscopic examination. Semithin sections were glued (Super Glue) to Araldite blocks and detached from the glass slide by repeated freezing (in liquid nitrogen) and thawing. The block with the flat semithin section was mounted in the ultramicrotome. Ultrathin sections were cut with a diamond knife and examined under a Jeol JEM 1010 electron microscope to determine which cell types incorporated ^3H -T [19].

Cell Counting and Image Analysis

^3H -T-Labeled cells were counted in representative semithin sections along the mediolateral axis in areas of 100 μm wide, up to 700 μm from the centre of the graft. Consistently in all the sections, width of the graft area was approximately 200 μm in total. Eight hundred four and six hundred sixty-three ^3H -T cells were counted in representative slices at 48 hours and 7 days postinjection, respectively. A cell was considered labeled if 10 or more silver grains overlaid the nucleus in three adjacent sections. Quantification of the percentage of GFP-labeled cells at various distances from the graft area was achieved via image analysis of GFP fluorescence using analysis FIVE image analysis software (Olympus Europa GmbH, Hamburg, Germany, <http://www.olympus.com>). Percentages were calculated referring to the total area of green fluorescence in each slice. Representative sections from three different animals of each group (48 hours and 7 days) were studied.

Quantitative Polymerase Chain Reaction

Analysis for the presence of graft transgene in host brain was performed using quantitative polymerase chain reaction (PCR) [13]. Briefly, quantitative PCR was carried out on genomic DNA in Taqman SYBR green universal mix PCR buffer using an ABI PRISM 770 (PerkinElmer Life Sciences, Boston, <http://www.perkinelmer.com>). Primer sequences are available upon request.

RESULTS

CldU-Labeled Neurons and Glia and Neural Precursors Are Found After Neonatal Transplantation of CldU-MAPCs

We transplanted MAPCs derived from ROSA26 mice labeled with CldU into the neonatal mouse brain. One to twelve weeks

Figure 1. CldU-labeled neurons, glia, and neural precursors were found after neonatal transplantation of CldU-MAPCs. (A): Widespread CldU labeling near ventricular area 12 weeks after i.c.v. injection. (B): CldU-labeled cells in the subventricular zone (1 week). Arrow: CldU-labeled cell co-expressing GFAP and nestin. (C): CldU-labeled cells in the olfactory bulb (5 weeks). Arrows: colocalization of CldU and NeuN. (D): CldU-labeled neurons (NeuN) and astrocytes (GFAP) in the dentate gyrus (12 weeks). Arrows: colocalization of CldU and NeuN; arrowheads: colocalization of CldU and GFAP. (E, F): CldU-labeled astrocytes and oligodendrocytes (CNPase) in corpus callosum (3 weeks). Arrows: colocalization of CldU and CNPase; arrowheads: colocalization of CldU and GFAP. Confocal series projections (A, C, E) and single optical section (B, D, F). Scale bars: 100 μm (A); 20 μm (B–F). Abbreviations: CldU, chlorodeoxyuridine; CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; GFAP, glial fibrillary acidic protein; NeuN, neuron-specific nuclear protein.

after transplantation of MAPCs into the lateral ventricle, large numbers of CldU-labeled cells were observed in the ventricular area (Fig. 1A, 1B), olfactory bulb (Fig. 1C), dentate gyrus (Fig. 1D), corpus callosum, and cortical areas surrounding the needle tract (Fig. 1E, 1F). Labeled cells were found to express region-appropriate markers, with mostly NeuN+ cells present in the olfactory bulb and the granule cell layer of the dentate gyrus, glia in white matter tracts, and nestin-positive cells in the SVZ. One week after transplantation, numerous CldU+ cells were visible within the RMS (supplemental online Fig. 1). Labeled cells present in the olfactory bulb did not express NeuN 1 week after transplantation (supplemental online Fig. 1) but did after

week 3, consistent with progressive migration and differentiation of the CldU-labeled cells. Transplantation of MAPCs into the neonatal cerebellum also resulted in numerous labeled granule, but not Purkinje, cells in parallel with normal neonatal neurogenesis of cerebellar granule cells (supplemental online Fig. 2). Continued proliferation of CldU-labeled cells was evidenced by colabeling with Ki67 at 7 days, and IdU [18] for up 12 weeks after transplantation (supplemental online Fig. 3).

BrdU and CldU Are Transferred from Donor to Host Cells in the Developing Brain

To independently test the donor identity of the CldU-labeled cells, we performed LacZ immunohistochemistry. To our surprise, no LacZ-positive cells could be identified by X-gal staining or by use of an antibody against β -galactosidase. We also performed similar experiments with MAPCs derived from GFP mice, and although rare GFP⁺ cells could be found in the ventricles three weeks after transplantation of CldU-labeled GFP-MAPCs (supplemental online Fig. 4), no GFP⁺ cells were detected in the brain parenchyma, where, again, large numbers of CldU-labeled neural cells could be detected. Because transgene expression can be lost or reduced after transplantation, we

also evaluated the brain tissue for the presence of transgene by PCR [13], which again yielded negative results. These findings suggested that the CldU-labeled cells were not of donor origin.

We next performed transplantations using CldU- or BrdU-labeled ROSA26-MAPCs that had been killed via repeated freeze-thaw. After transplantation of dead cells, we again found extensive labeling of neurons and glia with both BrdU and CldU (Fig. 2A). Transplantation of BrdU-labeled fibroblasts into E14.5 mouse embryos further yielded BrdU-labeled neurons not only in the olfactory bulb and hippocampus but also in the cortex (Fig. 2B), consistent with the prenatal cortical neurogenesis.

Although cells were washed multiple times prior to transplantation, we hypothesized that traces of extracellular CldU or BrdU remaining in the cell suspension could be responsible for the host labeling observed. We thus transplanted supernatant from cell preparation in parallel with cells ($n = 8$). BrdU labeling was not observed in any animals injected with supernatant (Fig. 2C), although substantial labeling was observed in all neonatal animals injected with labeled cells. To further verify this result, we directly injected 2 μ L of 20 μ M BrdU into two other animals; this concentration is double the *in vitro* BrdU concentration used to label cells for transplantation. Although

Figure 2. BrdU and CldU were transferred from donor to host cells in the developing brain. (A): Labeling of neurons (NeuN) in the olfactory bulb (left and right) and astrocytes (GFAP) in the corpus callosum (middle) 3 weeks after transplantation of dead CldU- or BrdU-labeled cells. (B): BrdU labeling in cortical, hippocampal, and olfactory bulb neurons in E14.5-injected mice (week 3). (C): BrdU labeling in the dentate gyrus after transplantation of BrdU-labeled cells but not supernatant. Direct infusion of 20 μ M BrdU produced minimal labeling relative to transplantation of labeled cells. All panels are confocal single optical sections. Scale bar: 20 μ m. Abbreviations: BrdU, bromodeoxyuridine; CldU, chlorodeoxyuridine; GFAP, glial fibrillary acidic protein; MAPC, multipotent adult progenitor cell; NeuN, neuron-specific nuclear protein.

some host cells were labeled via this technique, the amount of labeling was minimal compared with that obtained after injection of labeled cells (Fig. 2C).

Thymidine Analogs Are Transferred from Donor to Host Cells in the Adult Brain

We next tested whether or not transfer of thymidine analogs from donor to host cells may also occur in adult animals,

where proliferation of host cells occurs at a lower rate. Forty-eight hours and 7 days after transplantation of ^3H -T-labeled GFP rMAPCs into normal striatum, GFP-positive cells could be identified in the needle tract. These cells possessed characteristic MSC-like morphology by electron microscopy (Fig. 3B). No neuronal or glial markers were observed in these cells (data not shown). Only 5%–7% of the total number of GFP-positive cells were found outside of the

Figure 3. Thymidine analogs were transferred from donor to host cells in the adult brain. (A–J): ^3H -T-labeled GFP-rMAPCs transplanted into adult rat striatum. (A, F): GFP-rMAPCs remained confined to GA. Scale bars: 200 μm ; 20 μm (inset in A); 50 μm (inset in C). (B, G): ^3H -T-labeled cells were widely distributed up to and beyond 0.6 mm from GA (arrows). Scale bars: 100 μm . (C, H): number of ^3H -T and GFP cells in slice surrounding graft. (D–J): Ultrastructure of ^3H -T-labeled cells. Arrows in insets correspond to nuclei lettered in electron micrographs. Lower inset in E, intermediate filaments in astrocyte. Scale bars: 2 μm (D, E, I, J); 10 μm (insets); 0.2 μm (lower inset in E). (K): Astrocytosis surrounding cortical infarct. Top inset: GFP fibroblasts confined to GA; lower detail: colocalization of BrdU with astrocytes. Arrowheads indicate colocalization of BrdU with GFAP. Scale bars: 100 μm ; 20 μm (insets). (L, M): BrdU labeling in RMS (L) and olfactory bulb (M) 3 weeks after adult RMS injection of dead BrdU-labeled fibroblasts. Inset: colocalization of BrdU with NeuN. Arrowheads indicate colocalization of BrdU with NeuN. Scale bars: 200 μm ; 20 μm (inset). Abbreviations: BMC, donor-derived bone marrow cell; BrdU, bromodeoxyuridine; E, endothelium; GA, graft area; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; ^3H -T, tritiated thymidine; IF, intermediate filament; Mg, microglia; NeuN, neuron-specific nuclear protein; rMAPC, rat multipotent adult progenitor cell.

Figure 4. Labeled cells contained sufficient $^3\text{H-T}$ to label proliferating cells in vitro. (A, B): Negative and positive controls for autoradiography. (C–E): Previously unlabeled rMAPCs grown in noncontact coculture with $^3\text{H-T}$ -labeled rMAPCs after noncontact coculture with labeled rMAPCs. Coculture transwells contained live rMAPCs (C), dead rMAPCs (killed via repeated freeze-thaw) (D), or dead rMAPCs treated with DNase prior to coculture (E). Results shown are 2 days after start of coculture. Scale bar = 25 μm . Abbreviations: $^3\text{H-T}$, tritiated thymidine; rMAPC, rat multipotent adult progenitor cell.

graft area, all within 100 μm of the graft border (insets in Fig. 3A, 3F). By contrast, almost half of the total number of $^3\text{H-T}$ -labeled cells present in any given graft-containing slice were found outside of the graft area, at distances of up to 600 μm and beyond (Fig. 3C, 3H).

Ultrastructural analysis of individual $^3\text{H-T}$ -labeled cells revealed labeled astrocytes and microglia (Fig. 3E, 3I), consistent with their proliferative behavior in regions of injury [20]. $^3\text{H-T}$ -labeled endothelium was also found in 1 out of 8 animals analyzed (Fig. 3J). Consistent with thymidine analog labeling of glia in an area of injury, BrdU-labeled astrocytes were found after injection of BrdU-labeled GFP fibroblasts into the penumbra surrounding an infarct (Fig. 3K).

To determine whether adult-born neurons may also incorporate thymidine analogs, dead BrdU-labeled fibroblasts were injected into the SVZ or RMS of adult mice. Substantial BrdU labeling of NeuN $^{+}$ neurons in the olfactory bulb was observed after each transplant ($n = 6$; Fig. 3M), suggesting that dividing host cells may be capable of incorporating graft-derived thymidine analog label regardless of the age of the host animal, the cell type grafted, or the thymidine analog used.

Labeled Cells Contain Sufficient $^3\text{H-T}$ to Label Proliferating Cells In Vitro

Finally, to further demonstrate that the quantity of thymidine analog present within a suspension of labeled cells is sufficient to label dividing cells, $^3\text{H-T}$ -labeled cells (rMAPCs) were cocultured in vitro in transwells above unlabeled cells. A slight amount of labeling was observed in cells under transwells containing live labeled cells (Fig. 4C), with somewhat more labeling visible after coculture with dead cells (Fig. 4D). Overwhelming labeling, however, was observed if the dead cell lysate was first treated with DNase (Fig. 4E), suggesting that transfer of thymidine analogs from transplanted cells to host cells in vivo may be dependent upon endogenous DNase activity.

DISCUSSION

Although BrdU and tritiated thymidine have been used to track transplanted cells for many years [3–12, 21–25], false labeling of host neural cells has, to our knowledge, not been reported previously. It is well documented that more than 90% of cells transplanted into the CNS may die shortly after transplantation, which remains a significant limitation for neural transplantation. Consequently, there is potentially a large quantity of thymidine analog from cells progressively dying in the graft that may be processed via endogenous DNase and recycled for incorporation into dividing host cells. Many studies employing thymidine analogs as a marker of transplanted cells in the CNS do not include the controls described in this paper, such as independent confirmation of graft identity using multiple independent tracers or transplantation of dead cells or fibroblasts. However, among those that have included controls [3, 6, 8], this is the first report to our knowledge of the potential for thymidine analogs to be taken up by endogenous neural cells.

Recent studies in which nonneural cells were transplanted into the CNS have suggested that marrow stromal cells or mesenchymal stem cells may possess significantly greater plasticity than was previously realized, with the potential to differentiate into tissues from unrelated embryonic germ layers. In these studies, BrdU has been widely used as a sole marker of adult stem cells to assess their potential to integrate and differentiate in the developing or injured CNS [9–11].

Thymidine analogs are also routinely used to label dividing endogenous cells [26]. The most common fate of newly born cells in the adult brain is apoptosis [27, 28]. Whether BrdU from such apoptotic cells may again be released and thereby become available for incorporation into adjacent dividing cells has not been tested but could compromise the temporal resolution of birth date identification after single bolus injection of BrdU. Some authors have suggested that BrdU may label cells undergoing DNA repair as well as dividing cells [29], especially in the context of injury and prolonged thymidine analog administration. In the present study, however, labeling was observed in host cells undergoing normal development, as evidenced by continued proliferation and time-dependent migration and adoption of neuronal identity (supplemental online Figs. 1, 3).

CONCLUSION

In conclusion, we report here for the first time that thymidine analogs from transplanted cells are released in vivo and become

incorporated into dividing host neural cells. We suggest that interpretation of BrdU-, CldU-, or tritiated thymidine-labeled cell transplantation studies must be performed with the utmost care and that rigorous controls should be employed in the transplantation of thymidine analog-labeled cells. We suggest that, at a minimum, parallel transplantation of dead cells or labeled fibroblasts and independent confirmation of donor identity [5, 8] should be considered before drawing conclusions about the *in vivo* behavior of thymidine analog-labeled cells after transplantation.

ACKNOWLEDGMENTS

We thank Enrique Andreu, Mark Blackstad, Dominic Schomberg, and Zhenhong Nan for technical assistance. We also thank Íñigo Narvaiza who provided the Lentivirus-GFP and I. Weissman who provided the GFP mice used in this project. Support for this project was provided by an NIH Medical

Scientist Training Program grant (T.C.B., X.R.O.-G., C.D.K.), 3M (T.C.B.), University of Minnesota Biomedical Engineering Institute (T.C.B.), NIH National Research Service Award (X.R.O.-G., C.D.K.), Turner Family Fund (W.C.L.), Isaksen Fund (W.L.), Cornford Fund (W.C.L.), Cavin Fund (W.C.L.), Miller Family Fund (W.C.L.), Michael Charles Winery Fund (W.C.L.), NIH grants (C.M.V., W.C.L.), and grants from Fondo de Investigaciones Sanitarias, Gobierno de Navarra-Salud, and Fundación de Investigación Médica Mutua Madrileña del Automóvil. This work was also funded through the Unión Temporal de Empresas Project Centro de Investigación Médica Aplicada. T.C.B., X.R.O.-G., and M.G.-P. contributed equally to this work.

DISCLOSURES

The authors indicate no potential conflicts of interest.

REFERENCES

- McConnell SK. Migration and differentiation of cerebral cortical neurons after transplantation into the brains of ferrets. *Science* 1985;229:1268–1271.
- Altman J, Das GD. Post-natal origin of microneurons in the rat brain. *Nature* 1965;207:953–956.
- Shihabuddin LS, Horner PJ, Ray J et al. Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. *J Neurosci* 2000;20:8727–8735.
- Gage FH, Coates PW, Palmer TD et al. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc Natl Acad Sci U S A* 1995;92:11879–11883.
- Flax JD, Aurora S, Yang C et al. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. *Nat Biotechnol* 1998;16:1033–1039.
- Lundberg C, Martinez-Serrano A, Cattaneo E et al. Survival, integration, and differentiation of neural stem cell lines after transplantation to the adult rat striatum. *Exp Neurol* 1997;145:342–360.
- McDonald JW, Liu XZ, Qu Y et al. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat Med* 1999;5:1410–1412.
- Reubinoff BE, Itsykson P, Turetsky T et al. Neural progenitors from human embryonic stem cells. *Nat Biotechnol* 2001;19:1134–1140.
- Kopen GC, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc Natl Acad Sci U S A* 1999;96:10711–10716.
- Munoz-Elias G, Marcus AJ, Coyne TM et al. Adult bone marrow stromal cells in the embryonic brain: Engraftment, migration, differentiation, and long-term survival. *J Neurosci* 2004;24:4585–4595.
- Li Y, Chen J, Chopp M. Adult bone marrow transplantation after stroke in adult rats. *Cell Transplant* 2001;10:31–40.
- Mahmood A, Lu D, Yi L et al. Intracranial bone marrow transplantation after traumatic brain injury improving functional outcome in adult rats. *J Neurosurg* 2001;94:589–595.
- Jiang Y, Jahagirdar BN, Reinhardt RL et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002;418:41–49.
- Keene CD, Ortiz-Gonzalez XR, Jiang Y et al. Neural differentiation and incorporation of bone marrow-derived multipotent adult progenitor cells after single cell transplantation into blastocyst stage mouse embryos. *Cell Transplant* 2003;12:201–213.
- Wright DE, Cheshier SH, Wagers AJ et al. Cyclophosphamide/granulocyte colony-stimulating factor causes selective mobilization of bone marrow hematopoietic stem cells into the blood after M phase of the cell cycle. *Blood* 2001;97:2278–2285.
- Cunningham MG, McKay RD. A hypothermic miniaturized stereotaxic instrument for surgery in newborn rats. *J Neurosci Methods* 1993;47:105–114.
- Zhao LR, Duan WM, Reyes M et al. Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Exp Neurol* 2002;174:11–20.
- Maslov AY, Barone TA, Plunkett RJ et al. Neural stem cell detection, characterization, and age-related changes in the subventricular zone of mice. *J Neurosci* 2004;24:1726–1733.
- Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A. Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J Neurosci* 1997;17:5046–5061.
- Amat JA, Ishiguro H, Nakamura K et al. Phenotypic diversity and kinetics of proliferating microglia and astrocytes following cortical stab wounds. *Glia* 1996;16:368–382.
- Cao QL, Zhang YP, Howard RM et al. Pluripotent stem cells engrafted into the normal or lesioned adult rat spinal cord are restricted to a glial lineage. *Exp Neurol* 2001;167:48–58.
- Cao QL, Onifer SM, Whitemore SR. Labeling stem cells *in vitro* for identification of their differentiated phenotypes after grafting into the CNS. *Methods Mol Biol* 2002;198:307–318.
- Chen J, Li Y, Wang L et al. Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. *J Neurol Sci* 2001;189:49–57.
- Mokry J, Nemecek S. Immunohistochemical detection of cells labeled with bromodeoxyuridine after neural transplantation. *Mol Chem Neuro-pathol* 1995;25:235–245.
- Surendran S, Shihabuddin LS, Clarke J et al. Mouse neural progenitor cells differentiate into oligodendrocytes in the brain of a knockout mouse model of Canavan disease. *Brain Res Dev Brain Res* 2004;153:19–27.
- Kaplan MS, Hinds JW. Neurogenesis in the adult rat: Electron microscopic analysis of light radioautographs. *Science* 1977;197:1092–1094.
- Morshead CM, van der Kooy D. Postmitotic death is the fate of constitutively proliferating cells in the subependymal layer of the adult mouse brain. *J Neurosci* 1992;12:249–256.
- Sun W, Winseck A, Vinsant S et al. Programmed cell death of adult-generated hippocampal neurons is mediated by the proapoptotic gene Bax. *J Neurosci* 2004;24:11205–11213.
- Rakic P. Neurogenesis in adult primate neocortex: An evaluation of the evidence. *Nat Rev Neurosci* 2002;3:65–71.

See www.StemCells.com for supplemental material available online.