Journal of Visualized Experiments

Isolation and culture of adult neural stem cells from the mouse subcallosal zone

--Manuscript Draft--

Manuscript Number:	JoVE54929R2
Full Title:	Isolation and culture of adult neural stem cells from the mouse subcallosal zone
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Adult neural stem cell, Subcallosal zone, Primary culture, Neurosphere, Differentiation
Manuscript Classifications:	1.11.251: Cells, Cultured; 5.5.240.249.500: Primary Cell Culture
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Abstract:	Adult neural stem cells (aNSCs) can serve for the regeneration of damaged brain. Because NSCs have the potential for proliferation and differentiate to three types of cells, such as neuron, astrocyte, and oligodendrocyte. Identifying aNSCs-derived regions and characterizing the properties of aNSCs are critical for the potential use of aNSCs and elucidation of their role for neural regeneration. The subcallosal zone (SCZ), located between white matter and hippocampus, has been recently reported to contain aNSCs and continuously give rise to neuroblasts. While low percentage of aNSCs from the SCZ is differentiated to neurons, most of cells are differentiated to glial cells, such as oligodendrocytes and astrocytes. These cells are suggested to have a therapeutic potential for traumatic cortical injury. Here, this protocol describes in detail the process to generate SCZ-aNSCs from adult mouse brain. A brain matrix with intervals of 1 mm is used to obtain the SCZ-containing coronal slices and precisely dissect SCZ from the whole brain. These SCZ sections are initially subjected to neurosphere culture. Well-developed culture system allow us to confirm their characteristics and to expand study about NSCs. To understand the characteristics of aNSCs, expansion of aNSCs is required. Neurosphere culture system provides a useful tool to determine proliferation and collect the genuine NSCs. Monolayer culture is also an in vitro system to assay proliferation and differentiation. In addition, this culture system provides more homogenous environment to NSCs than neurosphere culture system. Thus, this new culture system using a discrete brain region will be helpful for expand our knowledge about aNSCs and their application for therapeutic uses.
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Dear Editor in-Chief of the JOVE Journal (Dr. Nandita Singh)

We greatly appreciate your interests in our study, and providing us a chance to submit the manuscript. Please find enclosed our manuscript entitled "**Isolation and culture of adult neural stem cells from the mouse subcallosal zone**" by Kim et al.

In this manuscript, we have described the detailed procedures for isolation, expansion, and differentiation of the subcallosal zone-adult neural stem cells (SCZ-aNSCs) *in vitro*. Cultures of aNSCs in Neurosphere or monolayer formats were relatively well-established methods. However, we experienced that many colleagues requested us to learn this technique, and we felt that your Journal is one of the best media to spread our technique to the community. Especially, precise dissection of desired brain regions is very important to address region-specific NSCs, and our procedure will provide general idea how adult brain regions can be properly isolated for culture.

I confirm that all authors have read and approved the submitted manuscript; the manuscript has not been published elsewhere, and is not under considerable for publication in any journal. Also please find the list of potential reviewers attached. We hope hearing favourably soon.

Sincerely yours,

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Isolation and culture of adult neural stem cells from the mouse subcallosal zone

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KEYWORDS:
Adult neural stem cell, Subcallosal zone, Primary culture, Neurosphere, Differentiation

SHORT ABSTRACT:
Establishing culture systems for the expansion of adult neural stem cells (aNSCs) allows for the examination and application of aNSCs for therapy. The subcallosal zone (SCZ) has recently been recognized as a novel neuroblast-forming region in adult mice. Here, methods for the isolation, expansion, and differentiation of SCZ-aNSCs are described.

LONG ABSTRACT:
Adult neural stem cells (aNSCs) can be used for the regeneration of damaged brain tissue. NSCs have the potential for differentiation and proliferation into three types of cells: neurons, astrocytes, and oligodendrocytes. Identifying aNSC-derived regions and characterizing the aNSC properties are critical for the potential use of aNSCs and for the elucidation of their role in neural regeneration. The subcallosal zone (SCZ), located between white matter and the hippocampus, has recently been reported to contain aNSCs and continuously give rise to

neuroblasts. A low percentage of aNSCs from the SCZ is differentiated into neurons; most cells are differentiated into glial cells, such as oligodendrocytes and astrocytes. These cells are suggested to have a therapeutic potential for traumatic cortical injury. This protocol describes in detail the process to generate SCZ-aNSCs from an adult mouse brain. A brain matrix with intervals of 1 mm is used to obtain the SCZ-containing coronal slices and to precisely dissect the SCZ from the whole brain. The SCZ sections are initially subjected to a neurosphere culture. A well-developed culture system allows for the verification of their characteristics and can increase research on NSCs. A neurosphere culture system provides a useful tool for determining proliferation and collecting the genuine NSCs. A monolayer culture is also an *in vitro* system to assay proliferation and differentiation. Significantly, this culture system provides a more homogenous environment for NSCs than the neurosphere culture system. Thus, using a discrete brain region, these culture systems will be helpful for expanding our knowledge about aNSCs and their applications for therapeutic uses.

INTRODUCTION:

NSCs have characteristics of self-renewal and multiple-lineage differentiation. To confirm these properties, a neurosphere culture system has widely been used. The neurosphere culture system was developed in the early 1990s and served as a standard stem cell culture system[1]. Depending on self-renewal potency, NSCs continuously proliferate and generate a cell mass in a suspension culture. The number of cells and the size of the neurosphere are considered to be closely associated with the proliferation properties of the NSCs. Monolayer cultures are also widely used for the maintenance and differentiation of NSCs. Compared to the neurosphere culture, the monolayer culture system provides better homogenous maintenance and expansion of NSCs[2]. These two well-developed culture systems have contributed to the characterization of aNSCs *in vitro*.

NSCs reside in different brain regions, such as the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampus[3-5]. The subcallosal zone (SCZ) of the caudal subcortical white matter is recognized as a novel neurogenic region[6-8]. It was recently reported that the SCZ-aNSCs have therapeutic potential in traumatic brain injury[9]. Compared to other neurogenic regions, the SCZ resides along the subcortical white matter. In the human brain, subcortical white matter occupies a larger region than in the mouse brain[10]. Therefore, an understanding of the characteristics of SCZ-aNSCs using an *in vitro* culture system is important in order to promote the potential use of these cells for neural regeneration. Precise dissection of the desired brain region is required to rule out possible contamination by unwanted regions containing active or quiescent NSCs. For instance, aNSCs in non-neurogenic regions can be activated and produce new neural cells in injured brains or during *in vitro* culturing[11]. To obtain NSCs from the SCZ, cells were collected from brain slices containing the SCZ. Then, a careful micro-dissection of the SCZ region was performed using a fine needle. To generate the neurosphere from the SCZ, micro-dissected SCZ tissue chunks were dissociated into single cells and then cultured as a suspension in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). After SCZ-aNSCs form neurospheres, they also can be maintained as neurospheres or monolayers for expansion. This procedure also demonstrates immunostaining processes with various markers for the detection of NSCs and

their progeny after their expansion and differentiation in a monolayer culture. Here, a visual protocol of the SCZ-aNSC culture system is presented. This protocol contains detailed instructions for the micro-dissection of the SCZ region and for the maintenance and passaging of the cells.

PROTOCOL:

1. Preparation of materials and culture medium

1.1) For the dissection and dissociation of the SCZ, wrap the brain matrix, double-edged razor blade, and forceps with aluminum foil, and then sterilize them by autoclaving.

1.2) Prepare 50 mL of cold PBS buffer to wash the whole mouse brain.

1.3) Set up a dissection microscope and prepare the surgical tools required for the dissection of the brain (autoclaved scissors and forceps) and the isolation of the SCZ (1-mL syringe, 30G needle, brain matrix, and fine forceps).

1.4) **Preparation of N2 medium**:

1.4.1) Prepare F-12/DMEM (+L-glutamin, +sodium bicarbonate) medium with 2% B27, 1% N2 supplements, and 1% Penicillin-Streptomycin.

Note: The growth medium consists of N2 medium and growth factors (20 ng/mL purified epidermal growth factor (EGF) and 20 ng/mL basic fibroblast growth factor (bFGF2)).

1.5) Prepare a digestion buffer (40 unit/mL papain, 2.4 unit/mL dispase II, and 2% penicillin-streptomycin in PBS) for tissue digestion.

1.6) **Preparation of coating plate and coverslip:**

1.6.1) Prepare poly-L-ornithine (PLO; 0.01%) and laminin (10 µg/mL dissolved in DH_2O). To coat 6-well plates for the maintenance of SCZ-aNSCs as a monolayer or 18-mm coverslips for immunostaining, incubate them with PLO overnight at 4 °C. Then wash them 3 times with DH_2O. Allow the plates and coverslips to dry after the last washing.

1.6.2) Next, incubate the plates with laminin overnight at 4 °C. Then, wash them 3 times with DH_2O.

Caution: Do not dry the laminin, which will affect cell attachment. **Note:** The coating solutions can be reused 3 times.

2. Isolation and dissociation of the adult SCZ

2.1) Prior to culture preparation, place the brain matrix and double-edged razor on ice.

Note: Do not freeze the brain matrix, because the brain could attach to it.

2.2) Sacrifice a mouse (8 weeks old) by CO_2 asphyxiation or cervical dislocation.

2.2.1) Cut off the head with sharp scissors after spraying 70% ethanol. To immobilize the head, hold both sides of the head tightly. Cut the skin with scissors at the midline in a caudal-rostral direction. This promotes the complete removal of the skin from the skull.

2.2.2) Remove the caudal part of skull (posterior to lambda) first, and then insert the forceps between the skull and brain at the dorsal midline position. Grab the left (or right) parietal bone and carefully peel it off. Repeat this procedure for the removal of the other parietal bone.
Note: Disconnection of optic nerve and removal of the meninges from the brain make it easier to detach the brain from the skull.

2.2.3) Transfer the brain to cold PBS buffer (25 mL) and rinse it twice to remove excess blood.

2.3) Place the brain into the brain matrix on ice and make coronal cuts to obtain 1-mm thick slices. Transfer the brain slices (1 mm) containing the SCZ regions that are located in the posterior part of the brain to a cold PBS in a 35-mm plastic petri dish.
Caution: In order to obtain a parallel plane of coronal sections, ensure that the brain fissure is placed in the midline of the brain matrix; it is critical to avoid unnecessary variations in the sections.

Note: Obtain brain slices at 2-3 mm posterior to bregma; this allows the separation of the SCZ from the SVZ.

2.4) Under the dissecting microscope with a low magnification, micro-dissect the SCZ from the white matter area of the cortex and hippocampus with a bent 30G needle[6,9]. Then, remove the cortex regions above the SCZ. Place the dissected SCZ region from the slices into a 35-mm plastic petri dish on ice without cold PBS.
Note: Contamination of extra regions containing mature neurons could affect the viability of aNSCs and neurosphere formation because they undergo cell death in aNSC culture conditions.

2.5) Using a bent 30G needle, immediately chop the dissected tissues into small pieces.
Note: If a longer time is required for dissecting the SCZ tissue, immerse the dissected tissues in cold PBS before chopping.

2.6) Re-suspend the chopped tissue with 1 mL of digestion buffer, transfer the tissue to a 15-mL tube containing 2 mL of digestion buffer, and incubate it for 30 min in a 37 °C water bath.
Note: Shake the tube every 10 min to mix well.

3. Subcallosal zone-derived adult neural stem cell culture

3.1) Tap the tube mildly to dissociate the digested tissue, and then centrifuge the tube at 145 x g for 5 min. Discard the supernatant, re-suspended the digested tissue with 1 mL of pre-warmed N2 medium to wash out the digestion buffer, and gently pipet the sample solution a maximum of 5 times using a P1000 pipette.
Note: Over-triturating with a narrow pipet tip can diminish cell viability and subsequent growth.

3.2) Centrifuge the tube at 145 x g for 5 min. After discarding the supernatant, re-suspend the cell pellet in 1 mL of N2 medium.

3.3) Prepare 1 mL of N2 medium in a non-coated 6-well plate and add 1 mL of the suspended cells to make a final volume of 2 mL.

3.4) Add EGF (20 ng/mL) and bFGF (20 ng/mL) into each well. Gently shake the 6-well culture dish by hand to mix the added growth factors with the plated cells. Keep the 6-well plate in a 37 °C and 5% CO_2 incubator.

3.5) Add EGF (20 ng/mL) and bFGF (20 ng/mL) to each well every day for 8 days. Every third day, add 200 µL of N2 media to maintain the approximate 2-mL volume of the medium.

4. Passaging of NSCs as neurospheres and to monolayer cultures

4.1) Gather the neurospheres and transfer them to a new 15-mL conical tube.

Note: The number of neurospheres (> 50-µm diameter) per well from the SCZ of a single mouse brain was 64.3 ± 7.31, which was less than that of the SVZ (190.5 ± 6.33)[9].

4.2) Incubate the neurospheres with 0.5 mL of digestion buffer for 5 min in a 37 °C water bath to dissociate the neurospheres into single cells. Primary neurospheres can be dissociated into single cells and maintained over several passages as neurosphere or monolayer cultures.

Note: Expanding SCZ-aNSCs as a monolayer is superior to neurospheres because SCZ-aNSCs can be passaged > 10 times in a monolayer culture format, but < 5 times in a neurosphere culture format.

Caution: SCZ-aNSCs exhibit strong aggregation, and it is difficult to dissociate them into single cells. Thus, the neurosphere culture format is not recommended for routine cell expansion.

4.3) Gently pipet the sample solution up and down with a P1000 pipette less than 5 times and centrifuge the tube at 145 x g for 5 min. Discard the supernatant and re-suspend the neurospheres with 1 mL of N2 medium.

Note: Over triturating with a narrow pipette tip can diminish cell viability and subsequent growth.

4.4) To count the cells, make a 1:1 mixture of the cell suspension (10 µL) and the 0.4% trypan blue solution, and then count the number of live cells on a hematocytometer. After coating a 6-well plate with PLO/laminin, plate the cells at 2.5 x 10^5 cells/mL with 2 mL of N2 medium for each well.

Note: Changes in cell density can affect their condition and differentiation potential.

4.5) Maintain the SCZ-aNSCs with a daily treatment of growth factors (2 mL, 20 ng/mL) for 5 days, and them passage them.

5. Differentiation of subcallosal zone-derived adult neural stem cells

5.1) Plate aNSCs onto a PLO/Laminin-coated 18-mm coverslip with 1 x 10^5 cells/mL in 1 mL N2 with growth factors (20 ng/mL) for differentiation of the SCZ-aNSCs.

5.2) The next day, when the cells are firmly attached to the coverslip, exchange the growth medium with N2 to remove the growth factors.

5.3) After 6 days, wash the differentiated cells with 1 mL of PBS to remove cell debris and fix them for immunostaining.

Note: BrdU can be incorporated into the newly-synthesized DNA of proliferating cells. Therefore, if desired, BrdU (10 µg/mL) can be added to live cells before the fixation of cells.

6. Immunostaining adult neural stem cells and differentiated progenitors

6.1) For immunostaining, wash the cells with PBS and fix them with 4% PFA for 20 min at room temperature.

Caution: PFA is highly toxic; avoid contact with the skin and eyes.

6.2) Remove the 4% PFA, rinse the fixed cells with PBS 3 times, and then store them at 4 °C until they are needed for immunostaining.

6.3) Incubate the cells on the coverslip with blocking solution (3% bovine serum albumin and 0.1% Triton X-100 in 1X PBS) for at least 30 min at room temperature.

6.4) Prepare the primary antibodies in fresh blocking solution and incubate the samples overnight at 4 °C.

6.4.1) **Immunostaining the aNSCs**:

6.4.1.1) Stain the aNSCs using anti-Nestin and anti-BrdU antibodies. Before fixation, add 20 µg of BrdU to the aNSCs and incubate them for 2 h. Perform the denaturing step using 2 N HCl for 20 min at 37 °C before performing the blocking step.

6.4.2) **Immunostaining the differentiated progenitors**:

6.4.2.1) Use anti-O4, anti-bIII-tubulin, and anti-glial fibrillary acidic protein (GFAP) antibodies to label the differentiated progenitors.

6.5) Wash the samples with PBS 3 times and incubate them with secondary antibodies conjugated to fluorescent dyes (1:500) in blocking solution for 30 min at room temperature. Then, wash the samples 3 times with PBS.

Note: Use secondary antibodies that match the hosts of the primary antibodies.
Hoechest33343 (1:2,000) is used for nuclear staining.

6.6) Add mounting solution to a slide glass and proceed with mounting. Observe and image the sample on a confocal microscope at multiple wavelengths: FITC (488 nm), Cy3 (543 nm), Cy5 (647 nm), and Hoechest33343 (405 nm).

REPRESENTATIVE RESULTS:

Defining the culture system for aNSCs from the unknown neurogenic region is essential for understanding these cells and for developing their potential use in brain repair[12]. It is known that NSCs in different developmental stages or in different regions behave differently[3,4]. Recently, it was reported that SCZ-derived cells exhibit differential potentials for neuronal differentiation *in vivo* and *in vitro* compared to SVZ-derived cells[7-9]. Therefore, to precisely isolate each neurogenic region, brain slices that include the SCZ were dissected using a 1-mm brain matrix (Figure 1A). After 8 days of culture with growth factors, aNSCs derived from the SCZ can form neurospheres, in which the cells can be subsequently maintained (Figure 1B).

Since a subset of NSCs in the neurospheres may be spontaneously differentiated[13], a monolayer culture system is also helpful for the maintenance of the relatively homogeneous population of SCZ-aNSCs. Growth factors and dissociation enzymes such as trypsin do not easily permeate deep inside of the neurosphere[14,15]. Monolayer cultures provide more even conditions for the expansion of NSCs. From primary neurospheres, SCZ-aNSCs were dissociated into single cells by a digestion buffer treatment. One day after cell seeding, aNSCs were attached to the coated plate and exhibited cell proliferation (Figure 2A). To confirm their potency of proliferation, BrdU was added into the media. After incubation with BrdU for 2 h, cells were readily stained with anti-BrdU (a marker for proliferation) and anti-Nestin (a marker for neural stem cells) antibodies, indicating that SCZ-aNSCs are actively proliferating and maintaining the key properties of stem cells (Figure 2B). Accordingly, they did not exhibit markers for differentiated cells, such as EGFR (expressed in transiently-amplifying cells) and DCX (expressed in neuroblasts) (Figure 2C).

To confirm the multiple differentiation potential of SCZ-aNSCs, growth factors were removed from the culture media. After 6 days, cells were immunostained with various makers for differentiated cells. To exhibit the different progenies of aNSCs, markers for neurons (Tuj1), astrocytes (GFAP), and oligodendrocytes (O4) were employed; all of these cell types were generated from the SCZ-aNSCs (Figure 3).

FIGURE LEGENDS:

[Place Figure 1 here]

Figure 1: Isolation of the SCZ region and formation of the neurosphere. A. Procedure for the dissection of the SCZ region from the adult mouse brain. To culture the SCZ-aNSCs, an 8-week-old mouse brain is placed onto a brain matrix (1-mm intervals). After sectioning, 1-mm brain slices that included the SCZ region (2-3 mm from bregma) were dissected (indicated by the red dotted line). **B**. Neurosphere formation. Eight days after the *in vitro* culture, primary neurospheres (passage 0) were formed and passaged.

[Place Figure 2 here]

Figure 2: Maintenance of the SCZ-aNSCs as a monolayer culture. A. One day after seeding the dissected cells, SCZ-aNSCs were attached and expanded onto a coated dish in a monolayer manner (left). Three days after maintenance, the number of SCZ-aNSCs was increased (right). **B**. Immunostaining with BrdU (red, a marker for proliferating cells), Nestin (green, a marker for neural stem cells), and Hoechest33343 (blue, a marker for nuclei). **C**. Immunostaining with neural stem/progenitor cell markers Nestin (red, a marker for type B neural stem cells), EGFR (green, a marker for type C transient-amplifying cells), and DCX (blue, a marker for type A neuroblasts). Nuclei were counterstained with Hoechest33343 (white).

[Place Figure 3 here]

Figure 3: Immunostaining of the differentiated cells from the SCZ-aNSCs. Immunostaining with differentiation markers Tuj1 (green, a marker for immature neurons), O4 (red, a marker for oligodendrocytes), and GFAP (yellow, a marker for astrocytes). Magnified images are shown as insets. Hoechest33343 (blue) was used for counter-staining the nuclei.

Table 1: List of the materials used in the SCZ-aNSC culture.

DISCUSSION:

This paper describes a detailed protocol to generate NSCs from the adult mouse SCZ and to maintain them for various applications. There are three critical steps for establishing the *in vitro* culture system needed to purify and expand SCZ-NSCs. First, it is important to ensure that the SCZ region is precisely dissected out from other potential neurogenic regions (Figure 1B). Thick and precise sections containing the SCZ regions were obtained with a 1-mm interval brain matrix, and then a fine needle was used for the micro-dissection of the SCZ from other cortical regions (Figure 1A). When non-NSCs from adjacent tissues, such as the cerebral cortex, are cultured with SCZ-aNSCs, catastrophic death occurs, which negatively affects the viability and sphere-formation of SCZ-NSCs[16-18]. The caudal SCZ (2-3 mm posterior to bregma) was confirmed as the best region for generating distinct SCZ-aNSCs. Second, the appropriate enzymatic treatment in the harvesting and passaging steps is critical for achieving a high yield of cells. Dispase II and papain were more effective for isolating aNSCs than trypsin. Dissociation of cells for passaging with dissociation buffer instead of trypsin enhanced their viability[19]. Mechanical dissociation and trituration with a pipette should be minimal. Third, a cell strainer is generally used in primary culture systems to remove cellular debris after tissue digestion. However, due to the localization of SCZ-aNSCs, these cells are obtained after the breakage of white matter by enzyme digestion and mechanical trituration. During filtration with a cell strainer, a substantial amount of cells would be lost. Therefore, culturing SCZ-aNSCs without using a cell strainer is a better way to get a high yield of cells.

While both neurosphere cultures and monolayer cultures can be applied to the maintenance of NSCs, one limitation of the neurosphere culture is that single NSCs dissociated after splitting can be randomly aggregated. The aggregation results in different sizes of neurospheres. When the size of a neurosphere reaches a certain critical value, the neurosphere grows as a heterogeneous structure, due to a lack of nutrients, growth factors, and oxygen at the core[20].

Furthermore, neurospheres with large sizes are not easily dissociated with dissociation buffer and require longer enzymatic treatment times with extensive mechanical trituration, leading to lower cell viability. Therefore, the monolayer culture system is recommended to maintain SCZ-aNSCs through multiple passages. In a monolayer culture system, aNSCs are stably maintained as NSCs (type B) without spontaneous differentiation into specified cells, such as progenitors (types C and A) (Figure 3A). SCZ-aNSCs were passaged for extended periods, < 5 passages in a neurosphere format and > 10 passages as a monolayer. This is consistent with previous results, which suggest that the monolayer culture system maintains NSCs *in vitro* in long-term cultures[21]. However, the proliferating speed decreased, and the portion of dying cells increased over 5 passages. Extended passaging affects the multipotency and neuronal differentiation with increased chromosome aberration[22]. Therefore, to avoid extended passaging effects, it is recommended to use early passage (< passage 5) cells for proliferation and differentiation analysis. Although the potential for self-renewal of SCZ-aNSCs is similar to SVZ-aNSCs, less neuronal differentiation was shown in SCZ-aNSCs[9].

Methods for isolating aNSCs from neurogenic regions of the adult brain, including the SVZ and dentate gyrus (DG), have been established[22]. Although such protocols have promoted the isolation and cultivation of aNSCs in vitro, there are several limitations to obtaining a high number of cells. Many protocols utilize a brain tissue chopper that may cause the loss of brain tissue during the chopping procedure. Another approach to isolate aNSCs from neurogenic regions uses a coronal cut through the brain using a scalpel. This is followed by the micro-dissection of the SVZ or of the DG along the longitudinal fissure[23]. The presence of other brain regions can cause other cell types to contaminate the aNSC culture, which might affect the viability of the cells *in vitro*. With the current protocol, many different samples can be managed in a single experiment, including controls versus a variety of experimental groups. Also, slicing using a brain matrix is superior to a brain chopping tool, as it allows for the attainment of NSCs from various brain regions with a high cell yield. It also enables the comparison of aNSCs from different regions of the same brain.

Transplantation and engineering of endogenous NSCs have been considered as possible strategies for stem cell therapy. For this, *in vitro* studies about the characteristics of aNSCs should also be comprehensively explored. Therefore, the establishment of a well-characterized *in vitro* culture system will be helpful for furthering the application of NSCs. In this culture system, the stem-cell properties of SCZ-aNSCs were well-maintained, as evidenced by self-renewal and multiple-lineage differentiation under the appropriate conditions. Therefore, this culture system can be used for the expansion of SCZ-aNSCs for biological studies and therapeutic applications.

ACKNOWLEDGMENTS:
This work was supported by the Brain Research Program through the National Research Foundation (NRF), funded by the Korean Ministry of Science, ICT, and Future Planning (Grants NRF-2015M3C7A1028790); and by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI14C3347).

DISCLOSURES:
The authors declare no conflicts of interest.

REFERENCES:

1 Reynolds, B. A. & Weiss, S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Developmental biology* **175**, 1-13, doi:10.1006/dbio.1996.0090 (1996).

2 Babu, H., Cheung, G., Kettenmann, H., Palmer, T. D. & Kempermann, G. Enriched monolayer precursor cell cultures from micro-dissected adult mouse dentate gyrus yield functional granule cell-like neurons. *PloS one* **2**, e388, doi:10.1371/journal.pone.0000388 (2007).

3 Alvarez-Buylla, A. & Garcia-Verdugo, J. M. Neurogenesis in adult subventricular zone. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **22**, 629-634 (2002).

4 Kempermann, G., Song, H. & Gage, F. H. Neurogenesis in the Adult Hippocampus. *Cold Spring Harbor perspectives in biology* **7**, a018812, doi:10.1101/cshperspect.a018812 (2015).

5 Gage, F. H. Mammalian neural stem cells. *Science* **287**, 1433-1438, doi:10.1126/science.287.5457.1433 (2000).

6 Seri, B. *et al.* Composition and organization of the SCZ: a large germinal layer containing neural stem cells in the adult mammalian brain. *Cereb Cortex* **16 Suppl 1**, i103-111, doi:10.1093/cercor/bhk027 (2006).

7 Kim, W. R. *et al.* Evidence for the spontaneous production but massive programmed cell death of new neurons in the subcallosal zone of the postnatal mouse brain. *The European journal of neuroscience* **33**, 599-611, doi:10.1111/j.1460-9568.2010.07557.x (2011).

8 Kim, H. J., Kim, J. Y. & Sun, W. Age-dependent changes in the subcallosal zone neurogenesis of mice. *Neurochemistry international* **61**, 879-884, doi:10.1016/j.neuint.2012.02.027 (2012).

9 Kim, J. Y. *et al.* Promotion of Cortical Neurogenesis from the Neural Stem Cells in the Adult Mouse Subcallosal Zone. *Stem Cells*, doi:10.1002/stem.2276 (2015).

10 Nunes, M. C. *et al.* Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. *Nature medicine* **9**, 439-447, doi:10.1038/nm837 (2003).

11 Gould, E. How widespread is adult neurogenesis in mammals? *Nature reviews. Neuroscience* **8**, 481-488, doi:10.1038/nrn2147 (2007).

12 Gage, F. H. & Temple, S. Neural stem cells: generating and regenerating the brain. *Neuron* **80**, 588-601, doi:10.1016/j.neuron.2013.10.037 (2013).

13 Shaker, M. R., Kim, J. Y., Kim, H. & Sun, W. Identification and characterization of secondary neural tube-derived embryonic neural stem cells in vitro. *Stem cells and development* **24**, 1171-1181, doi:10.1089/scd.2014.0506 (2015).

14 Jensen, J. B. & Parmar, M. Strengths and limitations of the neurosphere culture system. *Molecular neurobiology* **34**, 153-161, doi:10.1385/MN:34:3:153 (2006).

15 Suslov, O. N., Kukekov, V. G., Ignatova, T. N. & Steindler, D. A. Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 14506-14511, doi:10.1073/pnas.212525299 (2002).

16 Richards, L. J., Kilpatrick, T. J. & Bartlett, P. F. De novo generation of neuronal cells from the adult mouse brain. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 8591-8595, doi:10.1073/pnas.89.18.8591 (1992).

17 Reynolds, B. A. & Weiss, S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707-1710, doi:10.1126/science.1553558 (1992).

18 Gritti, A. *et al.* Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **19**, 3287-3297 (1999).

19 Wachs, F. P. *et al.* High efficacy of clonal growth and expansion of adult neural stem cells. *Laboratory investigation; a journal of technical methods and pathology* **83**, 949-962 (2003).

20 Xiong, F. *et al.* Optimal time for passaging neurospheres based on primary neural stem cell cultures. *Cytotechnology* **63**, 621-631, doi:10.1007/s10616-011-9379-0 (2011).

21 Lee, S.-W. *et al.* Optimization of Matrigel-based culture for expansion of neural stem cells. *Animal Cells and Systems* **19**, 175-180, doi:10.1080/19768354.2015.1035750 (2015).

22 Guo, W., Patzlaff, N. E., Jobe, E. M. & Zhao, X. Isolation of multipotent neural stem or progenitor cells from both the dentate gyrus and subventricular zone of a single adult mouse. *Nature protocols* **7**, 2005-2012, doi:10.1038/nprot.2012.123 (2012).\

23 Walker, T. L. & Kempermann, G. One mouse, two cultures: isolation and culture of adult neural stem cells from the two neurogenic zones of individual mice. *JoVE (Journal of Visualized Experiments)*, e51225-e51225, doi:10.3791/51225 (2014).

Figure 1

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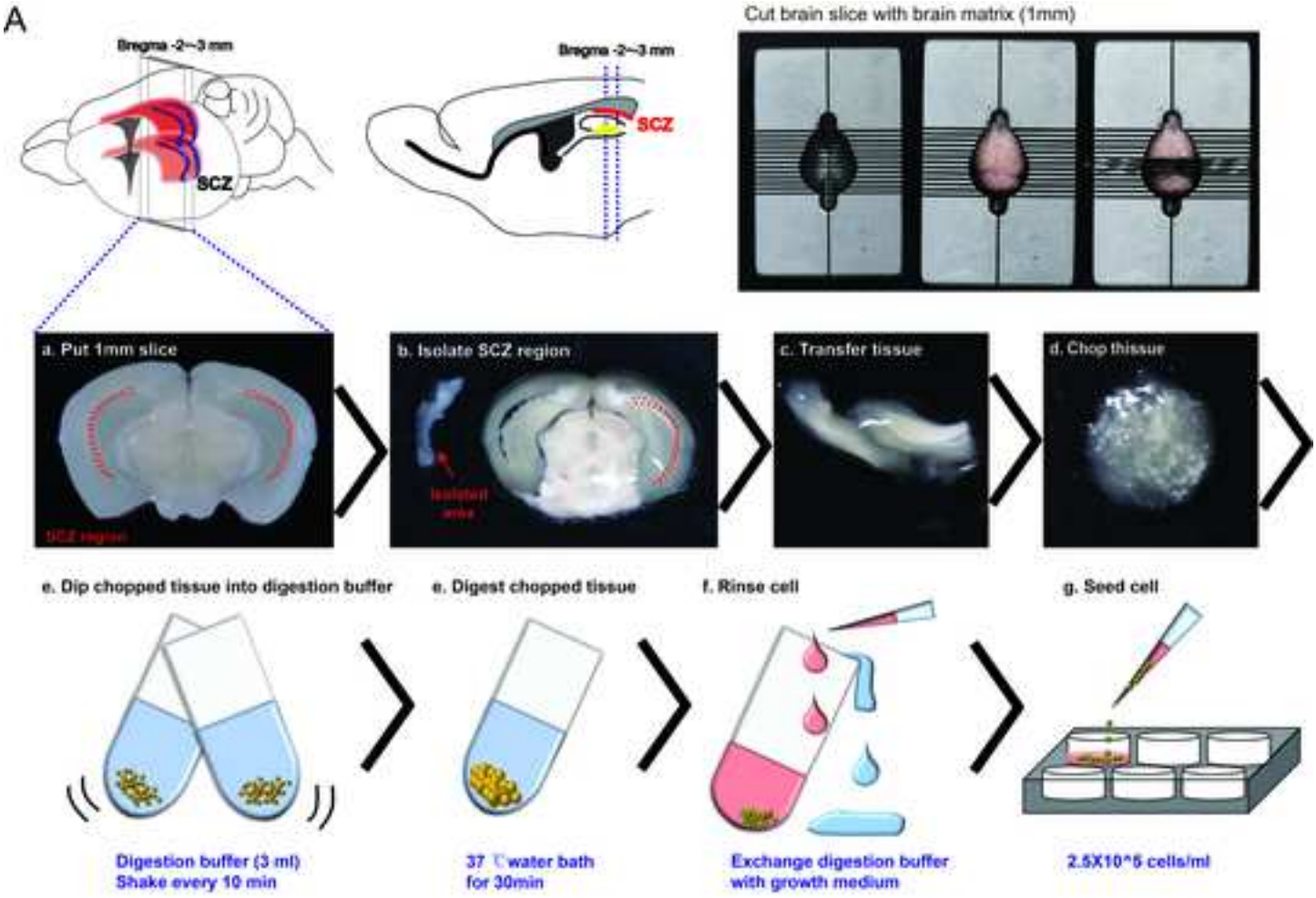

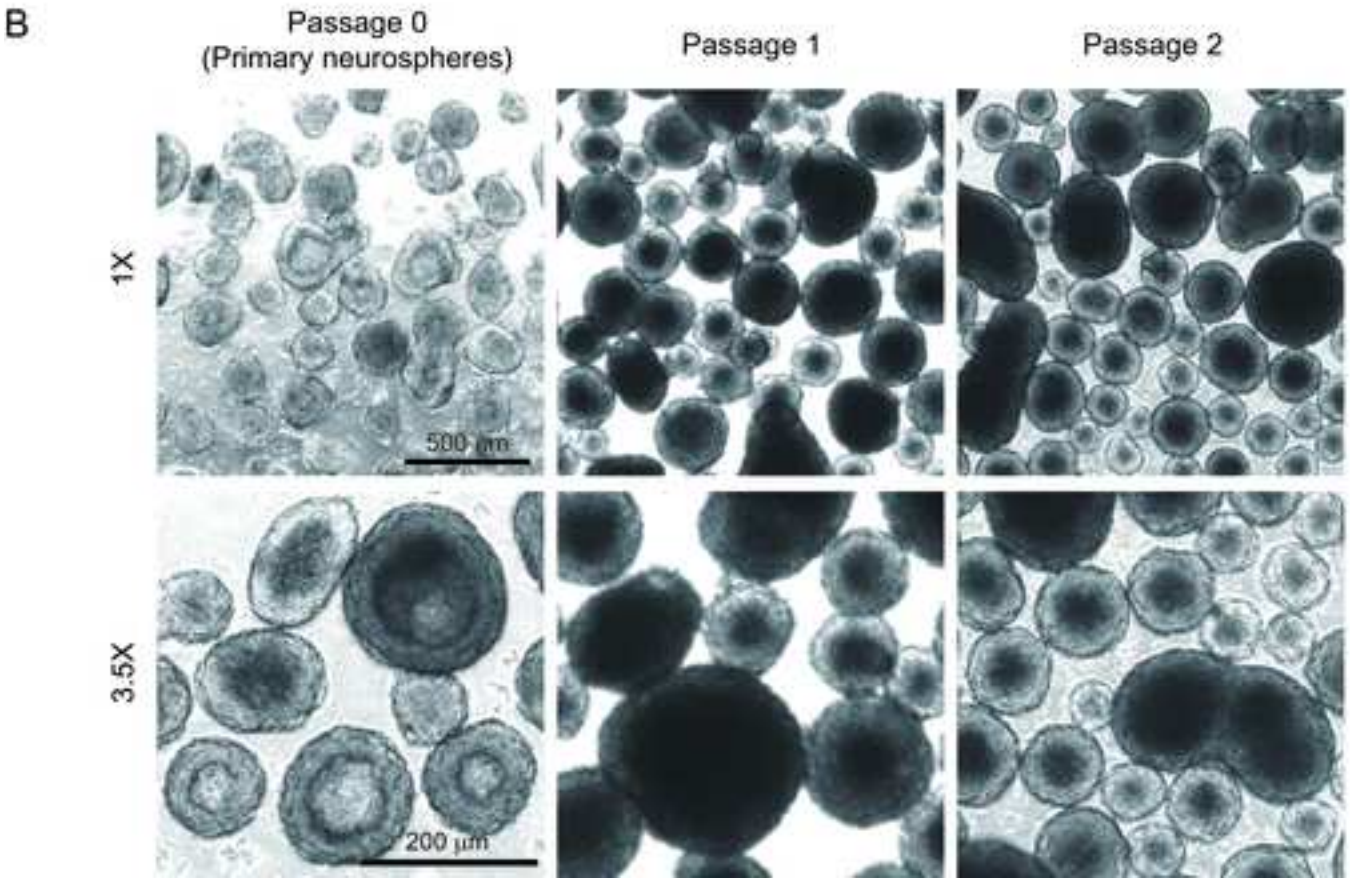

Figure 2

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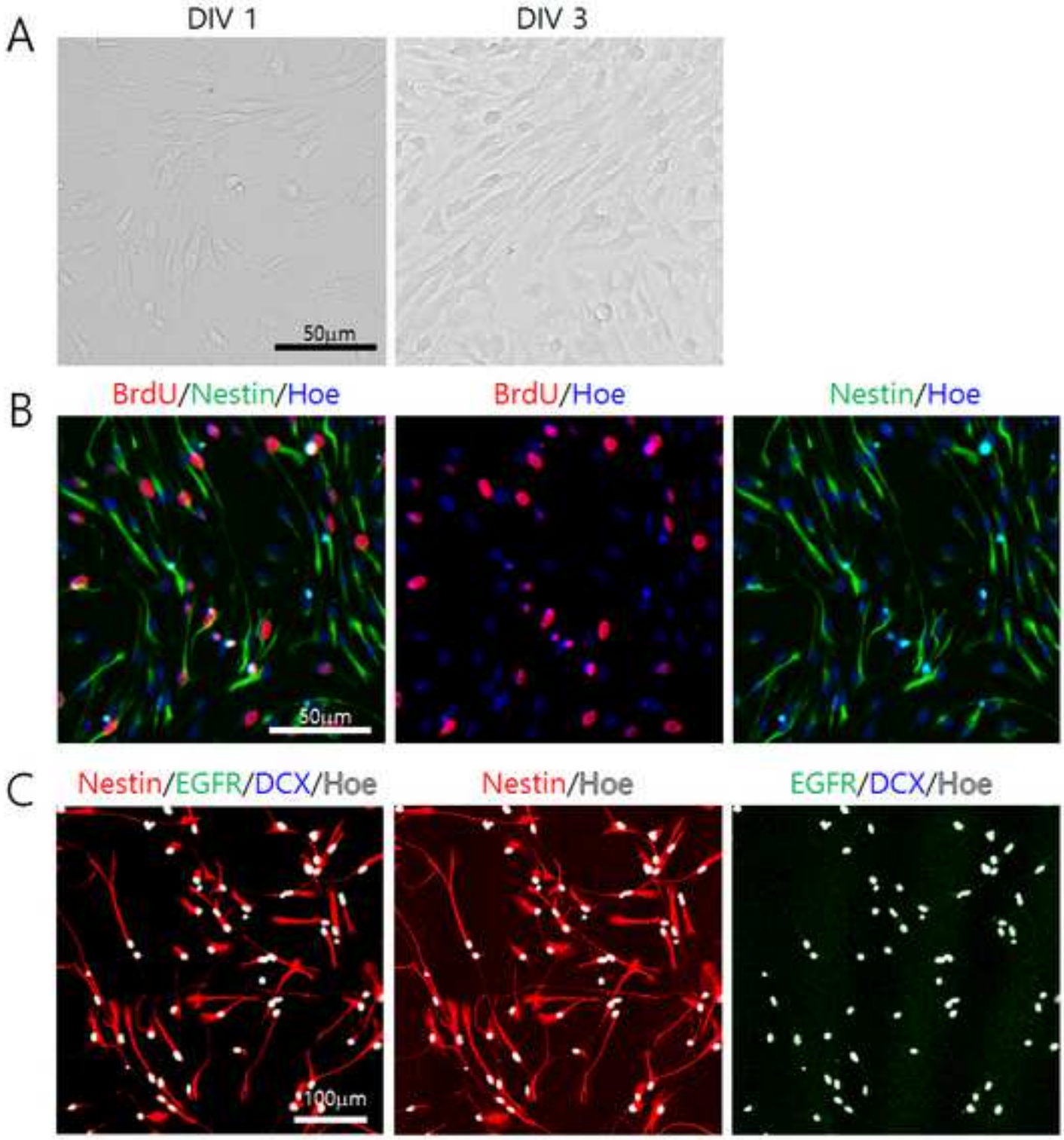

Figure 3

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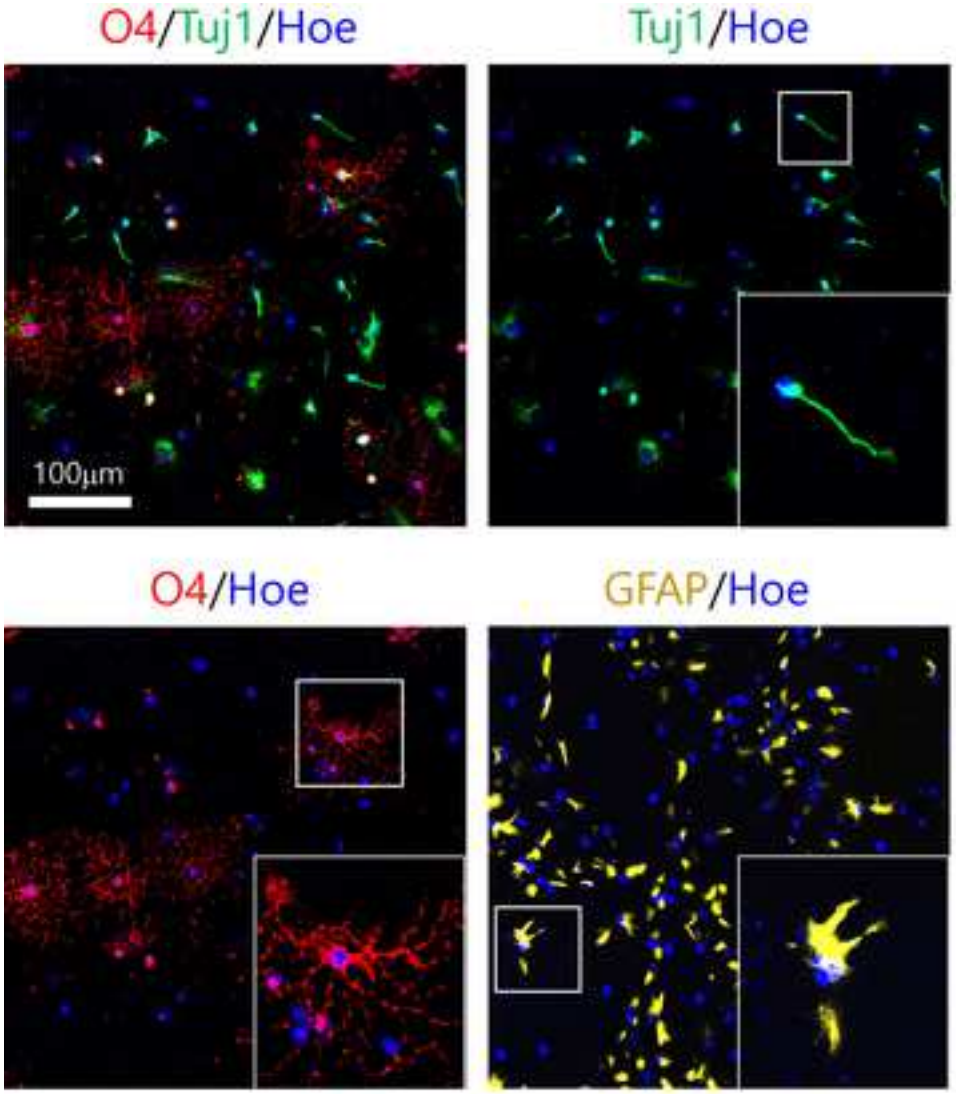

Excel Spreadsheet- Table of Materials/Equipment

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Type	Name	Company	Catalog number	Comments
Medium components	DMEM/F12	Gibco	11320-033	+L-glutamin, +Sodium bicarbonate
	Pen/Strep	Invitrogen	15140-122	
	N2 supplement	Gibco	17502-048	
	B27 supplement	Gibco	17504-044	
Growth factor	bFGF	R&D	233-FB	
	EGF	Gibco	PHG0313	
Buffer	PBS (10X)	BIOSOLUTION	BP007a	1X dilution
	HBSS	Gibco	14175-095	
Dissociattion buffer	Dispase II	Roche	04-942-078-001	
	Papain	Worthington	3126	
	Accutase	ICT	AT-104	
Tool	Fine forceps	WPI	555229F	
	Scissors	Storz	E3321-C	
	Brain matrix (1mm)	RWD	68707	
	Double-edged razor	DORCO	ST-300	
	30 gage needle	SUNGSHIM	N1300	
Materials	15 ml tubes	SPL	50015	
	50 ml tubes	SPL	50050	
	35mm dish	SPL	10035	petridish
	100mm dish	SPL	10090	petridish
	Cover slip (18mm)	Deckglaser	111580	
	12 well dish	SPL	32012	non-coating
	6 well dish	SPL	32006	non-coating
Coating materials	PLO	Sigma	P4957	0.01%
	Laminin	Gibco	23017-015	10 μg/ml
Primary antibodies	Nestin	Millipore	MAB353	mouse (1:1000)
	EGFR	Abcam	ab2430	rabbit (1:1000)
	DCX	Santa Cruz	SC8066	goat (1:500)
	Tuj1	Sigma	T2200	rabbit (1:2000)
	GFAP	Invitrogen	13-0300	rat (1:1000)
	O4	Millipore	MAB345	mouse (1:500)
	BrdU	Abcam	ab6326	Rat (1:500)
Secondary antibodies	anti-mouse 488	Invitrogen	A21202	1:500
	anti-mouse cy3	Jackson	715-165-151	
	anti-mouse 647	Jackson	715-606-150	
	anti-rabbit 488	Alexa	A21206	
	anti-rabbit cy3	Jackson	711-165-152	
	anti-rabbit 647	Jackson	711-605-152	
	anti-goat 488	Alexa	A11055	
	anti-goat cy3	Jackson	705-165-147	
	anti-goat 647	Invitrogen	A21447	
	anti-rat 488	Invitrogen	A21208	
	anti-rat cy3	Jackson	712-166-150	
	anti-rat 647	Jackson	712-605-153	
Immunostaining materials	BSA	Millipore	82-100-6	th 0.1% Triton X-100 in PBS
	Triton X-100	usb	22686	
	4% PFA	Biosesang	P2031	
	Hoechest33342	Life Technology	H3570	e for staining nuclei

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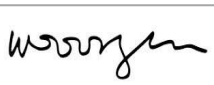
Signature:	
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•Formatting:
-Short abstract exceeds 50 words and must be reduced.

We have reduced the words in abstract to 50.

-Two tables are mentioned, but only one is provided. Please delete the reference to the tables in the figure legends section, as only the Table of Materials should be included with this manuscript. Please delete all references to Table 1.

Corrected.

•Grammar:
-2.2.1 – “Cut the off head”
-3.1 – “1000P”
-3.2 – “After discarding the soup” – do you mean discarding the supernatant? Please use “supernatant” rather than “soup”.
-Line 352 – “be also be”

We have modified these grammar errors. Now we used professional English editing service to correct typos and grammar errors.

•Additional detail is required:
-1.6 – Are the coverslips placed in the 6-well plate for coating? Or are the wells and coverslips coated separately?

For routines monolayer culture, we used coated 6-well plates. When immunostaining is required, 18-mm coverslip was inserted to each 12-well plate prior to coating, so that cells can attach the glass surface.

-2.2.1 – How is the skin removed?

We have added an additional description to clearly explain the skin removal step.

“To immobilize the head, hold both sides of the head tightly. Cut the skin with scissors from the midline in a caudal-rostral direction.”

-2.2.2 – How is the skull opened after making the incision?

We made clear statement how we removed skull to isolate brain.

“Remove the caudal part of skull (posterior to lambda) first, and then insert the forceps between the skull and brain at the dorsal midline position. Grab the left (or right) parietal bone and carefully peel it off. Repeat this procedure for the removal of the other parietal bone.”

-2.3 note – Please clarify “Ensure that the angle of the brain fits the brain matrix”. What angle of the brain?

The following sentence was added to the revised manuscript:

“In order to obtain parallel plane of coronal sections, ensure the brain fissure is placed in the midline of the brain matrix, it is critical to avoid unnecessary variations in the sections.”

-2.4 – How long can the tissue be stored on ice without solution?

Immediately we chopped dissected SCZ region in 35 mm plastic petri dish after tissue dissection. We recommend that this process is done within 1 min. However, if longer time was required for dissecting SCZ tissue, immerse dissected tissues in a cold PBS before chopping. Additional information was added in Step 2.5 as a Note.

“**Note**: If longer time is required for dissecting SCZ tissue, immerse dissected tissues in a cold PBS before chopping”

-2.4 note - What culture condition? It does not seem as though cells are cultured here; rather they are stored prior to digestion.

Sorry for the mistake, corrected.

-3.4 – Are cultures incubated with shaking? This is not clear.

We have added the following sentence to clarify this issue:

“With hand, shake gently the 6-well culture dish to mix well the added growth factors with plated cells.”

-4.3, 4.5, 5.1, 5.3 – What volumes are used?

We have added volume information to all steps.

-6.2.2.2 – What are secondary antibodies diluted in?

We have indicated it in Step 6.5.

"…incubate with secondary antibodies conjugated to fluorescent dyes (1:500) in blocking solution for 30 min at room temperature."

•Branding should be removed from 1.6, 4.1, Results, Discussion – Accutase

Removed.

•Results:
-Figure 2A – Please describe the two images in the legend.

We have described the two images in the legend.

Figure 3 – Please describe the insets in the legend. From which part of the original image are they taken?

We have now described the insets in the legend.

•Discussion: Please describe the other techniques that are mentioned when discussing significance and provide citations. Please discuss any modifications/troubleshooting that can be performed.

We have now described other techniques that we earlier mentioned in the discussion

"Methods for isolating aNSCs from neurogenic region of adult brain including SVZ and dentate gyrus (DG), have been established [22]. Although such protocols have promoted the isolation and cultivation of aNSCs in vitro, there are several limitations to obtain high number of cells. Many protocols utilize brain tissue chopper that may cause loss of brain tissue during chopping procedure. Another approach to isolate aNSCs from neurogenic regions uses a coronal cut through the brain with scalpel, followed with microdissection of SVZ, or cut along the longitudinal fissure with scalpel to micro-dissect the DG."

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Understanding of neural stem cells (NSC) behaviors in adult brain is critical for therapeutic development for various neuro-psychiatric disorders as well as for promotion of normal brain functions such as memory. Thus in vitro culture systems for adult NSCs (aNSCs) offer an important platform for those researches. aNSC culture methods have been previously demonstrated in many studies, but we cannot say that the aNSC culture is a commonly utilized technique up to date, mainly due to fastidious culture methods and lack of clarity in their protocol descriptions. The paper of Kim et al. describes the detailed methods for isolation, expansion, passaging, differentiation of aNSCs, specifically from subcallosal zone (SCZ), which has recently been shown as another neurogenic brain region, responding to injury at posterior brain regions. Considering that aNSCs in subcallosal zone (SCZ) are much less abundant than the other neurogenic brain regions such as SVZ and hippocampal SGZ, the protocol for successful SCZ-aNSC culturing described in this paper seems to be an advance in aNSC culture methods. Thus this article provides a description of a technique that will be of interest to researchers, especially who have been experiencing difficulties in aNSC cultures. Only several minor comments are made as follows:

Major Concerns:

N/A

Minor Concerns:

1. Page 2, line 4, 'white matter' can be changed into 'corpus callosum (white matter)'

We have changed.

2. An English editing service may be required, due to many awkward and unclear descriptions, the followings are the examples:
- P. 3, 1.2) 'For brain wash'
- P. 3 1.3) 'dissociation brain'
- P.3 1.4) 'growth medium by containing'
- P.3, 1.6) 'For the cell split'
- P.4, line 5, 'affect affects'
- P4, 3.1) 'below 5 times' (<5 times)
- P.4 3.3) 'non-coating' 'uncoated', 1ml suspended cells 1ml of suspended cells, make final 2ml growth medium make final volume to 2ml by adding growth medium

Thank you for the comments, and we utilized an English editor service to correct grammatical errors.

3. The following issues can be discussed at least in discussion section:
-How long (or How many passages) can the SCZ-aNSC be expanded in vitro?

We can culture SCZ-aNSCs over 10 passages. We have added this point in the discussion section.

"SCZ-aNSCs were passaged for extended periods, <5 passages in a neurospheres format and> 10 passages as a monolayer, this consistent with previous that suggests monolayer culture system maintains NSCs in vitro in long-term cultures."

- What is differentiation propensity of the SCZ-aNSCs in culture? (% of neurons, astrocytes, oligodendrocytes)

We published a study including this data. We have mentioned this point briefly in discussion.

"Extended passaging affects to multipotency and neuronal differentiation with increased chromosome aberration. Therefore, to avoid extended passaging effect, it is recommended to use early passage (< passage 5) cells for proliferation and differentiation analysis. Although the potential for self-renewal of SCZ-aNSCs is similar to SVZ-aNSCs, less neuronal differentiation was shown in SCZ-aNSCs"

- Description about the aNSC property changes during in vitro expansions (passages).

We have mentioned.

"Extended passaging affects to multipotency and neuronal differentiation with increased chromosome aberration. Therefore, to avoid extended passaging effect, it is recommended to use early passage (< passage 5) cells for proliferation and differentiation analysis. Although the potential for self-renewal of SCZ-aNSCs is similar to SVZ-aNSCs, less neuronal differentiation was shown in SCZ-aNSCs"

4. Fig. 1A: It is more comprehensive to indicate other brain regions (Lateral ventricle, corpus callosum, hippocampus…) alongside SCZ highlighted in red.

We have modified Fig 1A and included other brain regions (Lateral ventricle, hippocampus).

Additional Comments to Authors:
N/A

Reviewer #2:

Manuscript Summary:

This manuscript demonstrates a simple technique to isolate adult neural stem cells (NSCs) from subcallosal zone (SCZ). Since SCZ has recently been focused as a novel stem cells containing region, isolation, expansion, and analyzing detailed differentiation properties of these SCZ derived NSCs are important for potential therapeutic approach such as traumatic brain injury. The authors tried two well-established NSCs culture system, sphere culture, and monolayer culture, to show that isolated SCZ-NSCs possess ability to differentiate into three major types of neural cells.

Since protocol is simple and don't need any specialized instruments or fancy dissection tools, scientists who are interested in isolating these NSCs would easily try to follow this method. However, except for the utilization of brain matrix to separate SCZ, most of author's provided methods in here are more or less the copy of well-established systems that are used in these research fields. Detailed comparison of NSC's character of SCZ-NSCs with SVZ or SGZ-derived NSCs would help increasing the value of current studies.

Thank you for the comments. It is true that NSCs culture system is well established. However, the novelty of our method is the micro-dissection of SCZ out of a complex brain structure and study the behavior of cells of SCZ in vitro, as this reviewer commented. In addition, we also proposed the efficient way to expand SCZ-aNSCs by neurosphere formation (for isolation of aNSCs) and subsequent monolayer culture (to maintain and expand aNSCs). We have compared these cells to SVZ-aNSCs and recently published in Stem cells (Kim et al., 2016).

Major Concerns:

Is there any difference for the differentiation potential and proliferation speed of SCZ-derived NSCs, compared to others? (Beside authors declared in page 5 line 186.) How about general proliferation speed in monolayer cultures? When SCZ-NSCs are differentiated, what are the percentages of cells generating each type of cells (neurons, astrocytes, and oligodendrocytes).

We have added a description in the discussion section. We examined and demonstrated the characteristics of SCZ-aNSCs including proliferation and differentiation in recent Stem Cell journal (Kim et al., 2016).

“SCZ-aNSCs were passaged for extended periods, <5 passages in a neurospheres format and (> 10 passages as a monolayer), this consistent with previous that suggests monolayer culture system maintains NSCs in vitro in long-term cultures. However, the proliferating speed decreased, and the portion of dying cells increased over 5 passages. Extended passaging affects to multi-potency and neuronal differentiation owing with increased chromosome aberration. Therefore, to avoid extended passaging effect, it is recommended to use early passage (< passage 5) cells for proliferation and differentiation analysis. Although the potential for self-renewal of SCZ-aNSCs is similar to SVZ-aNSCs, less neuronal differentiation was shown in SCZ-aNSCs.”

Minor Concerns:

1. Protocol has to be arranged along the time series from beginning.
It would be good to demonstrate from 'coating procedures'. Since coating is important step, and coating material is sometimes very toxic to cells. I wonder more detailed protocol for this section.

In this protocol, coating step is required for maintenance of aNSCs with passage 1 after primary neurosphere culture is done. We have modified and added more detail protocol in coating section in Step 1.6, 1.6.1, and 1.6.2.

"**Preparation of coating plate and coverslip:**

Prepare poly-L-ornithine (PLO; 0.01%) and Laminin (10 µg/ml dissolved in DH_2O).

1.6.1) To coat 6-well plates for maintenance of SCZ-aNSCs as monolayer or 18-mm coverslips for immunostaining, incubate them with PLO overnight at 4 °C, followed 3 times washing with DH_2O. Allow plates and coverslips to dry after the last washing.

1.6.2) Next, incubate the plates with Laminin overnight at 4 °C, followed 3 times washing with DH_2O.

Caution: Do not dry Laminin, which affects cell attachment.

Note: Coating solutions can be re-used 3 times."

2. There are several grammar mistakes (eg. page 1 line 35, 'recognized as novel a neuroblast-' should be 'recognized as a novel neuroblast-') and appropriate expression (eg. page 4 line 169, What does the soup means?).

We have corrected grammatical errors according to your comments. The soup is the supernatant. For better understanding, we re-stated the word from soup to the supernatant.

3. It appears that author's English expression make it difficult to imagine their experimental procedure especially for page 3 and 4. English correction by a native-speaker would help understanding these things. Definitely movie will help a lot.

Thank you for the comment, and we have rephrased our paragraphs in page 3 and 4. In addition, we have used English editor service to overcome grammatical mistakes. As you stated, a video will definitely help to follow up with our procedures.

4. I found some incorrect information. (eg. page 2 line 80. Not many current protocols use whole brain to isolate NSCs. Even the paper they referenced, Walker et.al., they dissected out subventricular zone and dentate gyrus to isolate adult NSCs.

Sorry for the mistake, and we have revised and erased incorrect information from the revised manuscript.

5. There are several mistakes in concentration or units. Obviously, these information are important, thus author should be again point by point confirmation.
(page 5 line 179, 200 ml should be 200 ul?)

We are sorry for the error. Concentrations and units were correctly stated in the revised manuscript.

6. Table1. Coating materials, PLO, comments, 'Working concentration: 0.1%'. Is this correct? Should be 0.01%.

We have edited accordingly.

Additional Comments to Authors:
N/A

Reviewer #3:

Manuscript Summary:

Jun et al, present a method to isolate and culture subcallosal zone NSCs. This paper is in general well written and of interest to the scientific community. We have only minor concerns that should be addressed.

*On line 65, the sentence should be changed from "The number and size of the cells in the neurosphere…" to "The number of cells and size of the neurosphere…"

We have changed it according to your comments.

*The structure and wording of the paragraph starting on line 88 and continuing to line 92 should be edited since it is confusing (i.e. "Neurospheres could be formed from SCZ-aNSCs in the culture system and maintained continuously as neurospheres or monolayers")

We have edited.

"After SCZ-aNSCs form neurospheres, they also can be maintained as neurospheres or monolayers for expansion"

*Step 1.3 on line 105 should clarify what is the meaning of "prepare the surgical tools".

We have included "autoclaved surgical tools" in this section.

*Step 2.2.2 on line 132 should add a sentence clarifying the skull must be removed to extract the brain.

We have added a description of brain removal from the skull.

"Remove the caudal part of skull (posterior to lambda) first, and then insert the forceps between the skull and brain at the dorsal midline position. Grab the left (or right) parietal bone and carefully peel it off. Repeat this procedure for the removal of the other parietal bone."

*The note under step 2.4 on line 152 should have a reference if published.

We cited two references. And to culture SCZ-aNSCs, we modified the culture method of SCZ-aNSCs compared to other protocols to get a high yield of aNSCs *in vitro*.

*Step 3.2 on line 169 mentions the discarding of the "soup" when it should be supernatant.

Corrected

*Step 3.3 on line 172 should clarify if there is a specific number of cells that should be plated as stated on Figure 1.

We did not count the number of cells prior to plating SCZ-aNSCs. Instead, all single cells that were obtained from digested SCZ brain tissue were plated.

*Step 4.1 on line 182 should add that the neurospheres are transferred into a conical tube before the incubation on Accutase.

Yes, we have added this in Step 4.1.

"Gather neurospheres and transfer to a new 15 ml conical tube."

*The note under step 4.1 on line 185 should clarify if the number of neurospheres quantified is per well or total.

We have added "per well"

*A note needs to be added to Step 5.3 on line 215 indicating that BrdU must be added to live cells before fixation if BrdU staining is of interest.

We have added note that explain BrdU treatment.

"**Note**: BrdU can be incorporated into newly synthesized DNA of proliferating cells. Therefore, if desired, BrdU (10 μg/ml) can be added to live cells before fixation of cells."

*Step 6.2.1 should clarify the units of BrdU (i.e. ug/ml).

We have given the units of BrdU in note of Step 5.3.

"**Note**: BrdU can be incorporated into newly synthesized DNA of proliferating cells. Therefore, if desired, BrdU (10 μg/ml) can be added to live cells before fixation of cells."

*The duration of BrdU incubation should be clarified. The duration of the incubation is defined as overnight on line 265 while on line 232 it's defined as 2 hours.

Thank you for the comment. We have clarified the duration of BrdU incubation time. Two hours incubation is right. We have edited that in manuscript. And we have added this information in the note of Step 5.3.

"**Note**: BrdU can be incorporated into newly synthesized DNA of proliferating cells. Therefore, if desired, BrdU (10 μg/ml) can be added to live cells before fixation of cells."

*On line 318, the sentence discussing cell death occurring due to non-NSCs should be referenced if published.

We have added references.

*On line 337, the sentence discussing the lack of nutrients reaching the core of neurospheres should be referenced if published.

We have added references.

*On line 339, the sentence discussing multiple passages of NSCs should mention there's a decline in multipotency and/or neuronal differentiation after multiple passages if this is the case as it is for adult SVZ NSCs.

We have added accordingly.

Major Concerns:
N/A

Minor Concerns:
N/A
Additional Comments to Authors:
N/A