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ORIGINAL ARTICLE

In Vitro Maturation of Human iPSC-Derived Neuroepithelial Cells Influences Transplant Survival in the Stroke-Injured Rat Brain

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Stem cell transplantation is a promising strategy for brain tissue regeneration; yet, despite some success, cell survival following transplantation remains low. In this study, we demonstrate that cell viability is enhanced by control over maturation of neuronal precursor cells, which are delivered in an injectable blend of hyaluronan and methylcellulose. We selected three subpopulations of human neuronal precursor cells derived from a cortically specified neuroepithelial stem cell (cNESC) population based on differences in expression of multipotent and neuron-specific proteins: early-, mid-, and late-differentiated neurons. These cells were transplanted into an endothelin-1 stroke-injured rat brain and their survival and fate were investigated 1 week later. Significantly, more cells were found in the brain after transplanting early- or mid- differentiated cNESCs compared to the late-differentiated population. The mid-differentiated population also had significantly more β-III tubulin-positive cells than either the early- or late-differentiated populations. These results suggest that maturity has a significant impact on cell survival following transplantation and cells with an intermediate maturity differentiate to neurons.

Keywords: hydrogels, stroke, regenerative medicine, stem cell transplantation

Introduction

With 15 million people worldwide suffering from a stroke each year, finding a treatment is a significant clinical challenge.[1] Currently, tissue plasminogen activator is the only Food and Drug Administration-approved treatment for ischemic stroke; however, it has to be administered within 4 h to be effective and it cannot regenerate lost cells and tissue. One approach to promote recovery is cell transplantation into the injury site. These cells can be used to complement endogenous repair mechanisms and/or directly replace lost cells.[2,3] While some success has been demonstrated, most studies report a lack of cell survival, within an average range of 2–8%.[4] This poor survival is generally attributed to the hostile microenvironment of the lesion,[5–7] which limits graft cell integration and thereby survival.

To improve the efficacy of cell transplantation strategies, injectable hydrogels have emerged as cell delivery vehicles.[8] Hydrogels can be derived from extracellular matrix molecules, such as collagen and fibronectin, and can form a porous structure that fills the cavity left after stroke-induced cell death, providing a physical scaffold for transplanted cells that is permeable to oxygen and nutrients.[9,10] The physical blend of hyaluronan (HA) and methylcellulose (MC) (HAMC) has been studied for cell transplantation in rodent models of central nervous system disease and trauma[11–14] because it is bioresorbable and cytocompatible, and has tunable mechanical and chemical properties.[13,15] HAMC provides superior cell survival and distribution within the tissue when compared to conventional saline or artificial cerebrospinal fluid (aCSF), and consequently leads to improved functional recovery.[12]

Although the majority of stroke transplantation studies use stem or neural progenitor cells, a growing body of evidence suggests that an increasingly committed population, such as neuronal precursors, exhibit better survival and integration into the brain than neural stem cells.[16–19] Transplantation of

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neuronal precursors can potentially limit the amount of astrocytic differentiation, which may contribute negatively to the glial scar,[19–21] and the risk of tumor formation, which has been associated with the use of undifferentiated cells.[22,23] Furthermore, neuronal precursor cells, compared to undifferentiated progenitors, have been shown to better integrate appropriately into the existing neural circuitry for long-term functional improvement poststroke.[18,19] Despite the potential benefits of using neuronal precursors, little has been done to date to directly compare the effect of neuronal maturation from the same starting population of cells on transplant survival.

In this study, we transplanted cortically specified neuroepithelial stem cells (cNESC) previously derived from human pluripotent cells (Varga, B.V., *et al.*, under review), into the stroke-injured rat brain using the HAMC hydrogel. We chose to transplant into the injured brain rather than naive as it has been previously demonstrated that the injury environment can significantly influence the survival and phenotype of grafted cells.[6] To investigate the effect of cell maturity on survival *in vivo*, we first established three distinct states of cellular maturation in cNESCs *in vitro*. cNESCs initially expressed multipotent markers, which, after differentiation for 32 days *in vitro*, were lost in the majority of cells and replaced by expression of immature and mature neuronal markers. We describe NESCs at day 0 as early-differentiated cells, those at day 16 as mid-differentiated, and those at day 32 as late-differentiated cells. When cells corresponding to early-, mid-, and late-differentiated populations were injected into the stroke-injured brain, we observed significantly more cells present in the early- and mid-differentiated groups than the late-differentiated group and the mid-differentiated group had significantly more β-III tubulin-positive cells at 1 week post-transplantation. These findings demonstrate that cell maturity is an important consideration for transplantation studies.

Materials and Methods

In vitro *culture and differentiation of cNESCs*

Induced pluripotent stem cell-derived cortically specified neuroepithelial cells (cNESCs) were obtained and maintained in culture as previously described at a density of 100,000 cells/cm^2 (Varga, B.V., *et al.* ''Multiple signals control the full neocortical potential of human neuroepithelial stem cells,'' under review). To differentiate cNESCs, cells were dissociated from the culture plate using Accutase (Sigma-Aldrich), reseeded onto laminin-coated culture plates at a density of 30,000 cells/cm^2, and cultured with serum-free media containing 25 mL 10×DMEM/F12 with glutamine and HEPES, 25 mL Neurobasal (Gibco), 2% penicillin/streptomycin, 1 mL B27, and 50 μL bovine serum albumin (BSA) fraction V (Thermo Fischer) for up to 32 days, with half medium changes every 48 h. Cells for both *in vitro* experiments and transplantation were between passages 35 and 45.

Immunocytochemistry and quantification

At 0, 4, 8, 12, 16, 24, and 32 days of differentiation, medium was removed and cells were fixed in 4% paraformaldehyde (PFA) for 20 min and washed twice in phosphate-buffered saline (PBS). Cells were then blocked for 1 h with blocking solution (0.1% Triton-X-100 and 5% BSA in PBS) and incubated with primary antibody diluted in blocking solution overnight at 4°C. The following antibodies were used: Sox2 (1:500; Abcam 137385); Nestin (1:200; Abcam ab105389); DCX (1:500; Abcam ab18723); β-III tubulin (1:1000; Abcam ab41489); MAP2 (1:500; Sigma Aldrich m1406); and CD44 (1:200; Abcam ab97478). Cells were washed thrice for 5 min each with PBS and incubated with the secondary antibodies diluted in the blocking buffer for 2 h at room temperature. Alexa-tagged secondary antibodies (Molecular Probes; Invitrogen) were used at 1:500 dilutions: Alexa Fluor® 488 goat anti-rabbit IgG (A11034, Invitrogen), Alexa Fluor 546 goat anti-mouse IgG (A11003, Invitrogen), and Alexa Fluor 633 goat anti-chicken IgG (A21103, Invitrogen). Wells were washed thrice for 5 min each with PBS and left in PBS for imaging. Images were captured on an Olympus FV1000 laser scanning microscope. Protein expression was evaluated in five areas of each well to count cells and expressed as a percentage of total cells.

HAMC preparation

A blend of HA (1200–1900 kDa; Novamatrix, Drammen, Norway) and MC (300 kDa; Shin-Etsu, Tokyo, Japan) was used to prepare HAMC. HA and MC were dissolved in aCSF[24] at a concentration of 1% HA (w/v) and 1% MC (w/v). This 1%/1% HAMC was then mixed using a SpeedMixer (DAC 150 FVZ, Siemens) for 30 s at 3500 RPM, centrifuged, and allowed to fully dissolve at 4°C for 14 h. The gel was then mixed again using a SpeedMixer for 30 s at 3500 RPM and stored on ice.

In vitro *cNESC injection survival assay*

To determine if HAMC can reduce the number of cells lost due to injection through a Hamilton syringe, cNESCs were cultured in a maintenance medium or differentiated for 16 or 32 days and dissociated into a cell pellet. Cells were counted and ~50,000 cells/μL were suspended in an equal volume of 1 wt%/1 wt% HA/MC, resulting in a final concentration 0.5%/0.5% (w/v) HAMC or aCSF. Two microliters of this solution was injected through a Hamilton 10 μL Gastight syringe #1701 fitted with a 26G 45° beveled needle at a rate of 0.25 μL/min. The number of live cells was determined immediately following injection into a culture plate by using calcein AM and ethidium homodimer (Thermo Fisher) and capturing images on an Olympus FV1000 laser scanning microscope. Cell viability was expressed as the percentage of live cells normalized to the initial cell number.

In vitro *cNESC survival in HAMC*

To determine the influence of HAMC on cell survival in 3D culture, cNESCs were cultured in maintenance medium or differentiated for 32 days and dissociated into a cell pellet. Cells were counted and ~250 cells/μL were suspended in an equal volume of 1.5%/1.5% HAMC reconstituted with a differentiation medium [final concentration 0.75%/0.75% (w/v)]. A volume of 200 μL of cell-loaded gels was pipetted into a 16-well chamber slide. As a control, an equal number of cells were seeded onto 2D poly-D-lysine-coated wells. The number of live cells was determined after 48 h of cell culture by using calcein AM and ethidium homodimer (Thermo Fisher) and capturing images on an Olympus FV1000 laser scanning microscope with *xy* scans every 5 μm in the *z*-direction. Quantification was conducted using Imaris Bitplane and cell

viability was expressed as the percentage of live cells normalized to the initial cell number.

Animal use and stroke model

Experimental procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals and approved by the Animal Care Committee at the University of Toronto. Twenty-eight 10-week-old male Sprague-Dawley rats (Charles River) were anesthetized, the skull was shaved, and the skin cleaned and a rostral–caudal incision was made on the skull. Using sterile technique, the skull was exposed and three holes were drilled at coordinates: (1) AP 0.0, ML 3.0; (2) AP 2.3, ML 3.0, and (3) AP 0.7, ML 3.8). Animals were subjected to an endothelin-1 (Et-1, Calbiochem San Diego CA)-induced stroke in the cortex and striatum, as previously described.[25–27] An injection of 1 μL of 400 pmol/μL Et-1 was given at a rate of 0.25 μL/min through each of the drilled holes into the brain, 2.3 mm ventral from the surface of the skull for locations 1 and 2, and 7.0 mm ventral for location 3, using a 10 μL Hamilton Gastight syringe (#1701) fitted with a 26G 45° beveled needle. The needle was left in place for 3 min after Et-1 injection and then slowly withdrawn to prevent backflow. The incision was sutured closed and animals were given Ketoprofen (3 mg/kg) and saline for recovery.

Cell transplantation

Seven days following stroke surgery, cells were transplanted into the cortex of rats by adapting a previous method.[12] Cells from three groups (early, mid-, and late differentiated) were suspended in 0.5%/0.5% w/v HAMC and loaded into a Hamilton 10 μL Gastight syringe #1701 fitted with a 26G 45° beveled needle. The needle tip was inserted 2.2 mm below the surface of the skull and 2 μL of cells in HAMC was injected into two of the three previously drilled locations corresponding to cortical lesion sites: AP 0.0 mm, ML 3.0 mm; and AP 2.3 mm, ML 3.0 mm at a concentration of 170,000 cells/μL for a total of 680,000 cells per animal. The rate of injection was 0.25 μL/min and the needle was left in place for 3 min before being slowly withdrawn. Rats received daily subcutaneous injections of cyclosporine A (CsA) (15 mg/kg) for 2 days leading up to cell transplantation, and during the transplantation surgery, a subcutaneous osmotic minipump (Alzet, 2ML4) with CsA (15 mg/kg/day) was implanted in all animals. Both incisions were sutured closed and animals were given ketoprofen (3 mg/kg) and saline for recovery.

Immunohistochemistry and quantification

Rats were sacrificed 7 days later by CO_2. Brains were fixed by immersion in 4% PFA for 10 days followed by cryoprotection in 30% sucrose, as previously described.[12] Brains were flash frozen using dry ice-cooled 2-methyl-butane and serially sectioned to 30 μm using a cryostat (Leica CM3050S).

Every fourth section from each brain was used for staining and quantification. The following antibodies were used for analysis: human nuclear antigen (HuNu, 1:500; Millipore MAB1281); Ki67 (1:500; Abcam ab15580); DCX (1:500; Abcam ab18723); and β-III tubulin (1:1000; Abcam ab41489). Images were captured using an Olympus FV1000 laser scanning microscope at 20× magnification to generate 25-μm-thick z-stacks. Quantification of total cell number per brain was conducted by using Image J (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/, 1997–2017) to measure the number of HuNu-positive pixels in each brain and dividing this value by the average pixel number in one HuNu-positive cell. A validation of this method in comparison to manual counting is provided in Supplementary Figure S1 (Supplementary Data are available online at www.liebertpub.com/tea). To quantify the number of cells expressing Ki67, DCX, or β-III tubulin, cells coexpressing HuNu and the antibody of interest were counted according to the optical fractionator method with the use of Stereo Investigator v10.03 (MicroBrightField, Inc., Colchester, VT).

Statistical analysis

All data are reported as mean ± standard deviation unless otherwise indicated. Statistical analysis was performed using GraphPad Prism 5 (GraphPad, La Jolla, CA). For comparisons between *in vitro* protein expression and the *in vivo* study, a one-way ANOVA with Tukey's multiple comparisons test at 95% confidence intervals was used. Significance was set at $p < 0.05$.

Results

In vitro *characterization of cNESC differentiation*

We used a population of self-renewing cNESCs, derived from an hiPSC line (Varga, B.V., *et al.*, under review), to study the effects of cell maturity on transplant survival in the stroke-injured rat brain. These cells are cultured as a monolayer, remain highly proliferative in the presence of FGF and inhibitors of BMP, TGFβ, and GSK3 (maintenance medium) for at least 50 passages, and do not express the pluripotent factor OCT4 or ventral forebrain marker NKX2.1 (Varga, B.V., *et al.*, under review). When the growth factors are removed, these cells adopt a forebrain-specific neuroectodermal phenotype in the differentiation medium (Fig. 1A).

Cells were differentiated in culture for up to 32 days (Fig. 1A), at which point, the majority of cells expressed neuronal markers (Fig. 1B, C). At day 0, before exposure to differentiation conditions, 80% of the cells expressed the stem cell marker, Sox2, and 80% of the cells expressed Nestin. The cells exhibited a flat round morphology, with some small projections visualized with cytoskeletal Nestin staining (Fig. 1C). At day 16, no Sox2 expression was seen and only 20% of the cells expressed Nestin. At this time, the cells primarily expressed immature neuronal proteins, DCX (60%) and βIII-tubulin (40%), and some cells expressed the mature neuronal protein, MAP2 (20%). The morphology of DCX-, βIII-tubulin-, and MAP2-expressing cells showed the presence of long fine processes that extended out to other clustered groups of cells (Fig. 1C). At day 32, neither Sox2 nor Nestin expression was observed. Eighty percent of cells continued to express the immature neuronal marker βIII-tubulin and 45% expressed MAP2. The morphology of cells at day 32 was similar to that at day 16, with long processes extending from cells, many of which exhibited the expected bipolar morphology of neurons (Fig. 1C). Many cells coexpressed certain markers such as Sox2 and Nestin, and DCX and βIII-tubulin, and therefore there is overlap with the proportion of cells expressing each marker. This continuum of cell maturation observed *in vitro* allowed us to establish three cell

populations, with significantly different protein expression profiles, to investigate the effect of maturation state on transplant survival *in vivo*: (1) day 0, early differentiated cNESCs; (2) day 16, mid-differentiated neurons; and (3) day 32, late-differentiated neurons (Fig. 1D).

In vitro *cell viability following syringe injection with HAMC*

We investigated the effect of the HAMC hydrogel relative to aCSF on cNESC viability following injection through a needle. The number of viable cells immediately following injection was visualized *in vitro* and quantified for each group using a calcein AM/ethidium homodimer live/dead assay (Fig. 2A, B). Cells encapsulated in HAMC generally exhibited higher viability in comparison to aCSF after injection (Fig. 2B); however, this difference was not significant. Interestingly, more viable cells were seen in the early- and mid-differentiated groups compared to the late-differentiated group, irrespective of injection vehicle, indicating that late-differentiated cells may be more susceptible to cell death during the preparation and injection process. *In vitro* cNESC survival in a 3D HAMC gel was similar to that of cNESCs cultured on a 2D poly(D-lysine)-coated surface, demonstrating cytocompatibility of the HAMC hydrogel (Supplementary Fig. S2). Last, we investigated the expression of CD44, an important receptor for HA binding involved in cell adhesion and survival,[28] in cNESCs and their progeny. We observed that there was no CD44 expression in mid- and late-differentiated cells, and only diffuse nuclear staining in the early differentiated cells (Supplementary Fig. S3).

Cell number and fate following transplantation into the stroke-injured brain

To determine the effect of cell maturity on survival following a stroke, cNESCs were differentiated for 0, 16, or 32 days, encapsulated in HAMC and transplanted into the stroke-injured rat brain 7 days after injury (Fig. 3A). Seven days following injury was chosen for transplantation as it represents a time point in the endothelin-1 stroke model where the cavity has reached its maximum size and the environment is considered more amenable to grafted cell survival.[29] Cells were injected directly into the two cortical stroke-injured cavities in HAMC based on previous success in mice.[11] Seven days following cell transplantation (and 14 days after Et-1 stroke injury), HuNu expression was analyzed. This time frame was chosen to provide data on the survival of cNESCs as this study is the first to transplant these cells in an injury model. Although surviving cells were found in all brains, animals that were injected with early- or mid-differentiated cells had significantly more cells present than those injected with late-differentiated cells (Fig. 3B). No significant difference was seen in viability between early- and mid-differentiated cells where there were an average of 2.0×10^5 ($\pm1.2\times10^5$) cells and 2.5×10^5 ($\pm1.1\times10^5$) cells, respectively, 1 week after transplantation. This contrasts with only 0.5×10^5 ($\pm0.3\times10^5$) cells from the late-differentiation group, which is significantly different from the other groups ($p=0.0261$ and $p=0.0041$ when compared to the mid- and early-differentiated group, respectively). Of the initial number of cells injected, 30%, 35% and 7.5% were viable for the early, mid-, and late-differentiated groups, respectively. Cells were generally localized to the two areas of injection (Fig. 3C).

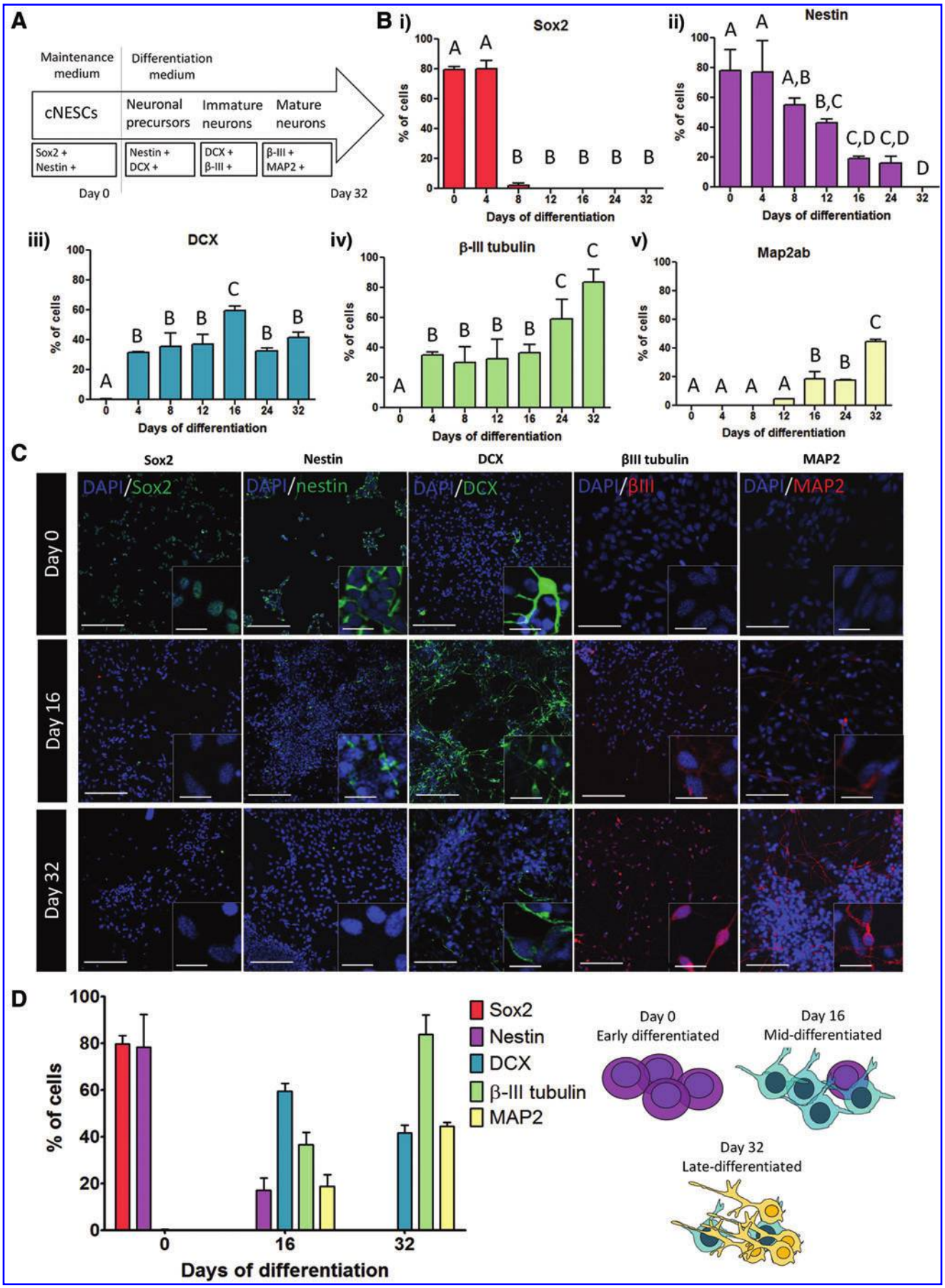
Next we aimed to determine whether the differences in viable cells after transplantation were due to survival or proliferation. Given the presence of Nestin-positive cells within the transplanted cell populations from early or mid-differentiated groups, we hypothesized that the increased numbers of viable cells would be attributed to proliferation.[30,31] To test this hypothesis, the number of proliferating cNESCs was quantified using Ki67 and HuNu co-expression. Interestingly, there was no significant difference in the percentage of double-labeled HuNu/Ki67-positive cells between the three cell groups (Fig. 4A), although there were more HuNu/Ki67+ cells in terms of absolute number in the early and mid-differentiated groups. This indicates that the difference in number of HuNu+ cells is likely attributed to survival of the initial population delivered rather than their subsequent proliferation.

It has previously been shown that mature cells do not survive as well as undifferentiated cells following transplantation.[18,32] To investigate this possibility, the phenotype of surviving transplanted cells was characterized using DCX and β-III tubulin staining. DCX-positive cells were present in transplanted cells from all groups. However, the early differentiated cNESCs had significantly more DCX-positive cells than the mid- or late-differentiated groups ($\sim$50% of the cells were DCX+ [Fig. 5A]). Similarly, β-III tubulin expression was observed in all groups; however, the transplantation of mid-differentiated cells showed a significantly higher number of β-III tubulin-positive cells compared to early- and late-differentiated cells (Fig. 5B), suggesting that cells continued to mature *in vivo* over this 7-day period. It is unlikely that the β-III tubulin-positive population survived better as the late differentiated should have had a higher number than the mid-differentiated group after transplantation. In all transplant groups, there were DCX- and β-III tubulin-double positive cells: 56% of HuNu+ cells for early differentiated; 67% for mid-differentiated; and 34% for late differentiated.

Discussion

Stem cell transplantation for stroke recovery, while a promising strategy, currently results in poor cell survival and minimal integration.[5–7] In this study, we investigated

FIG. 1. *In vitro* differentiation of cNESCs. **(A)** cNESCs were cultured in a maintenance medium and then in a differentiation medium over 32 days. **(B)** Expression of stem cell markers Sox2 and Nestin and neuronal markers DCX, β-III tubulin, and MAP2 in cNESCs ($n\geq5$ biological replicates, mean ± standard deviation plotted; *different letters* represent significance of at least $p<0.05$). **(C)** Representative images of differentiating cells at day 0, 16 and 32, stained with DAPI (*blue*), Sox2 (*green*), Nestin (*green*), DCX (*green*), β-III (*red*), and MAP2 (*red*) Scale bar = 100 μm. *Inset* scale bar = 25 μm. **(D)** Data from **(A)** isolated to compare expression of markers from early-differentiated (sox2+/nestin+/DCX–), mid-differentiated (sox2–/DCX+/ β-III tubulin+), and late-differentiated (nestin–/β-III tubulin+/MAP2+) populations. cNESC, cortically specified neuroepithelial stem cell. Color images available online at www.liebertpub.com/tea



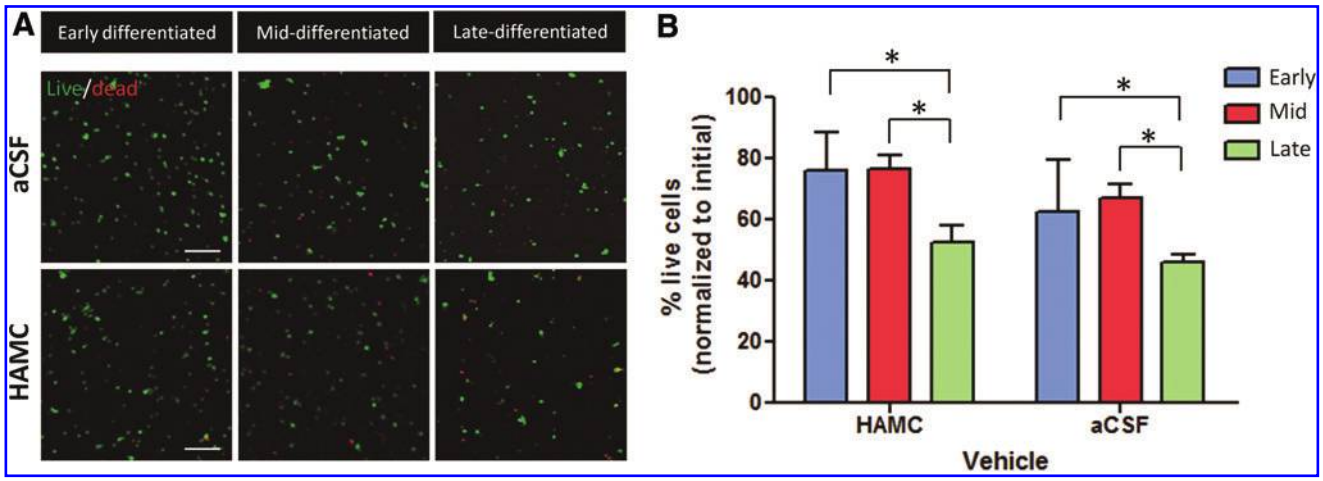


FIG. 2. Comparison of cell viability following injection in HAMC or aCSF. **(A)** Representative images of live (calcein AM, *green*) and dead (ethidium homodimer, *red*) staining of cells of different maturities immediately following injection through a Hamilton syringe. Scale bar = 100 μm. **(B)** Percentage of live cells normalized to the total number of cells injected ($n = 3$, mean ± standard deviation plotted, $^*p < 0.05$). aCSF, artificial cerebrospinal fluid; HAMC, hyaluronan (HA) and methylcellulose (MC). Color images available online at www.liebertpub.com/tea

the effect of cell maturity on survival after transplantation of cNESCs and their progeny in a hydrogel delivery system into the stroke-injured brain. By injecting all cell groups with HAMC, any differential effect of the hydrogel on survival of cells is minimized and makes it possible to draw conclusions predominantly based on the effect of cell maturity. The cells were derived from iPSCs and exhibit self-renewal and maintenance of multipotency when cultured in the presence of inhibitory factors (Varga, B.V., *et al.*, under review). Previous characterization of these cells showed that the majority are committed to a neuronal lineage, express cortical layer-specific markers, and are either glutamatergic or GABAergic (Varga, B.V., *et al.*, under review), similar to the cortical neurons that undergo cell death following stroke injury to the motor cortex.

We differentiated cNESCs over 32 days to obtain three populations with distinct maturation phenotypes, determined by their expression of Sox2, Nestin, DCX, β-III tubulin and

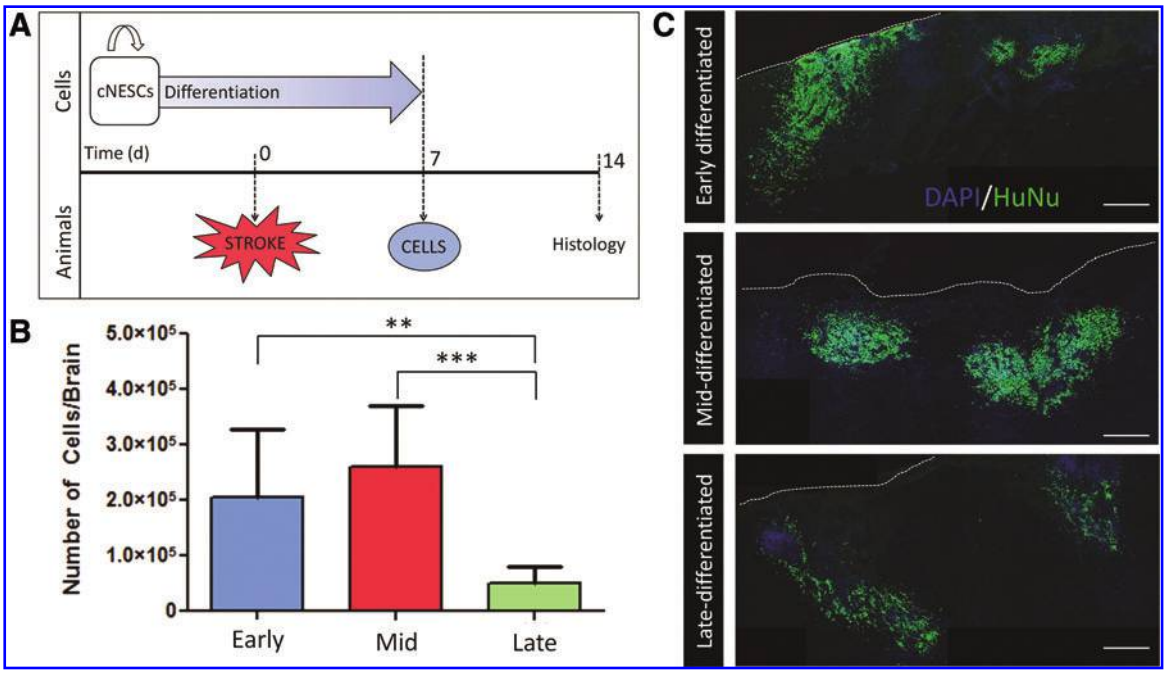


FIG. 3. Transplantation of cNESCs encapsulated in HAMC into the stroke-injured rat brain. **(A)** Study paradigm. **(B)** HuNu expression in transplanted cells ($n \geq 6$, mean ± standard deviation plotted, $^{**}p < 0.01$; $^{***}p < 0.001$). **(C)** Representative images of cells 7 days after transplantation: HuNu (*green*)/DAPI (*blue*). *Dotted line* indicates surface of the brain. Scale bar = 500 μm, *inset* scale bar = 50 μm. HuNu, human nuclear antigen. Color images available online at www.liebertpub.com/tea

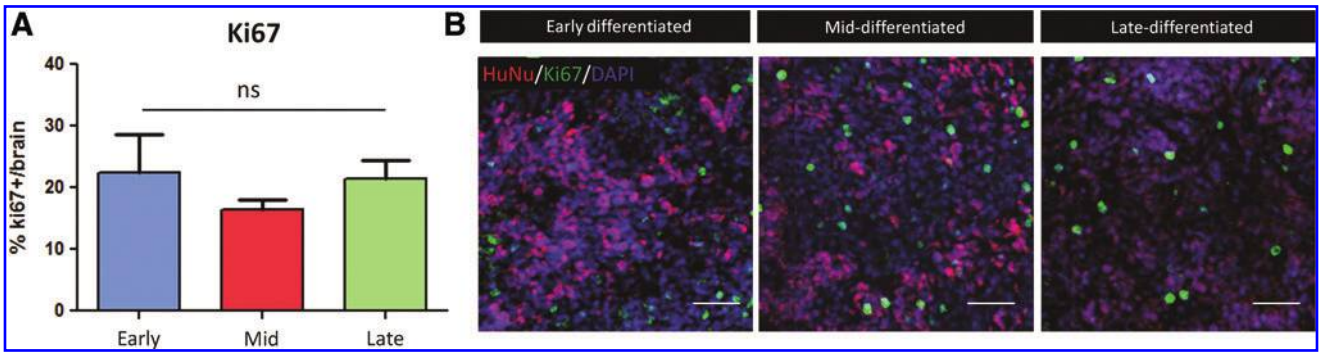


FIG. 4. Proliferation of cNESCs and progeny following transplantation. **(A)** Percentage of Ki67+ cells ($n \geq 6$, mean ± standard error of the mean, ns = not significant, $p > 0.05$). **(B)** Representative images of HuNu (*red*)/Ki67+ (*green*)/DAPI (*blue*) cells. Scale bar = 50 μm. Color images available online at www.liebertpub.com/tea

MAP2. This allowed us to compare the effect of cell maturity on survival of cells derived from the same starting population of cells, thereby eliminating the confounding factor of multiple cell sources.

The HAMC delivery system has previously been shown to increase cell survival[11–14] and distribution[11,12] using a range of stem and progenitor populations. Thus, for this study, HAMC was chosen as the sole delivery vehicle, allowing the role of maturity of human cNESCs to be investigated. In this study, we tested, for the first time, the effect of HAMC on cell viability immediately following injection through a fine-gauge needle and observed that cNESCs and their progeny had similar viability following injection in HAMC compared to aCSF. We hypothesize that the linear shear stress produced by flow through the needle as well as extensional flow of cells produced by the transition from the syringe to the needle during injection are reduced in HAMC, as has been demonstrated with other shear-thinning hydrogels.[33] However, the needle gauge used in this study may not have been narrow enough to generate sufficient sheer stress above the tolerance of cells to observe a beneficial effect of HAMC (Supplementary Data). Interestingly, we observed that regardless

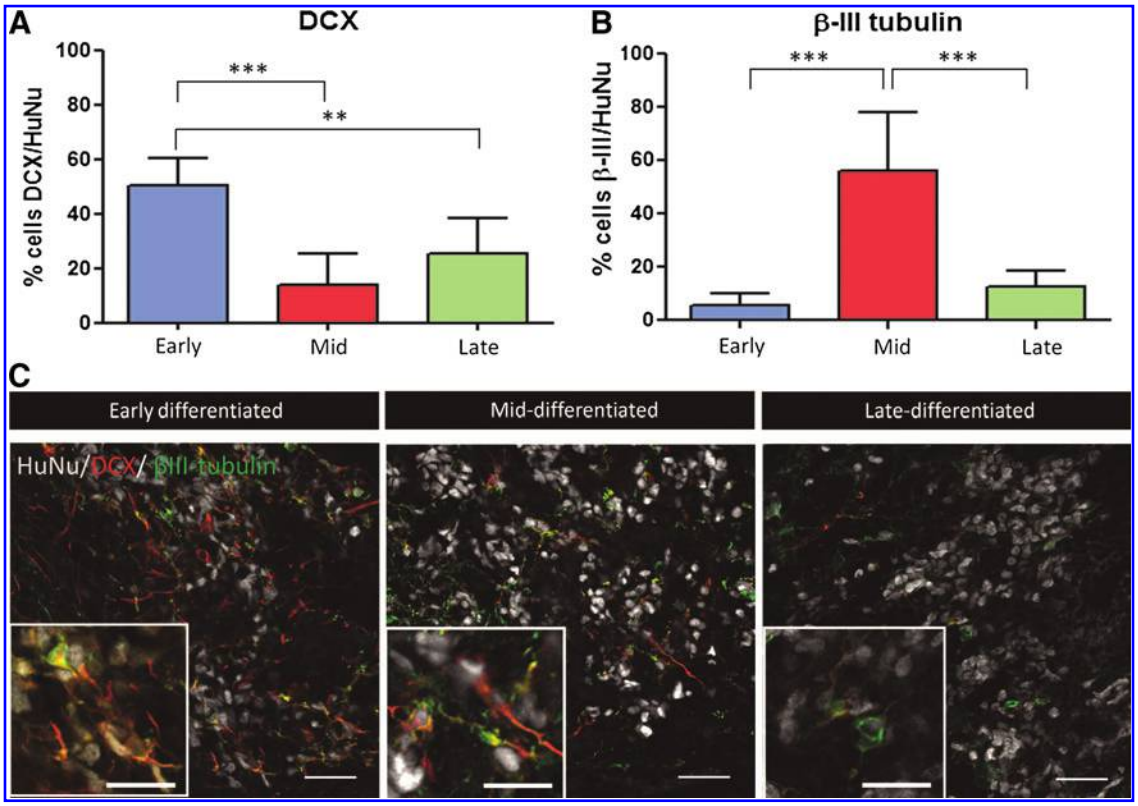


FIG. 5. Characterization of cNESCs and progeny following transplantation. **(A)** Percentage of HuNu+ cells expressing DCX, **(B)** percentage of HuNu+ cells expressing β-III tubulin ($n \geq 6$, mean ± standard error of mean, $^{**}p < 0.01$, $^{***}p < 0.001$). **(C)** Representative colocalization images of cells expressing HuNu (*white*), DCX (*red*), and β-III tubulin (*green*). Scale bar = 50 μm, *inset* scale bar = 25 μm. Color images available online at www.liebertpub.com/tea

of vehicle used, late-differentiated cNESCs exhibited lower *in vitro* cell survival following injection compared to less differentiated cell populations. With increasing maturation, susceptibility to apoptosis and damage to the cellular processes may also increase during the pretransplantation procedure, which requires dissociation and resuspension of cells, resulting in damage to cell processes that are more highly developed in mature populations.

Cells transplanted at different stages of maturity into the stroke-injured brain showed an increase in the number of cells present after 7 days in the early- and mid-differentiated groups. We hypothesize that this difference is likely not due to variability in cell proliferation as there was no difference in the proportion of cells expressing Ki67 after 1 week; however, the contribution of proliferation differences before our histological analysis on day 7 cannot be ruled out. Based on our *in vitro* injection survival data (Fig. 2), despite loading the syringe with the same number of live cells per group, only 50% of cells in the late-differentiated group survive after injection through the syringe needle, which likely contributed to the lower number seen 7 days later, regardless of proliferative potential.

While the level of Ki67 expression in all three cell groups suggests that the transplanted cells remain proliferative *in vivo* and this may affect transplant outcome, others have found comparable levels of Ki67 expression at early times after transplantation without tumor formation.[18,19] A study that transplanted human neural progenitor cells (NPCs) into the injured spinal cord found a similar increase in the number of proliferating cells initially following transplantation (6 h to 1 day time points), which then decreased in subsequent days (10–21 day time points),[34] suggesting that after the initial survival of proliferative cells, there was a decrease in proliferation as cells differentiated over time.

It is likely that the differences in cell number are a result of variation in cell survival. The lower cell viability seen in the late-differentiated group following injection through the needle may have resulted in a disproportionate increase in the number of dead cells that were delivered. Transplantation of dead cells has been shown to have an adverse effect on graft survival and behavioral outcome.[35] In addition, it has been demonstrated that HAMC can mediate a prosurvival effect through the CD44 receptor,[12] which, although found on neural stem/progenitor cells and astrocyte-restricted precursor cells, is not known to be expressed by mature cortical neurons.[36,37] We also found no evidence of CD44 expression in our cell populations (Supplementary Fig. S2).

Previous studies that have directly compared cell groups of varying maturity have reported conflicting results. Some found higher survival after transplantation into the brain when cells are predifferentiated,[16,19] while others found the opposite or no effect.[18,32] These differences may be due, in part, to differences in methodology; others injected their cells at an earlier time point poststroke (e.g., 48 h poststroke)[18] or transplanted cells derived from a different source than our own (e.g., embryonic neural progenitor cells versus iPSC-derived cells). Furthermore, contrary to our observations, Tornero *et al.* reported that there was a difference in proliferation between the immature and mature cells,[18] therefore their finding that predifferentiation results in higher cell survival may be influenced by proliferation differently. Fricker-Gates *et al.* compared two embryonic-derived progenitor groups with a more mature postnatal group and found that there were no differences in cell survival between the embryonic-derived groups, but that the postnatal group was significantly worse,[16] aligning well with our results reported in this study. This team also reported that the older (E19) embryonic-derived group had the best efficiency at creating long-distance axonal projections, similar to our observation that there was significantly more βIII-tubulin in our mid-differentiated group. To the best of our knowledge, our study is unique in investigating and testing for cell death during the injection paradigm, which, given differences in injection methodology such as vehicle used, may account for differences in reported cell numbers between studies. By investigating the effect of passage through a syringe on cell survival at different maturities, and delivering these cells to the brain, we are able to provide a more complete understanding of the affect of cell maturity on survival.

There are several possible explanations for the substantial differences in the expression of β-III tubulin seen between the cell groups *in vivo*. The transplantation paradigm may have resulted in the selective survival of immature (DCX+) cells, while more mature cells underwent apoptosis. This hypothesis is supported by the observation *in vitro* that ~80% of the late-differentiated cells before transplantation expressed β-III tubulin, whereas *in vivo* less than 20% did. In the mid-differentiated group, the DCX+ cells may have continued to differentiate following transplantation, resulting in a higher number of β-III tubulin+ cells in that group than the other groups after 1 week *in vivo*. It is unclear to what extent the injured brain environment influences cNESC differentiation and fate. Others have reported that the majority of undifferentiated progenitors differentiate into astrocytes following transplantation,[38–40] whereas neuronally committed cells are reported to retain higher levels of neuronal markers following stroke.[18,19] Future work may include the incorporation of a growth factor or peptide into the HAMC hydrogel to better control cell fate, as we have previously successfully demonstrated with oligodendrocyte precursors delivered to the injured spinal cord in an HAMC hydrogel covalently modified with platelet-derived growth factor and arginine-glycine-aspartic acid-based peptides.[13]

Conclusions

We demonstrated that maturation stage is an important variable to consider when choosing a cell population for transplantation into the stroke-injured brain. Early-differentiated or mid-differentiated cNESC-derived cells survive better than late-differentiated cells following transplantation in HAMC into the stroke-injured brain and, in the case of the mid-differentiated population, the majority of cells are positive for β-III tubulin. Future studies will investigate functional recovery of animals following cell transplantation.

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Disclosure Statement

No competing financial interests exist, although we do hold a patent on HAMC for cell delivery.

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