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GM-free generation of blood-derived neuronal cells

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TITLE:

GM-free generation of blood-derived neuronal cells

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[*]**Dedicated to the memory of Dr. Rainer Saffrich**

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KEYWORDS:

Peripheral blood, reprogramming, signaling, pluripotent stem cells, neural stem cells and cell replacement therapies

SUMMARY:

We present a genetically modified-free (GM-free) method to obtain cells with a neuronal phenotype from reprogrammed peripheral blood cells. Activation of a signaling pathway linked to novel human GPI-linked protein reveals an efficient GM-free method for obtaining human pluripotent stem cells.

ABSTRACT:

Many human neurological disorders are caused by degeneration of neurons and glial cells in the brain. Due to limitations in pharmacological and other therapeutic strategies, there is currently no cure available for the injured or diseased brain. Cell replacement appears as a promising therapeutic strategy for neurodegenerative conditions. To this day, neural stem cells (NSCs) have been successfully generated from fetal tissues, human embryonic cells (ES) or induced pluripotent stem cells (iPSC). A process of dedifferentiation was initiated by activation of the novel human GPI-linked glycoprotein, which leads to generation of pluripotent stem cells. These blood-derived pluripotent stem cells (BD-PSCs) differentiate in vitro into cells with a neural phenotype as shown by brightfield and immunofluorescence microscopy. Ultrastructural analysis of these cells by means of electron microscopy confirms their primitive structure as well as neuronal-like morphology and subcellular characteristics.

INTRODUCTION:

Development of basic and pre-clinical stem cell research methods encourages the clinical application of stem cell-based therapies for neurological diseases. Such potential therapy critically depends on the method for generation of human neural cells leading to functional recovery[1].

Neural stem cells (NSCs) self-renew and differentiate into new neurons throughout life in a process called adult neurogenesis. Only very restricted brain areas harbor NSCs competent to generate newborn neurons in adulthood. Such NSCs can give rise to mature neurons, which are involved in learning and memory, thus replacing lost or damaged neurons. Unfortunately, these NSCs are present in restricted amounts and this limited neurogenesis decreases rapidly during juvenile development[2]. Therefore, other sources of neural cells must be considered in a cell therapy objective.

Degenerative neurological diseases are difficult to cure using standard pharmacological approaches. New therapeutic strategies for embracing many immedicable neurological disorders are based on cell replacement therapies of diseased and injured tissue. NSC transplantation could replace damaged cells and provide beneficial effects. Other sources for neural cell replacement include human embryonic stem cells (ESC), which are derived from the inner cell mass of mammalian blastocysts[3], as well as iPSCs[4], which have extensive self-renewal capacity like ESCs and are capable to differentiate into various cell lineages. NSCs can also be generated by direct reprogramming from human fibroblasts avoiding pluripotent state[5].

Cell replacement therapy is still a challenging issue. Though ESC, fetal, or iPS can be a source for generation of neuronal cells for treating many incurable neurological diseases, autologous adult SCs cell replacement of damaged tissues is a better alternative that circumvents immunological, ethical and safety concerns.

Activation of human GPI-linked protein by antibody-crosslinking *via* phosphorylation of PLCγ/PI3K/Akt/mTor/PTEN initiates a dedifferentiation of blood progenitor cells and generation of blood-derived pluripotent stem cells (BD-PSCs)[6]. These cells differentiate in vitro toward neuronal cells as confirmed by means of brightfield, immunofluorescence and transmission electron microscopy (TEM) analysis.

In this work we describe the GM-free generation of BD-PSCs and their successful re-differentiation into cells with neuronal phenotype.

PROTOCOL:

Ethic approvals were obtained when performing the experiments.

1. Isolation of human peripheral blood mononuclear cells (PBMNCs)

1.1 Ensure that all donors signed informed consent before blood withdrawing in compliance with institutional guidelines.

1.2 Take 30 mL of blood from healthy donors by trained medical personnel according to the standard protocol.

1.3 Isolate PBMNCs by density gradient media. Use 10 mL of media with 25 mL of 1:1 blood diluted with phosphate buffer saline (PBS), and centrifuge at 300 x *g* for 30 min.

1.4 Isolate the interphase layer between the plasma and the density gradient media by pipetting. Wash the isolated cells with 5 mL of sterile PBS and centrifuge at 300 x g for 10 min. Repeat twice.

1.5 Count the number of cells by standard methods using a counting chamber.

2. Activation of human GPI-anchored glycoprotein by antibody crosslinking on the surface of PBMNCs

2.1 Place the 6 x 10^6 mononuclear cells (MNCs) in 15 mL tubes and perform antibody crosslinking by incubating the cells with human GPI-linked membrane protein-specific antibody (30 µg/mL) for 30 min in PBS with 1% bovine serum albumin (BSA) at 37 °C.

2.2 Replace incubation medium with Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (FBS).

2.3 Grow cells in 15 mL polystyrene tubes, put the tubes in an incubator at 37 °C and 5% CO_2 for 8-10 days (without shaking). On D5, add an additional 1-2 mL of Iscove's medium supplemented with 10% FBS to each 15 mL tube.

3. Sorting of newly generated dedifferentiated cells

3.1 Count cells with an automated cell counter (18 µL cell suspension + 2 µL fluorescence dye) or in a counting chamber.

3.2 Centrifuge cultured cell suspension (5-7 x 10^6) at 300 x *g* for 10 min and aspirate the resulting supernatant with a sterile Pasteur pipette.

3.3 Re-suspend the cell pellet in 90 µL of pre-cooled PBS pH 7.2, 0.5% BSA and 2 mM EDTA.

3.4 Add CD45 positive nano-sized magnetic beads (80 µL) to the cell suspension and incubate on ice for 15 min.

3.5 Wash the cells by adding 2 mL of PBS buffer and centrifuge at 300 x *g* for 10 min.

3.6 Re-suspend the cells in 500 µL of PBS buffer.

3.7 Wash the column with 500 µL of pre-cooled PBS buffer and place it in the magnetic field.

3.8 Place the cell suspension on the column and wash it with 500 µL of PBS buffer (two times) and the centrifuge flow containing CD45 negative cells. Collect them in Iscove's medium supplemented with 1% BSA.

3.9 Count the cells in the counting chamber.

4. Preparing cell culture dishes for neuronal differentiation of newly generated stem cells

4.1 Coat the culture vessels with poly-L-ornithine and laminin for growing neuronal cells.

4.2 Place the glass coverslips in 4-well plates and coat it with 1:5 diluted poly-L-ornithine (0.1 mg/mL in ddH_2O) in ddH_2O. Place the coverslips into a 37 °C incubator for 1 h. Then wash with ddH_2O.

4.3 Slowly thaw laminin (0.5-2.0 mg/mL) and add to the top of coverslips. Incubate it at 37 °C for 2 h.

4.4 Prepare neural induction medium N2 consisting of 49 mL of D-MEM/F12, 500 µL of N2 supplement, 400 µL of non-essential amino acids (NEAA), basic FGF solution at 20 ng/mL final concentration (prepared from 100 µg/mL stock solution), and heparin at 2 ng/mL final concentration.

4.5 Remove excess laminin by pipetting and add neuronal medium N2 to culture dishes.

5. Culturing of neuronal dedifferentiated blood cells

5.1 Culture BD-derived CD45 negative cells on laminin/ornithine-coated glass coverslips for 2 days in an incubator at 37 °C and 5% CO_2 in N2 medium to initiate a neuronal differentiation of newly BD-generated cells.

5.2 Culture cells further in neuronal differentiation medium consisting of 48 mL of Neurobasal medium, 500 µL of L-glutamine, 1 mL of B27 Supplement, 500 µL of NEAA, 50 µL of recombinant human glial-derived neurotrophic factor (GDNF) at 5 µg/250 µL in PBS/0.1% BSA, and 50 µL of recombinant human brain derived neurotrophic factor (BDNF) at 5 µg/200 µL in PBS/0.1% BSA and 50 µL of ascorbic acid solution 2.9 g/50 mL in PBS. Place plates in an incubator at 37 °C and 5% CO_2.

6. Immunofluorescence microscopy analysis of blood-derived neural cells

6.1 Culture the cells as described above for 16 days and remove the media.

6.1.1 Incubate with pre-warmed fixative consisting of 75 mL of sterile water, 4 g of paraformaldehyde. Add 10 N NaOH as needed and stir until the solution clears. Then add 10 mL of 10x PBS, 0.5 mL of $MgCl_2$, 2 mL of 0.5 M EGTA, and 4 g of sucrose. Titrate to pH 7.4 with 6 N HCl, and bring to 100 mL of sterile water for 15 min, according to Marchenko et al.[7].

6.1.2 Discard the fixative and wash the cells 3 times for 5 min each time. Immediately add a freshly made 0.3% Triton X-solution and permeabilize the cells for 5 min. Wash 3 times with PBS and add a blocking solution made by PBS and 5% BSA.

6.1.3 Block the cells at room temperature on a rocker plate for 1 hour.

6.1.4 Prepare appropriate dilution of antibodies in 1% BSA/PBS and incubate the cells with antibody dilutions on rocker plate for 1.5 h at room temperature. Wash the cells 3 times with PBS for 5 min each, incubate the cells with DAPI and mount the coverslips with mounting media for visualization on a microscope.

NOTE: Directly labeled antibodies used in this experiment are listed in **Table of Materials**.

7. Transmission electron microscopy analysis of newly generated cells

7.1 Seed the cells for TEM in 8-well chamber slides.

7.2 Fix the cells in 3.5% glutaraldehyde for 1 h at 37 °C, post-fix in 2% OsO_4 for an additional hour at room temperature and stain in 2% uranyl acetate in the dark at 4 °C for 2 h 30 min.

7.3 Finally, rinse the cells in distilled water, dehydrate it in ethanol and embed in epoxy resin overnight. The following day transfer the samples to a 70 °C oven for 72 h for resin hardening.

7.4 Detach the embedded cell cultures from chamber slide and glue to araldite blocks.

7.5 Cut serial semi-thin sections (1.5 µm) with a machine, mount onto glass-slides and lightly stain with 1% toluidine blue.

7.6 Glue selected semi-thin sections to araldite blocks and detach them from the glass slide by repeated freezing (in liquid nitrogen) and thawing.

7.7 Prepare ultrathin sections (0.06-0.08 µm) with a machine and further contrast with lead citrate.

7.8 Obtain micrographs by using electron scanning microscope with digital camera.

REPRESENTATIVE RESULTS:

The results provide evidence that this novel GM-free method is capable of reverting blood progenitor cells to their most primitive state without directly acting on the human genome.

We have previously shown that GPI-linked protein specific antibody crosslinking initiates *via* PLCγ/IP3K/Akt/mTOR/PTEN upregulation of highly conserved developmentally relevant genes such as WNT, NOTCH and C-Kit, thus initiating a process of dedifferentiation that leads to the first step to generation of HSCs and a second and final to a generation of BD-PSCs[6,8].

Activated MNC cultures were subjected to immunomagnetic sorting using CD45 microbeads. Mature blood cells that cannot be reprogrammed with this method (e.g., CD45 positive cells) were retained on the column, whereas the negative fraction containing reprogrammed cells (CD45 negative cells) was used for generation of various neuronal lineage cells.

We first studied the morphological aspects of peripheral BD-dedifferentiated cells by means of light and TEM. As shown in **Figure 1**, specific GPI-anchored glycoprotein antibody crosslinking of human MNCs generates a steady growing new population of cells (**Figure 1A**). We analyzed these cells by means of TEM. BD-dedifferentiated cells are small in size and show the characteristics of immature agranular cells, with gradually less organelles and large nuclei with condensed chromatin, similar to ESCs (**Figure 1B**). Non-treated cultures showed a trend towards a gradual disappearance.

BD-CD45 negative cells were subjected to neuronal differentiation in two steps. We initiated the differentiation towards neuronal lineages by seeding the CD45 negative cells on poly-L-ornithine/laminin coated culture plates for 2 days in N2 medium following culture in neuronal differentiation medium. Brightfield pictures were acquired at days 4, 8, 10, 14 and 30 respectively upon starting neuronal differentiation of BD-generated stem cells.

As early as 4 days after starting the targeted differentiation of newly generated cells, the first neuronal-like cells with long branching structures could be detected. We observe the morphological changes from D2 to D30 with a more complex structure including ramification, implying an active process toward differentiation to neuronal lineages throughout the culture time period (**Figure 2**). To confirm the neuronal features of re-differentiated cells after culturing them in neuronal medium for 16 days, cells were fixed according to a previous protocol[7], and immunocytochemistry (ICC) was performed using antibody detection to nestin, glial fibrillary acidic protein (GFAP), microtubule-associated protein 2 (MAP2) and neuron-specific class III beta-tubulin (Tuj1).

GFAP is the protein that constitutes a portion of cytoskeleton in astrocytes representing the principal intermediate filament of mature astrocytes. As shown in **Figure 3**, the antibody to GFAP recognizes these structures in the newly generated neuronal cells, confirming that BD-PSCs are capable of re-differentiation towards human astrocytes[9].

MAP2 is a cytoskeleton protein that binds to tubuli and stabilizes microtubules. It is expressed within axons, dendrites and cell bodies and this expression is tissue- and developmentally-specific. The immunofluorescence microscopy results confirm the expression of this protein in re-differentiated cells[10].

Tuj1 is typical neuronal cell marker. Its function is to stabilize microtubili in neuronal cell body and axons. It is also implicated in axonal transport[11]. Newly re-differentiated cells clearly confirmed the expression of this protein at D16 upon starting the neuronal differentiation under the condition described here.

Nestin was first characterized in NSCs and represents a neuro epithelial stem cell protein, which belongs to intermediate filament (IF) protein[12] distinguishing neuronal progenitor cells from more differentiated neuronal cells. These IF proteins are expressed mostly in nerve cells where they are involved in the radial growth of the axon, but they are also present in a number of additional tissues. Nestin as a marker of predominantly NSCs is weakly expressed in the cells already on the path to differentiate into specific neuronal lineages as it is the case with BD-re-differentiated cells at D16.

Figure 1: Generation of dedifferentiated (pluripotent) stem cells

(A) Ficoll-isolated mononuclear cells were grown in Iscove's medium supplemented with 10% FBS. Micrographs of activated cultured cells were taken at days 1, 5 and 10, respectively. Non-activated MNCs were studied as a control. Scale bar: 50 μm. **(B)** TEM analysis of newly generated cells throughout culture time shows that organelles of mature cells (D1) gradually disappear (D8), leading to generation of completely dedifferentiated cells resembling ESCs.

Figure 2: Neuro re-differentiation of BD-PSCs

(A) BD-dedifferentiated cells were placed in ornithine/laminin coated culture dishes and cultured for 30 days as described in protocol. Micrographs are taken at days 4, 8, 10, 14, and 30 respectively, after growing in neuronal differentiation conditions. Most cells in the culture changed their morphology from small spherical shapes to larger, elongated shapes and in some cases branched cells. Scale bar: 100 μm. **(B)** BD-dedifferentiated cells were grown for 16 days in neuronal medium, fixed in glutaraldehyde and EM analysis performed as described in protocol section. The cell body and processes of these cells showed a higher complexity than those of undifferentiated cell in terms of organelles and cytoskeleton presenting a high density of stacked cisternae of rough endoplasmic reticulum and abundant bundles of actin filaments **(a, b)**. Unlike undifferentiated BD-cells, cells growing in differentiation media frequently established cell-to-cell contacts. Some of these specialized contacts involved cellular body **(c)** while others involved cellular processes in neurite-like fashion **(d).** Scale bars: **(a)** 20 μm; **(b-d),** 500 nm.

Figure 3: Immunophenotyping of newly generated neuronal cells

BD-dedifferentiated cells were cultured as described in the protocol for 16 days and immunocytochemistry analysis was performed using antibodies to neuronal markers nestin, GFAP, MAP2 and Tuj1. Shown are brightfield micrographs of re-differentiated cells accompanied with immunofluorescence pictures with DAPI as nuclear staining, as well as staining with relevant antibodies. Depicted are the fields showing a particular population that expresses one of the specific neuronal marker characteristic for specific lines. Scale bar: 100 μm. Control is presented in **Supplementary Figure 1**.

Supplementary Figure 1: BD-dedifferentiated cells control. BD-derived undifferentiated cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% FBS for 16 days as described in protocol and stained with antibody to nestin GFAP, Tuj1 and MAP2. DAPI was used for nuclear staining. Scale bar: 100 μm.

DISCUSSION:

The non-GM method of reprogramming human cells described in this work is based on membrane to nucleus activation of signaling(s) machinery behind the GPI-linked human membrane glycoprotein that initiates the process of dedifferentiation leading to the ex vivo generation and expansion of self-renewing PSCs obtained from non-manipulated human peripheral blood. These cells when cultured in appropriate media are capable of re-differentiation into cells belonging to different germ layers[1].

The data presented in this work show that GM-free generated BD-PSCs cells when cultured in a neuronal differentiation media acquired a completely different phenotype, with

elongated shapes, higher development of their organelles and established more complex interactions between cells. Moreover, re-differentiation using condition described here, implies neuronal differentiation towards various neuronal lineages.

To obtain the optimal number and the best quality of reprogrammed cells for their use in re-differentiation studies, fresh MNC preparations might be advantageous when compared to frozen MNC preparations. The method of immunomagnetic sorting that separated BD-PSCs from terminally differentiated cells that cannot be reprogrammed by this method could be incomplete requiring that the procedure be repeated, which is very stressful for cells and results in their premature deaths.

The critical step within the protocol relates to the number and quality of MNCs that could be obtained by the method described. Modification of the neural differentiation media as well as culture time can improve the differentiation potential of BD-PSCs, thus leading to generation of specific types of neuronal cells.

A limitation of this method is the non-teratogenic nature of these reprogrammed cells as it is not possible to generate the cell lines with this method. Once the dedifferentiated cells have reached the final stage, that of pluripotency, they become mostly quiescent and a new portion of MNCs must be dedifferentiated again to obtain a higher number of BD-PSCs.

Reprogramming described here relies on antibody crosslinking activation on the surface of blood progenitor cells. This paradigm provides numerous potential advantages with respect to clinical safety when compared to GM methods. The goal of achieving autologous stem cells for generation of neural tissue(s) can be achieved by minimally ex vivo manipulation; therefore strongly suggesting that this cell therapy could be a promising candidate for efficient and safe clinical approach in neurology.

Parkinson's disease, Alzheimer's disease and cerebral ischemia are among the diseases with the highest social and economic burden for the society in Europe and worldwide. The burden of neurodegenerative disorders is expected to increase with the aging population, becoming an important socio-economic problem and creating a desperate need for an answer to the problem. The presented method opens a new avenue for non-invasive therapeutic strategies by utilizing a simple and cost-effective procedure for generating suitable autologous stem cell populations holding a hope for the cure of currently intractable neurological diseases.

ACKNOWLEDGEMENTS:

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DISCLOSURES:

The corresponding author declares that she is a patent holder related to Novel Human GPI-linked Protein as well as she co-founded and works for ACA CELL Biotech. The other authors declare that they do not have any conflict of interest.

REFERENCES:

1 Peng, J. and Zeng, X. The Role of Induced Pluripotent Stem Cells in Regenerative Medicine: Neurodegenerative Diseases. *Stem Cell Research and Therapy.* **2** (4), 32 (2011).

2 Sorells, S. F. et al. Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature*. **555** (7696), 377-381 (2018).

3 Thomson, J. A. et al. Embryonic Stem Cell Lines Derived from Human Blastocysts. *Science*. **282** (5391), 1145-1147 (1998).

4 Takahashi, K., et al. Induction of Pluripotent Stem Cells From Adult Human Fibroblast by Defined Factors. *Cell*. **131** (5), 861-872 (2007).

5 Liu, G.-H., Yi, F., Suzuki, K., Qu, J., Izpisua Belmonte, J. C. Induced neural stem cells: a new tool for studying neural development and neurological disorders. *Cell Research*. **22** (7), 1087-1091 (2012).

6 Becker-Kojić, Z. A. et al. Activation by ACA Induces Pluripotency in Human Blood Progenitor Cells. *Cell Technologies in Biology and Medicine*. **2**, 85-101 (2013).

7 Marchenko, S., Flanagan, L. Immunocytochemistry: Human Neural Stem Cells. *Journal of Visualized Experiments*. **7**, 267 (2007).

8 Becker-Kojić, Z. A. et al. A novel glycoprotein ACA is upstream regulator of human heamtopoiesis. *Cell Technologies in Biology and Medicine*. **2**, 69-84 (2013).

9 Li, D. et al. Neurochemical Regulation of the Expression and Function of Glial Fibrillary Acidic Protein in Astrocytes. *Glial*. **68** (5), 878-897 (2020).

10 Melková, K. et al. Structure and Functions of Microtubule Associated Proteins Tau and MAP2c: Similarities and Differences. *Biomolecules*. **9** (3), 105 (2019).

11 Menezes, J. R. and Luskin, M. B. Expression of neuron-specific tubulin defines a novel population in the proliferative layers of the developing telencephalon. *Journal of Neuroscience*. **14** (9), 5399-5416 (1994).

12 Bernal, A. and Arranz, L. Nestin-expressing progenitor cells: function, identity and therapeutic implications. *Cellular and Molecular Life Sciences*. **75** (12), 2177-2195 (2018).

Figure

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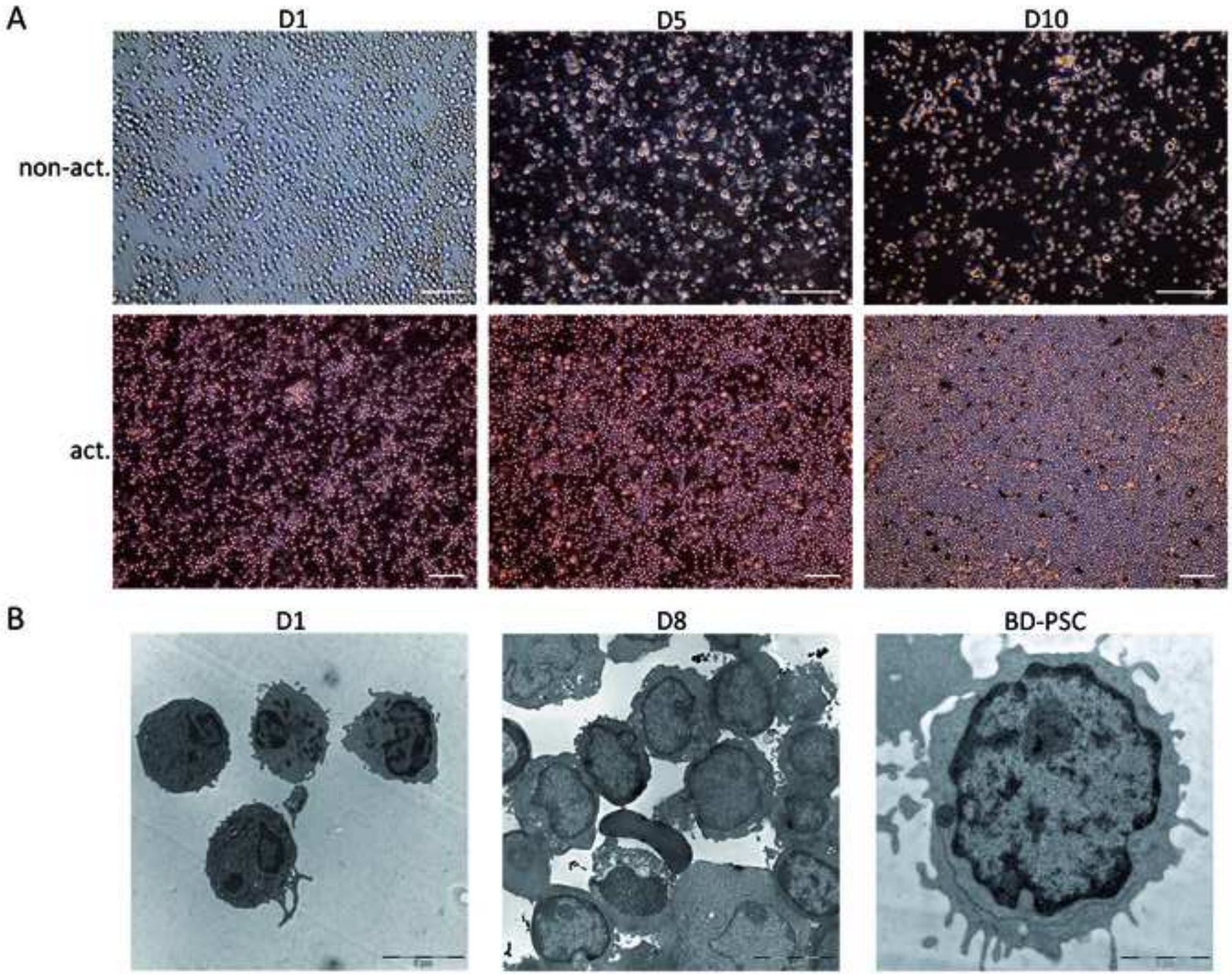
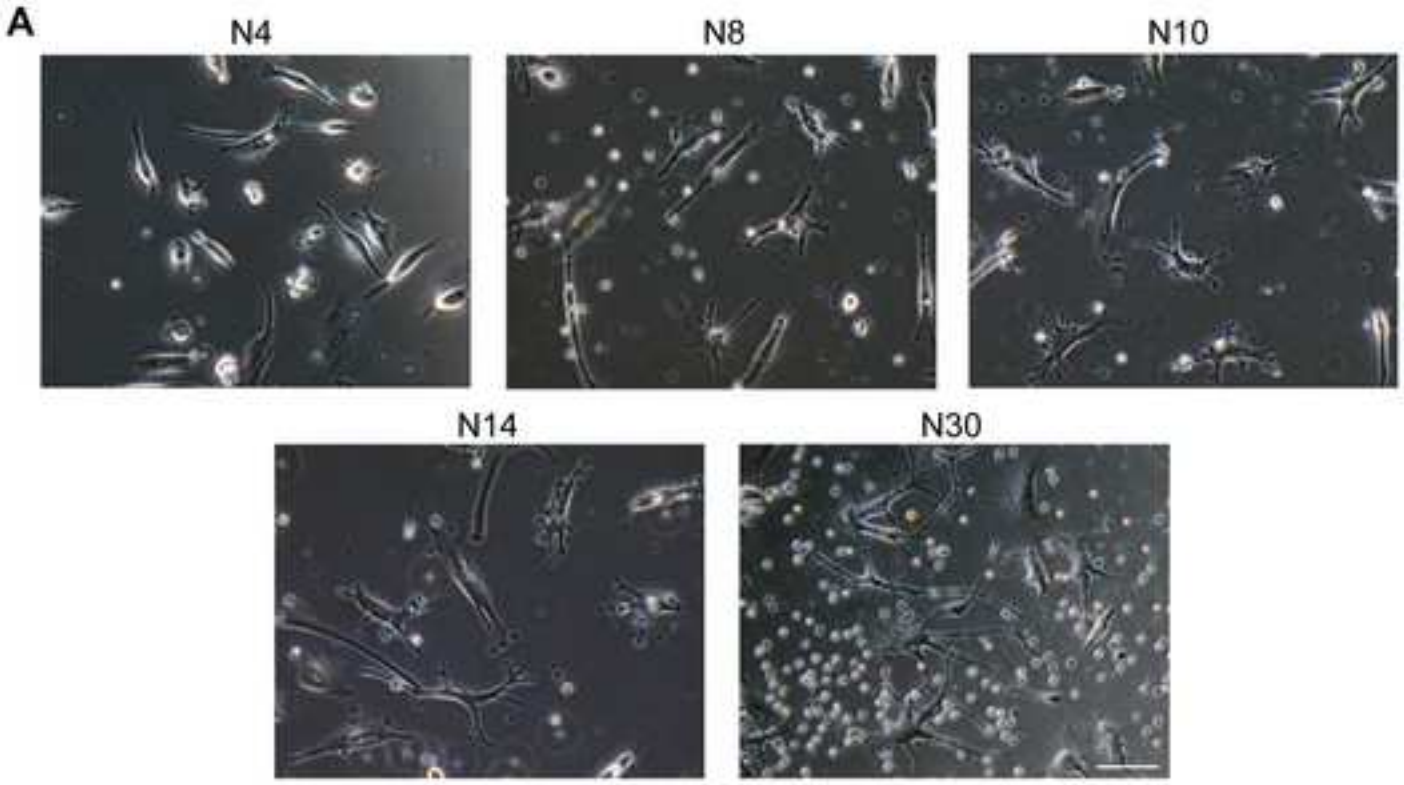
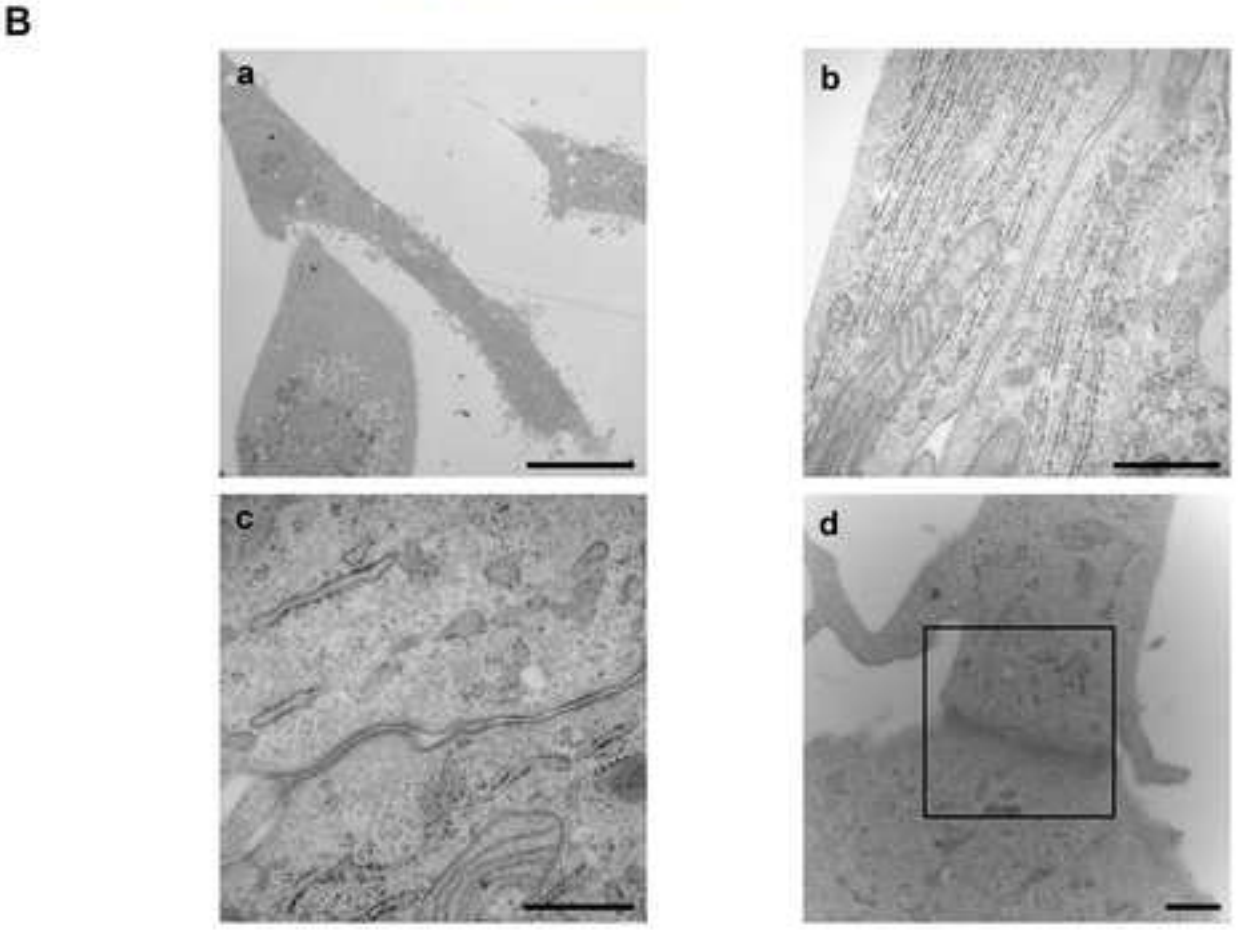


Figure





Figure

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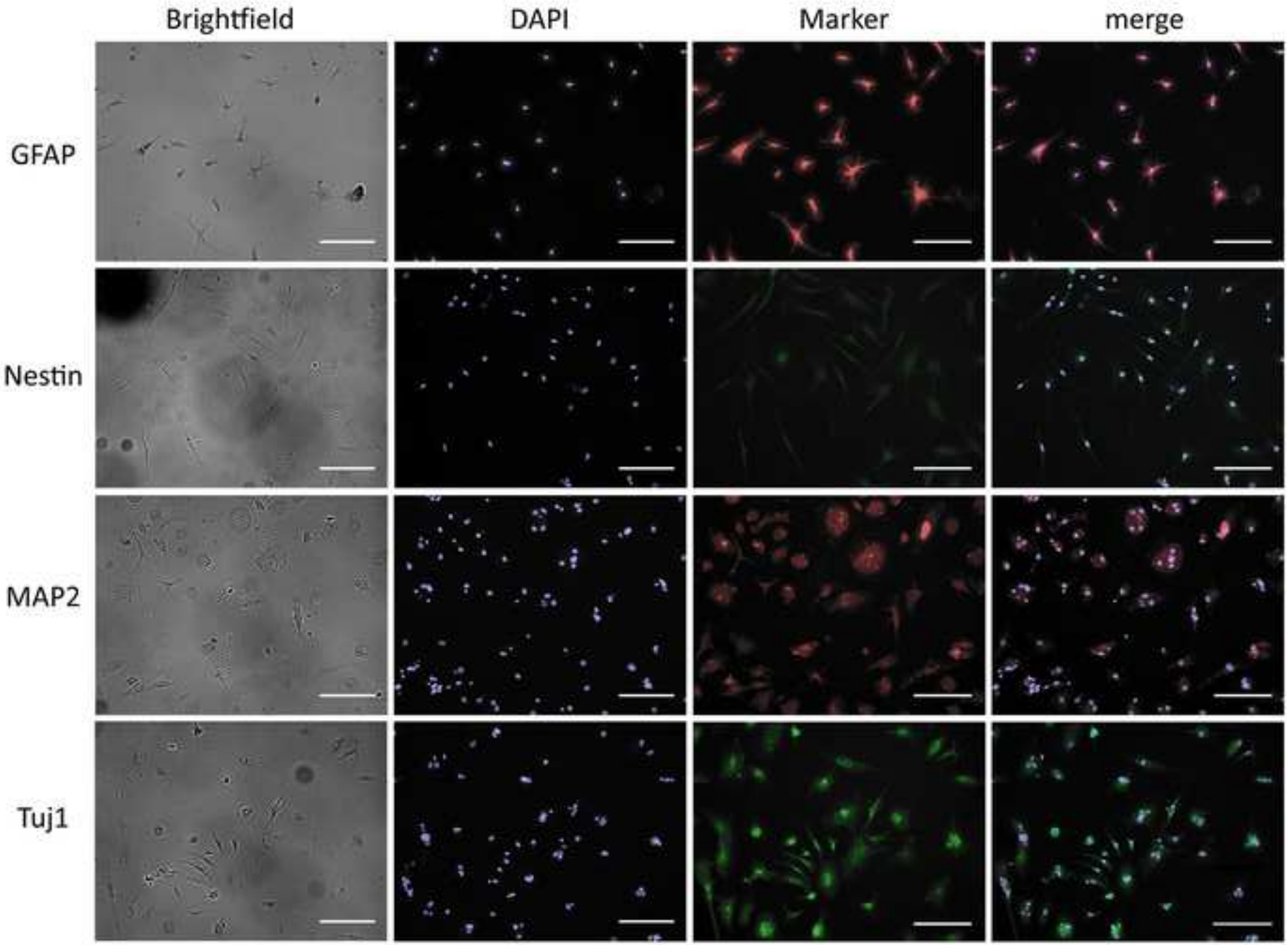

Table of Materials

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
Albumin Fraction V	Roth	T8444.4	
Anti-GFAP Cy3 conjugate	Merck Millipore	MAB3402C3	
Anti-MAP2 Alexa Fluor 555	Merck Millipore	MAB3418A5	
Anti-Nestin Alexa Fluor 488	Merck Millipore	MAB5326A4	
Anti-Tuj1 Alexa Fluor 488	BD Pharmingen	560381	
AO/PI Cell Viability kit	Biozym	872045	Biozym discontinued. The product produced by Logos Biosystems.
Ascorbic acid 2-phosphate sequimagnes	Sigma Aldrich	A8960-5G	
B27 Serum free 50x	Fisher Scientific (Gibco)	11530536	
Basic FGF solution	Fisher Scientific (Gibco)	10647225	
Biocoll	Merck Millipore	L6115-BC	density gradient media
BSA Frac V 7.5%	Gibco	15260037	
CD45 MicroBeads	Miltenyi	130-045-801	nano-sized magnetic beads
Cell counting slides Luna	Biozym	872010	Biozym discontinued. The product produced by Logos Biosystems.
Chamber Slides Lab-Tek	Fisher Scientific	10234121	
D-MEM/F12	Merck Millipore	FG4815-BC	
Durcupan	Sigma Aldrich	44610	epoxy resin
FBS	Merck Millipore	S0115/1030B	Discontinued. Available under: TMS-013-B
GDNF recombinant human	Fisher Scientific (Gibco)	10679963	
GlutaMax 100x	Gibco	35050038	L-glutamine

Glutaraldehyde grade	Sigma-Aldrich	G5882-50ML	
Heparin sodium cell	Sigma-Aldrich	H3149-50KU	
Human BDNF	Fisher Scientific (Gibco)	11588836	
Iscove (IMDM)	Biochrom	FG0465	
Laminin mouse	Fisher Scientific (Gibco)	10267092	
Lead citrate	Sigma-Aldrich	15326-25G	
Luna FL Automated Cell Counter	Biozym	872040	Biozym discontinued. The product produced by Logos Biosystems.
MACS Buffer	Miltenyi	130-091-221	
MEM NEAA 100x	Gibco	11140035	
MiniMACS Trennsäulen	Miltenyi	130-042-201	
Morada digital camera	Olympus		
Multiplatte Nunclon 4 wells	Fisher Scientific	10507591	
N2 Supplement 100x	Fisher Scientific (Gibco)	11520536	
Neurobasal Medium	Gibco	10888022	
PBS sterile	Roth	9143.2	
Poly-L-ornithine	Sigma-Aldrich	P4957-50ML	
Super Glue-3 Loctite	Henkel		
TEM FEI Technai G^2 Spirit	FEI Europe		
Ultracut UC-6	Leica		
Uranyl acetate C	EMS	22400	

Dear Dr. Nam Nguyen

We have corrected the manuscript according to editorial requirements. Please find the changes below.

Response to editorial comments

1. Please revise the following lines to avoid previously published text.

38-41: A process of dedifferentiation was initiated by activation of the novel human GPI-linked glycoprotein, that leads to generation of pluripotent stem cells[1]. These blood-derived pluripotent stem cells (BD-PSCs) differentiate *in vitro* into cells with neural phenotype as shown by brightfield and immunofluorescence microscopy.

47-48: Development of basics and pre-clinical stem cell research methods encourages the perspective for the clinical application of stem cell-based therapies for neurological diseases.

60-64: Degenerative neurological diseases are difficult to cure using standard pharmacological approaches. New therapeutic strategies for embracing many immedicable neurological disorders are based on cell replacement therapies of diseased and injured tissue.

65: damaged cells and provide

71-74: Cell replacement therapy is still a challenging issue. Though ESC, fetal, or iPS can be a source for generation of neuronal cells for treating many incurable neurological diseases, autologous adult SCs cell replacement of damaged tissues is better alternative that circumvent immunological, ethical and safety concerns.

76-80: Activation of human GPI-linked protein by antibody-crosslinking *via* phosphorylation of PLCγ/PI3K/Akt/mTor/PTEN initiates a dedifferentiation of blood progenitor cells and generation of blood-derived pluripotent stem cells (BD-PSCs)[1.] These cells differentiate *in vitro* toward neuronal cells as confirmed by means of brightfield, immunofluorescence and transmission electron microscopy (TEM) analysis.

248-249: We observe the morphological changes from D2 to D30 with more complex structure including ramification, implying an active process toward differentiation to neuronal lineages throughout culture time period.

265-267: Tuj1 is typical neuronal cell marker. Its function is to stabilize microtubili in neuronal cell body and axons. It is also implicated in axonal transport[11].

271-273: Nestin was first characterized in NSCs and represents a neuro epithelial stem cell protein, which belongs to intermediate filament (IF) protein[12] distinguishing neuronal progenitor cells from more differentiated neuronal cells.
274: involved instead of implicated

2. „Method for “is removed from the title.

3. Ethics statement
91: (ethic approvals were obtained when performing the experiments).

4.
1.3 Isolate PBMNCs by density gradient media, use 10 mL of media with 25 mL of 1:1 blood diluted with phosphate buffer saline (PBS), centrifuge at 300 x *g* for 30 min.

5.
1.4 Isolate the interphase layer between plasma and density gradient media by pipetting and wash the isolated cells with 5 ml sterile PBS and centrifuge at 300 x g for 10 min. Repeat twice.

6.
2.1 Place the 6 x 10^6 mononuclear cells (MNCs) in 15 mL tubes and perform antibody crosslinking by incubating the cells with human GPI-linked membrane protein-specific antibody (30µg/mL) for 30 min in PBS with 1% bovine serum albumin (BSA) at 37 °C.

7.
3.1 is removed and following steps renumbered.

8.
3.3 is replaced by 3.2 Centrifuge cultured cell suspension (5-7 x 10^6) at 300 x *g* for 10 min and aspirate the resulting supernatant with sterile Pasteur pipette.

9.
5.1 Culture BD-derived CD45 negative cells on laminin/ornithine-coated glass cover-slips for 2 days in incubator at 37 °C, 5% CO_2 in N2 medium to initiate a neuronal differentiation of newly BD-generated cells.

5.2 Culture cells further in neuronal differentiation medium consisting of 48 mL of Neurobasal medium, 500µL L-glutamine, 1 mL B27 Supplement, 500 µL NEAA, 50 µL recombinant human glial-derived neurotrophic factor (GDNF) at 5 µg/250 µL in PBS/0.1 % BSA, and 50 µL recombinant human brain derived neurotrophic factor (BDNF) at 5 µg/200µL in PBS/0.1 % BSA and 50 µL ascorbic acid solution 2.9 g/50 mL in PBS, place plates in incubator at 37 °C, 5% CO_2.

10. Abbreviations incorporated into the manuscript

252: immunocytochemistry (ICC)

253: Glial fibrillary acidic protein (GFAP), microtubule-associated protein 2 (MAP2) and neuron-specific class III beta-tubulin (Tuj1)

429-456 deleted

Manuscript changes

Protocol

128: 3.3 Re-suspend cell pellet in 90 µL of pre-cooled PBS pH 7.2, 0.5 % BSA and 2 mM EDTA.

Discussion

335: Moreover, re-differentiation using condition described here, implies neuronal differentiation towards various neuronal lineages.

345: specific types

Figure 3 legend updated

312: Depicted are the fields showing a particular population that expresses one of the specific neuronal marker characteristic for specific lines.

Table of Materials updated (see Reviewer #4)

Response to comments from Peer-Reviewer:

Reviewer #2

Major Concerns:

1. The cells shown in Figure 3 represent the different populations of cells. Depicted are the populations expressing either Tuj1 or GFAP showing that differentiation of BD-PSCs using the conditions described in the manuscript go towards various neuronal lineages.
2. The images shown in Appendage II were the results from different protocol and differentiation time therefore it cannot be used in this manuscript.

Reviewer #4

Major concerns:

Regarding Figure 1, the complete geno- and pheno- type analysis, *in vitro* and *in vivo* differentiation of BD-PSCs into three germ-layers as well as a mechanism of action of GPI-protein has been published and cited in the manuscript (citation: 1 and 8). We did ultrastructure analysis of BD-PSCs versus neuronal differentiated cells in order to compare morphological changes and capability of the cells originated from blood, to re-differentiate showing the changes at the structural level.

Regarding Figure 3, our intention is to show that re-differentiation using the condition described in this manuscript, enable the differentiation towards different neuronal lineages. The cells shown in this Figure represent the various populations of the cells expressing the specific marker. So, it is possible to drive the differentiation toward **single** specific lineages, but that requires longer different time and adjusted culture conditions.

Minor concerns:

1-4. The detailed information regarding line 115, line 160, line 171, as well as line 181 is provided in the corrected manuscript.

5. Table of Materials was updated

row 8: comment added: Biozym discontinued. The product produced by Logos Biosystems

row 10: company adjusted to Fisher Scientific (Gibco), hyphen removed in catalog number column
row 11: company adjusted to Fisher Scientific (Gibco), hyphen removed in catalog number column
row 12: BC added in catalog number column
row 13: hyphen removed in catalog number column
row 15: comment added: Biozym discontinued. The product produced by Logos Biosystems
row 16: Thermo removed in company column
row 19: comment added: discontinued. new catalog number: TMS-013-B
row 20: company adjusted to Fisher Scientific (Gibco), hyphen removed in catalog number column
row 21: hyphen removed in catalog number column, comment added: L-glutamine
row 24: company adjusted to Fisher Scientific (Gibco), hyphen removed in catalog number column
row 26: company adjusted to Fisher Scientific (Gibco), hyphen removed in catalog number column
row 27: catalog number adjusted to 15326-25G
row 28: comment added: Biozym discontinued. The product produced by Logos Biosystems
row 30: hyphen removed in catalog number column
row 33: Thermo removed in company column
row 34: company adjusted to Fisher Scientific (Gibco), hyphen removed in catalog number column
row 35: hyphen removed in catalog number column
row 36: A removed in catalog number column

Reviewer #5

Gene expression profiling indicating expression of pluripotency associated genes at RNA and protein level, *in vitro and in vivo* differentiation into cells of all three germ layers are demonstrated in the previous publications (see in References 1 and 8). Described is also the molecular mechanism behind the specific activation of GPI-linked receptor (Reference 8).

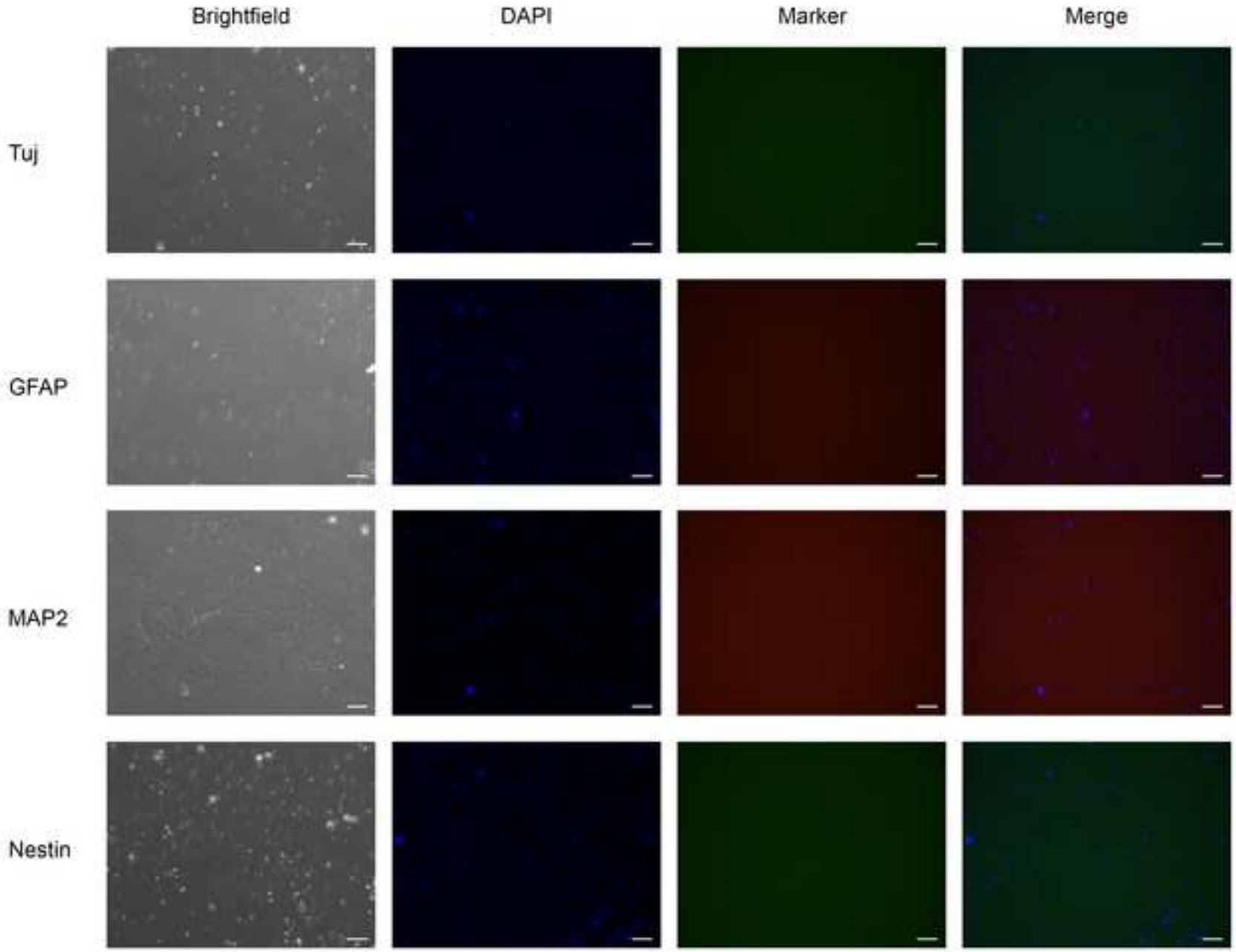
We have provided the protocol for enabling differentiation towards various neuronal lineages. The variation of media and differentiation time could provide the re-differentiation of BD-PSCs towards **single** specific neuronal cells and therefore the functional assays should be a topic for further analysis of neuronal generated cells using the technique described in this manuscript.

Thank you for your consideration!

Best regards,

Dr. Zorica Becker-Kojić

Supplementary Figure 1 - Negative Control For Submitted Figure 3
D16



Appendage I

Manuscript changes

Abstract:

40: citation 1 Becker-Kojić, Z. A. *et al*. Activation by ACA induces pluripotency in human blood progenitor cells. *Cell Technologies in Biology and Medicine*, **2**, 85-101 (2013).

43: neuronal-like morphology

Introduction:

50: citation 1 into 2

57: citation 2 into 3

66: citation 3 into 4

67: citation 4 into 5

69: citation 5 into 6

79: citation 1 instead of 6, toward neuronal cells

83: GM-free generation of BD-PSC

84: neuronal phenotype

Protocol:

87-216: Protocol section is changed into imperative and brand names removed

117: 3. Sorting of newly generated dedifferentiated cells

172:) eliminated

Representative results:

224,225: that leads to the first step to generation of HSCs and a second and final to a generation of BD-PSCs [1,8].

230: was used for generation of various neuronal lineage cells

243, 244: 4, 8, 10, 14 and 30 respectively

250: neuronal features

256: As shown in Figure 3

257: neuronal cells

258: towards human astrocytes, citation 8 into 9

263: citation 9 into 10

265: Tuj 1

268, 269: expression of this protein at D16 upon starting the neuronal differentiation under the condition described here[10]. Citiation 10 into 11

immunofluorescence staining showed that the majority of differentiated cells were Tuj1 positive neurons while GFAP glial cells were also present - eliminated

269: citation 11 into citation 12

274-276: as a marker of predominantly progenitor cells is weakly expressed in the cells already on the path to differentiate into specific neuronal lineages as it is the case with BD-re-differentiated cells at D16.

Figure legends:

Figure 1

285: days 1, 5 and 10

Figure 2

292-304: Figure 2 legends changed completely

Figure 3

309: neuronal marker

Supplementary Figure 1 added

317-319: controls are presented in supplementary Figure 1

Discussion:

325-371: Discussion is newly written according to editorial comments

Literature:

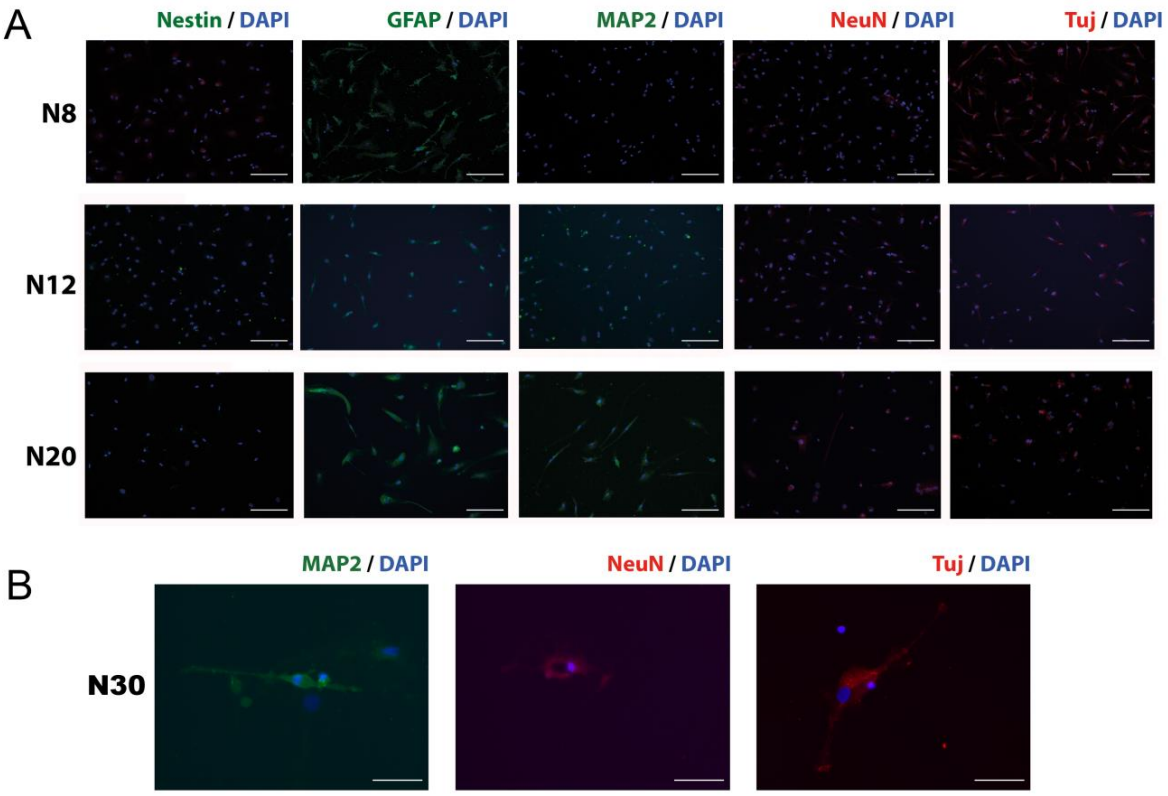
390-391: citation 1 added: Becker-Kojić, Z. A. et al. Activation by ACA Induces Pluripotency in Human Blood Progenitor Cells. *Cell Technologies in Biology and Medicine*. **2**, 85-101 (2013).

393-427: Citations newly numbered and Journals fully written

421: Literature 11 exchanged Menezes, J. R. and Luskin, M. B. Expression of neuron-specific tubulin defines a novel population in the proliferative layers of the developing telencephalon. *Journal of Neuroscience*. **14**, 5399-5416 (1994).

431-456: List of Abbreviations added

Appendage II



ACA blood-derived pluripotent stem cells (BD-PSCs) were subjected to neuronal differentiation in appropriate culture medium and re-differentiation to neuronal cells was observed in time course modus.

Graphic shows the expression of cell-type specific markers used for immunocytochemical (ICC) analysis of neuronal subtypes.