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Isolation, Propagation and Prion Protein Expression During Neuronal Differentiation of Human Dental Pulp Stem Cells

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Dear Editor in Chief,

I would like to submit the revised manuscript entitled "**Isolation, propagation and prion protein expression during neuronal differentiation process of Humand Dental Pulp Stem Cells"**, by Martellucci S. *et al.*, to be considered for publication as research paper in the **"Journal of Visualized Experiments".** We believe these findings will be of interest to the readers of your journal. We declare that this manuscript is original, has not published before and is not currently being considered for publication elsewhere. As corresponding author, I confirm that the manuscript has been read and approved for submission by all the named authors.

With my best regards
sincerely yours

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TITLE

Isolation, Propagation and Prion Protein Expression During Neuronal Differentiation of Human Dental Pulp Stem Cells

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KEYWORDS

Dental pulp, adult stem cells, mesenchymal stem cells, differentiation process, prion protein, prions.

SUMMARY

Here we present a protocol for human Dental Pulp Stem Cells isolation and propagation in order to evaluate the prion protein expression during neuronal differentiation process.

ABSTRACT

Bioethical issues related to the manipulation of embryonic stem cells have hindered advances in the field of medical research. For this reason, it is very important to obtain adult stem cells from different tissues such as adipose, umbilical cord, bone marrow and blood. Among the possible sources, dental pulp is particularly interesting because it is easy to obtain in respect of bioethical considerations. Indeed, human Dental Pulp Stem Cells (hDPSCs) are a type of adult stem cells able to differentiate in neuronal-like cells and can be obtained from the third molar of healthy patients (13-19 ages). In particular, the dental pulp was removed with an excavator, cut into small

slices, treated with collagenase IV and cultured in a flask. To induce the neuronal differentiation, hDPSCs were stimulated with EGF/bFGF for 2 weeks. Previously, we have demonstrated that during the differentiation process the content of cellular prion Protein (PrP^C) in hDPSCs increased. The cytofluorimetric analysis showed an early expression of PrP^C that increased after neuronal differentiation process. Ablation of PrP^C by siRNA PrP prevented neuronal differentiation induced by EGF/bFGF. In this paper, we illustrate that as we enhanced the isolation, separation and *in vitro* cultivation methods of hDPSCs with several easy procedures, more efficient cell clones were obtained and large-scale expansion of the mesenchymal stem cells (MSCs) was observed. We also show how the hDPSCs, obtained with methods detailed in the protocol, are an excellent experimental model to study the neuronal differentiation process of MSCs and subsequent cellular and molecular processes.

INTRODUCTION

Mesenchymal stem cells have been isolated from several tissues, including bone marrow, umbilical cord blood, human dental pulp, adipose tissue, and blood[1-6]. As reported by several authors, hDPSCs show plastic adherence, a typical fibroblast-like morphology. These represent a highly heterogeneous population with distinct clones and differences in proliferative and differentiating capacity[7,8]. hDPSCs express specific markers for mesenchymal stem cells (*i.e.* CD44, CD90, CD73, CD105, STRO-1), they are negative for some hematopoietic markers (such as CD14 and CD19) and are capable of *in vitro* multilineage differentiation[9-11].

Several authors have shown that these cells are able to differentiate into neuron-like cells using different protocols, that include the addition of NGF, bFGF, EGF or combinations of the specific media and supplements[7,12]. Also, many proteins are involved during neuronal differentiation process and, among these, several papers show a relevant role and significant expression of cellular prion protein (PrP^C), both in embryonic and adult stem cells[13,14]. PrP^C represents a pleiotropic molecule capable of performing different functions inside cells as copper metabolism, apoptosis, and resistance to oxidative stress[15-22].

In our previous paper[23], we investigated the role of PrP^C in the hDPSCs neuronal differentiation process. In fact, hDPSCs express precociously PrP^C and, after neuronal differentiation, it was possible to observe an additional increase. Other authors hypothesized a possible role of PrP^C in neuronal differentiation processes of stem cells. Indeed, PrP^C drives the differentiation of human embryonic stem cells into neurons, oligodendrocytes, and astrocytes[24]. The purpose of this study was to emphasize the methodology for obtaining stem cells from dental pulp, its differentiation process and the role of PrP^C during neuronal differentiation.

PROTOCOL

Third molars used in the study were excised from patients (13-19 years old) with no prior history of drug or alcohol consumption, all non-smoking and with appropriate oral hygiene. On the day of explanation, at the Department of Science Dentistry and Maxillofacial of “Sapienza” University of Rome, informed consent was obtained from the patients or the parents. Informed consent was obtained based on ethical considerations and approval of the ethics committee.

1. Tooth and dental pulp extraction

1.1) Preparation of appropriate medium for the conservation or transportation.

1.1.1) Prepare Dulbecco's Modified Eagle's medium low concentration of glucose (DMEM-L) with L-glutamine (494.5 mL).

1.1.2) Add 5 mL of penicillin/streptomycin (1%) and 0.5 mL of amphotericin (0.1%).

1.2) Extract the third molar from the patient, quickly rinse it with PBS, put in a 15 mL test tube with the medium and transfer it to the laboratory in less than 2 h.

1.3) Under a biohazard hood, open the tooth with a cutter by coronal cutting pass parallel and tangent through the roof of the pulp chamber.

1.4) Gently remove the pulp with a small excavator and place it in a test tube.

1.5) Wash with PBS three times and centrifuge at 2,500 x g for 10 min at RT.

2. Processing of the dental pulp and stem cell release

2.1) Remove the supernatant, resuspend the pellet in Hank's solution and place it in a petri dish. Incubate for 2 h at 37 °C in 5% CO_2.

2.2) Type IV collagenase solution preparation.

2.2.1) Prepare 0.8 mL of DMEM-L.

2.2.2) Melt 1 mg of type IV collagenase in 0.8 mL of DMEM-L and vortex for several min.

2.2.3) Add DMEM-L up to 1 mL to have a final concentration 1 mg/mL.

2.2.4) Filter the solution with a 0.22 µM filter.

2.3) Remove Hank's solution by centrifugation at 2,500 x g for 10 min at RT and divide the pulp into small slices approximatively 1 mm each one with a disposable scalpel.

2.4) Place the pulp slices in a petri dish and incubate with 1 mL of type IV collagenase for 15 min at 37 °C in 5% CO_2.

2.5) Medium culture preparation (500 mL).

2.5.1) To 445 mL of DMEM-L with L-glutamine.

2.5.2) Add 50 mL of Fetal Bovine Serum (FBS) (10%).

2.5.3) Add 5 mL of penicillin/streptomycin (1%).

2.6) Centrifuge the sample at 2500 x g for 10 min at RT, remove the supernatant, resuspend the pellet in the medium (step 2.5) and culture in T25 flask specific for stem cell at 37 °C in 5% CO_2.

3. Stem cell culture and propagation

3.1) Every day check the culture and, after 3 days of growth, observe different clones of adherent cells within the flask.

3.2) Every 3 days change the culture medium.

3.3) Between 7 and 12 days, once the adherent cells have reach confluence, detach them by treating the cells with 1 mL of trypsin-EDTA for 3 min at 37 °C or gently using a cell scraper.

3.4) Add 4 ml (ratio 1:5) of the culture medium (step 2.5) to stop trypsin action.

3.5) Centrifuge the cell suspension for 6 min at 259 x g, remove the supernatant and place the cells in a T25 flask to propagate.

Note: Every 3 days the cells reach confluence.

3.6) Propagate the cells up to 21 or 28 days (approximately 6-8 passages) to avoid the presence of non-stem like cells in the culture.

3.7) Detach the cells with 1 mL of trypsin-EDTA for 3 min at 37 °C or gently scraping. Centrifuge the cell suspension for 6 min at 259 x g.

3.8) Remove the supernatant and test the cells for cytofluorimetric analysis (step 6).

4. Transient PrP^C silencing by siRNA

4.1) Culture the hDPSCs in 6-well plates (2 x 10^5 cell/mL) with 2 mL of culture medium (step 2.5) for 24 h.

4.2) The day after, prepare siRNA PrP medium (400 µL).

4.2.1) To a sterile test tube, add 384 µL DMEM-L.

4.2.2) Add 1 µL for each type of siRNA PrP to DMEM-L to have a final concentration of 5 nM (4 siRNA PrP were used and verified by the supplier to guarantee a knockdown efficiency ≥70%).

4.2.3) Add 12 µL of transfection reagent to DMEM-L.

4.2.4) Vortex the mixture and incubate for 10 min at RT to allow the formation of transfection complexes.

4.3) Add 400 µL of siRNA PrP medium to each sample and incubate for 6 h at 37 °C.

4.4) Without discarding siRNA PrP medium, add 1.6 mL (ratio of 1 to 5) of culture medium (step 2.5).

4.5) Leave the cells for 72 h at 37 °C.

4.6) Remove the supernatant and wash 3 times with 2 mL of PBS at RT.

4.7) Add 2 mL of neuronal culture medium for 7 and/or 14 days (step 5.1).

4.8) Change the neuronal culture medium every 3 days.

Note: Replace the siRNA PrP solution each time replace the neuronal culture medium.

4.9) At end of the time, wash 3 times with 2 mL of PBS at RT and test for neuronal surface antigens by Western Blot analysis.

5. Neuronal induction process of hDPSCs

5.1) Neuronal culture medium preparation (500 mL).

5.1.1) Prepare 444.7 mL of basal media formulated to neuronal cells.

5.1.2) To the medium add 50 mL of serum-free supplement used for supporting the long-term viability of embryonic and adult neuronal stem cells (10%).

5.1.3) Add 200 µL of basic Fibroblast Growth Factor (bFGF) (final concentration 40 ng/mL) and 100 µL of Epidermal Growth Factor (EGF) to the medium (final concentration 20 ng/mL).

5.1.4) Add 5 mL of penicillin/streptomycin (1%) to the medium.

5.2) Culture hDPSCs in 6-well plates (2 x 10^5 cell/mL) up to 28 days from the pulp separation and stimulate them with 2 mL of neuronal culture medium.

5.3) Every 3 days discard the supernatant, wash 3 times with 2 mL of PBS at RT and replace 2 mL of the neuronal culture medium.

5.4) After 7 and/or 14 days, detach the cells with 1 ml of trypsin-EDTA for 3 min at 37 °C or gently

with a scraper.

5.5) Add 4 ml (ratio 1:5) of culture medium (step 2.5) to stop trypsin action.

5.6) Test for the presence of neuronal surface antigens (step 6) or prion protein expression (step 7) by flow cytometry analysis.

6. Characterization of hDPSCs by flow cytometry

6.1) Select mesenchymal stromal (MSC)-specific or neuronal surface antigens.

6.2) Culture the hDPSCs in 6-well plates (2 x 10^5 cell/mL) with 2 mL of culture medium (step 2.5).

6.3) Detach hDPSCs at 28 days from dental pulp separation or after 14 days of neuronal culture medium (step 5.1) with 1 mL of trypsin-EDTA for 3 min at 37 °C or gently scraper.

6.4) add 4 ml (ratio 1:5) of culture medium (step 2.5) to stop trypsin action and centrifugate at 259 x g for 6 min at RT. Wash another 2 times with 2 mL of PBS at RT.

6.5) Fix the untreated or treated hDPSCs with 4% paraformaldehyde in PBS for 10 min at 4 °C.

6.6) Permeabilize hDPSCS with 0.1% (v/v) non-ionic surfactant in PBS for additional 10 min at RT.

6.7) Perform the blocking with 5% nonfat dried milk in 1 mL of PBS for 1 h at RT.

6.8) Wash 3 times with 1 mL of PBS and incubate the cells with anti-CD105 (1:100/5x10^5 cells), anti-CD44 (1:100/5x10^5 cells), anti-STRO-1 (1:100/5x10^5 cells), anti-CD90 (1:100/5x10^5 cells), anti-CD73 (1:100/5x10^5 cells), anti β3-Tubulin (1:100/5x10^5 cells), anti-NFH (1:100/5x10^5 cells) and anti-GAP43 (1:100/5x10^5 cells) mAb for 1 h at RT.

6.9) Wash the cells 3 times with 1 mL of PBS and incubate with anti-mouse PE (1:50/5x10^5 cells) or anti-rabbit CY5 (1:50/5x10^5 cells) mAb for additional 1 h at RT.

6.10) Use the secondary antibodies for gating the immunopositive cells (anti-mouse PE or anti-rabbit CY5 mAb) and analyze all samples with a Flow cytometer and acquire at least 20,000 events.

7. Evaluation of PrP^C expression in hDPSCs by flow cytometry analysis

7.1) Culture the hDPSCs in 6-well plates (2 x 10^5 cell/mL) with 2 mL of culture medium (step 2.5).

7.2) Detach hDPSCs at 21 and 28 days from dental pulp separation and after 7 and/or 14 days with neuronal culture medium (step 5.1) with 1 mL of trypsin-EDTA and stop the trypsin action as described in step 6.4. Fix the specimens with 4% paraformaldehyde in PBS for 10 min at 4 °C.

7.3) Permeabilize with 0.1% (v/v) non-ionic surfactant in PBS for 10 min at RT. Remove the supernatant and stain the cells with rabbit anti-PrP mAb EP1802Y (1:50/5x10^5 cells) mAb for 1 h at RT. Incubate with anti-rabbit CY5 (1:50/5 x 10^5 cells) mAb for additional 1 h at RT.

7.4) Analyze all samples with a Flow cytometer and acquire at least 20,000 events.

REPRESENTATIVE RESULTS

The isolation and separation procedures of hDPSCs from dental pulp, obtained from the third molar, are complex processes in which small changes can lead a ruinous result. In this paper, we use the protocol of Arthur et al.[12] with several improvements. A representative scheme of procedures is shown in **Figure 1**.

hDPSCs represents a heterogeneous population of cells with distinct clones and differences in proliferative and differentiating capacity[7,8]. After pulp separation and seeding of tiny fragments of pulp, we observed clusters of cells expanding on the periphery. **Figure 2** shows a small cluster of cells at 1 day (left panel), 4 days (central panel) and 7 days (right panel) from dental pulp separation. Generally, these clusters of cells grow up to reach the confluence approximately between 7 and 12 days.

These cells, after neuronal differentiation induced by EGF/bFGF, reduce their growth and, after two weeks it was possible to observe significant changes in the cell morphology and neurites outgrowth (**Figure 3**).

In **Figure 4**, we show that untreated hDPSCs express multipotent mesenchymal stromal specific surface antigens such as CD44, CD90, CD105, CD73, and STRO-1[5,9]. Otherwise, after appropriate neuronal induction stimuli, hDPSCs express specific neuronal markers such as β3-Tubulin, NFH, and GAP43. hDPSCs, untreated or treated with neuronal induction stimuli, do not express hematopoietic markers such as CD14 and CD19.

In **Figure 5**, we show that hDPSCs express precociously PrP^C (21 and 28 days) and, after neuronal differentiation process induced by EGF/bFGF for additional 7 and 14 days, the PrP^C content increased (**Figure 5A**). Since several authors reported that PrP^C is involved in the cellular neuronal differentiation, we evaluated the role of endogenous PrP^C in this process. Therefore, a small interfering RNA (siRNA PrP) was applied to ablate PrP^C and its function. Pretreatment with siRNA for 72 h before EGF/bFGF stimuli for 14 days prevents the expression of neuronal markers B3-tubulin and NHF (**Figure 5B**). The data show that silencing of PrP^C by siRNA affected the neuronal differentiation process of hDPSCs, induced by EGF/bFGF after 2 weeks.

FIGURE AND TABLE LEGENDS

Figure 1. Scheme of Dental pulp separation from the third molar. The tooth has been opened with a cutter by coronal cutting pass parallel and tangent through the roof of the pulp chamber and the pulp was gently removed under sterile conditions with a small excavator and placed in a

test tube. The pulp, after hank's solution treatment, was cut into slices and treated with collagenase IV for 15 min and propagated in a T25 flask.

Figure 2. hDPSCs morphology at different days from dental pulp separation by phase contrast microscope. Morphology of hDPSCs from dental pulp at different days (1, 4, 7) from dental pulp separation. Scale bars 100 µm.

Figure 3. Neurite outgrowth of hDPSCs by phase contrast microscope. Morphology of hDPSCs from dental pulp untreated or treated with EGF/bFGF for 14 days. Scale bars 100 µm.

Figure 4. hDPSCs characterization. Flow cytometry analysis of CD44, CD90, CD105, CD73, STRO-1, CD14, CD19, β3-Tubulin, NFH and GAP43 expression in hDPSCs untreated or treated with EGF/bFGF for 14 days. Histograms represent log fluorescence *vs* cell number, gated on cell population of a side scatter/forward scatter (SS/FS) histogram. Each panel was compared with the corresponding secondary antibodies as a negative control. A representative experiment among 3 is shown.

Figure 5. Role of PrP^C during neuronal differentiation of hDSPCs. (A) Cytofluorimetric analysis of PrP^C expression untreated (21 and 28 days from dental pulp separation) and after additional 7 and 14 days with neuronal induction media EGF/bFGF. Each panel was compared with the corresponding IgG negative isotype control. A representative experiment among 3 is shown. (B) Western blot analysis of neuronal markers β3-Tubulin and NFH expression (28 days from dental pulp separation) and after additional 14 days with neuronal induction media EGF/bFGF in the presence or absence of siRNA PrP. This figure 5 (A, B) has been modified from "Role of Prion protein-EGFR multimolecular complex during neuronal differentiation of human dental pulp-derived stem cells" [23].

DISCUSSION

In this work, we focused on methodology for isolation and neuronal differentiation of hDPSCs; moreover, we evaluated the role of PrP^C in this process. There are several methods to isolate and differentiate hDPSCs in neuron-like cells and critical steps during the process. hDPSCs are able to differentiate in several lineages such as chondroblasts, adipocytes, osteoblasts, and neurons. In our paper, we investigated the mechanisms of neuronal differentiation and the presence of PrP^C. As discussed above, these cells express typical mesenchymal stromal-specific surface antigens such as CD44, CD90, CD105, CD73, and STRO-1[10,25,26].

In the protocol, several critical steps can cause the failure of the separation procedure. The first critical step is represented by the choice of patients[27]. In our experience, we found that increasing age of donors (> 20) gradually reduces the ability of proliferation and differentiation of stem cells. In our experiments, we used third molars excised from patients aged 13-19 years old and with no prior history of drug or alcohol consumption, all non-smoking and with appropriate oral hygiene.

The second critical step is represented by the choice of the enzyme to separate the cells from the pulp tissue, which could represent the main hot step of stem cells separation procedure. In fact, we realized that a wide use of collagenase I and II, enzymes that can be too aggressive, could damage the cells present in the pulp tissue during the separation process. For this reason, we decided to use collagenase IV because this kind of collagenase as lower tryptic activity than collagenase type I and II. Following exactly the procedure, it is possible to obtain stem cells in 90% of the treated teeth.

After the separation, the solution containing hDPSCs is cultured in appropriate flasks until the 6° passage (approximatively 21-28 days) to avoid the presence of non-stem cells in the culture. At 28 days, they were tested for the presence of typical mesenchymal antigens (as referenced above) and used for experiments only when they were positive. In fact, despite the progress made throughout years on hDPSCs, there are still important limitations represented by the extreme heterogeneity of the population.

As shown by several authors, there are different cell population types in the dental pulp and, to date, it's unknown what is the best phenotypes able to differentiate into each mesenchymal lineage (chondroblasts, adipoblasts, osteoblasts or neurons). To avoid the current limitations of our and other procedures, the next step will be to select cellular populations with specific phenotypes using specific monoclonal antibodies.

In previous work, we showed that PrP^C is expressed from hDPSCs. In fact, it is possible to observe a weak positivity at 21 days and an increase of PrP^C content at 28 days. Furthermore, we observed that during EGF/bFGF-mediated neuronal induction, PrP^C content was further increased. Moreover, we investigated the role of endogenous PrP^C in neuronal induction process of hDPSCs. The transient silencing of PrP^C prevented the differentiation process of hDPSCs induced by EGF/bFGF[23].

We fine-tuned and improved the methods of isolation, separation and *in vitro* cultivation of hDPSCs with simple and versatile procedures. These innovations allow obtaining more efficient cell clones and the large-scale expansion of the mesenchymal stem cells. Also, we suggest that the hDPSCs are an excellent experimental model to study cellular and molecular mechanisms of MSCs neuronal differentiation process.

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Figure 5 (A, B) reprinted by permission of the publisher Taylor & Francis Ltd from: Role of Prion protein-EGFR multimolecular complex during neuronal differentiation of human dental pulp-derived stem cells. Martellucci, S., Manganelli V., Santacroce C., Santilli F., Piccoli L., Sorice M., Mattei V. *Prion*. 2018 Mar 4. Taylor & Francis Ltd.

DISCLOSURES

The authors have nothing to disclose.

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Figure 1

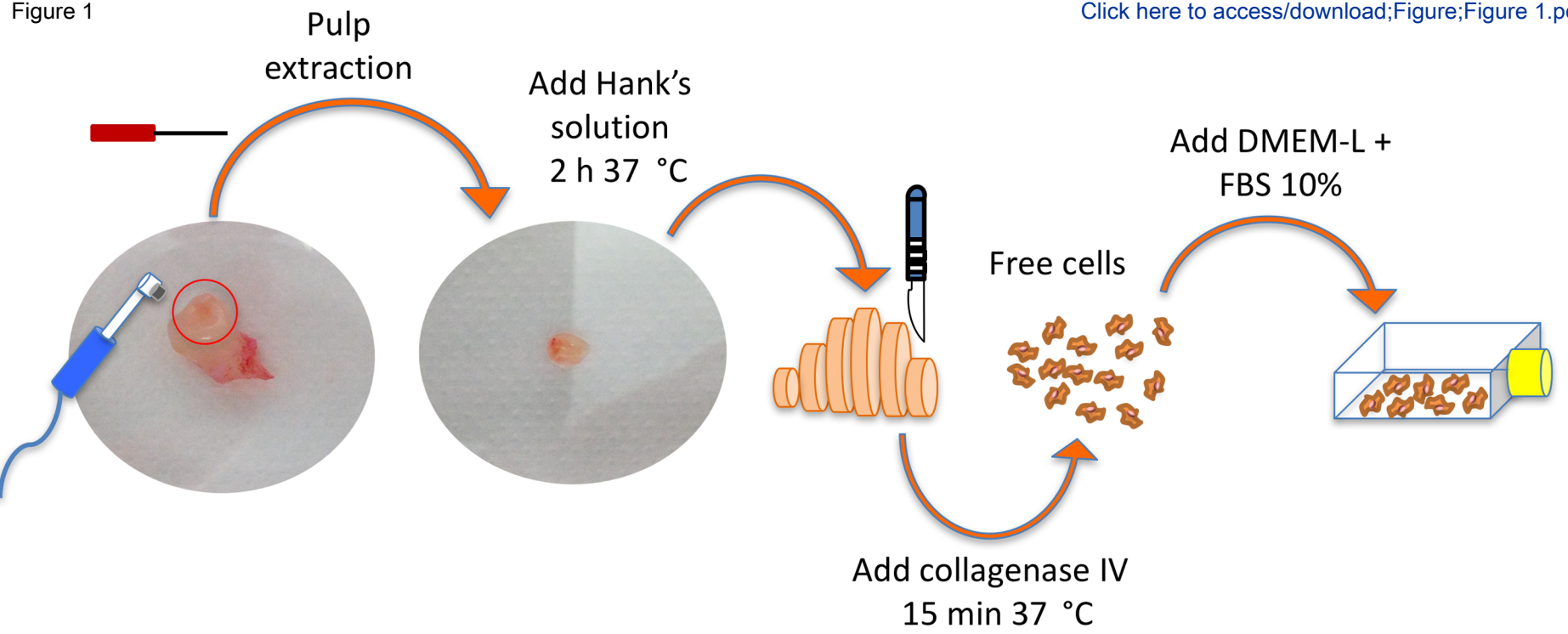
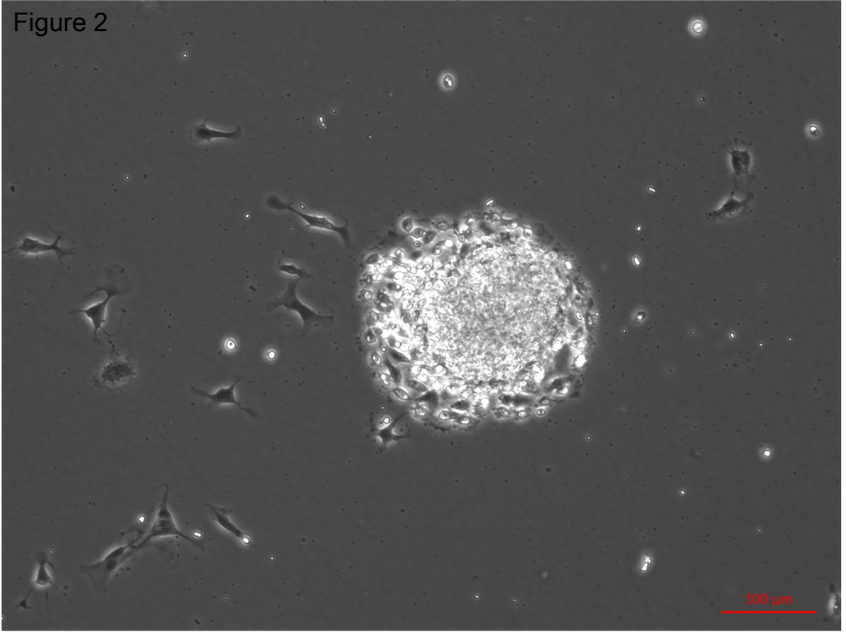


Figure 2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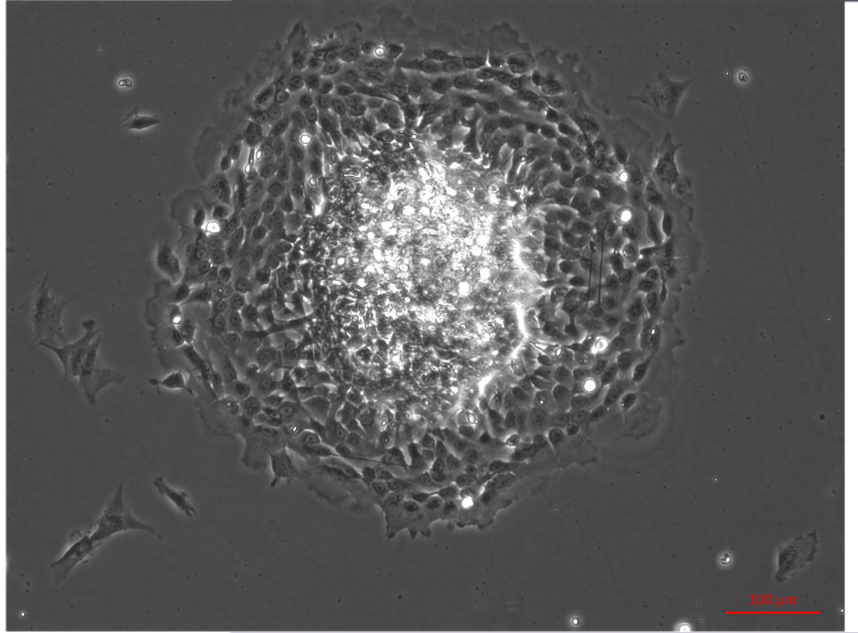
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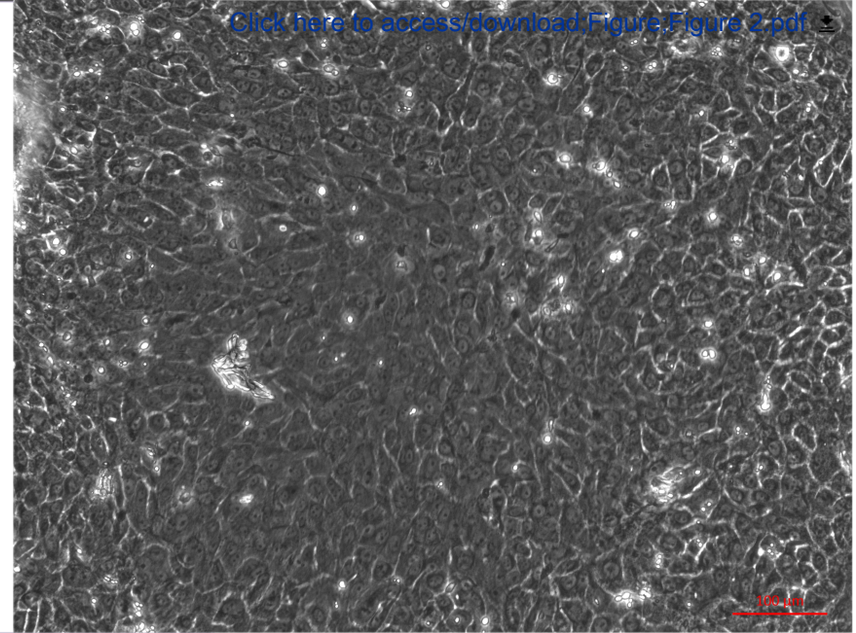
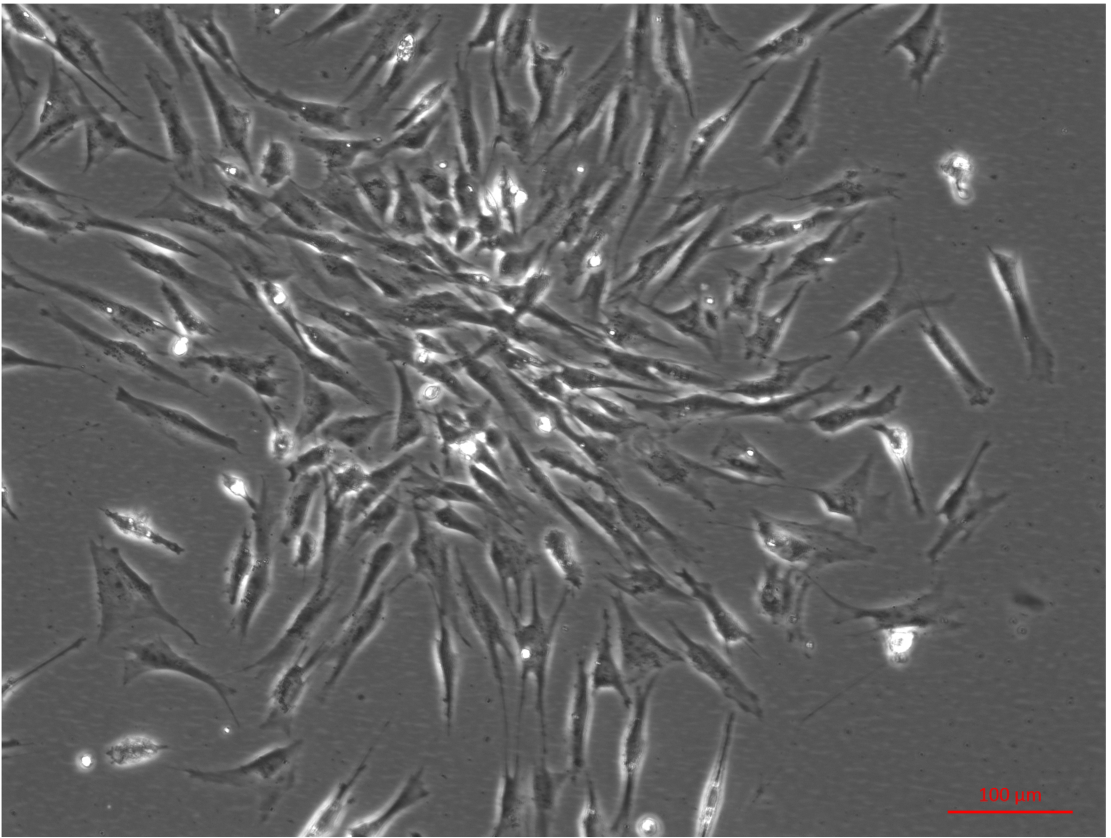

Click here to access/download;Figure;Figure 2.pdf
100 µm

Figure 3



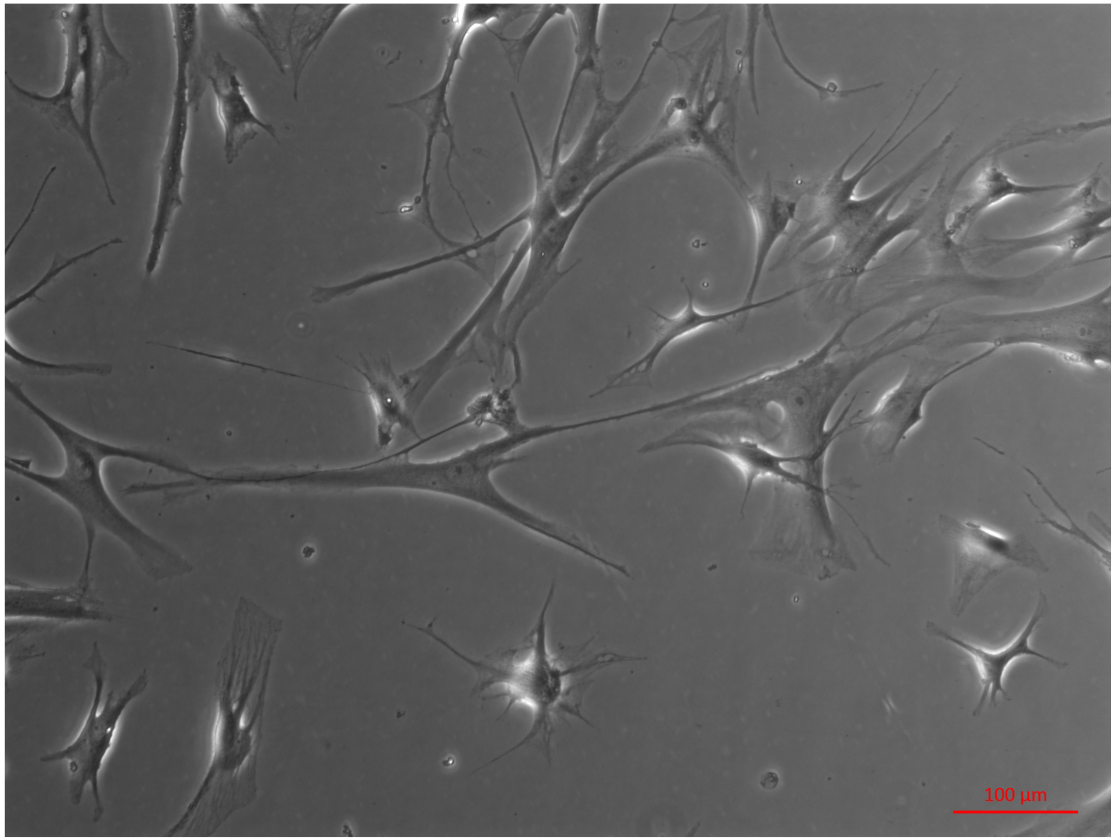
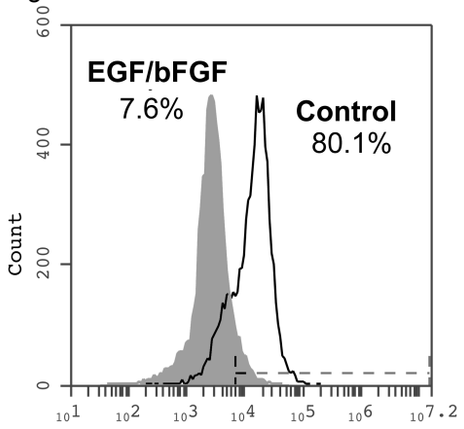
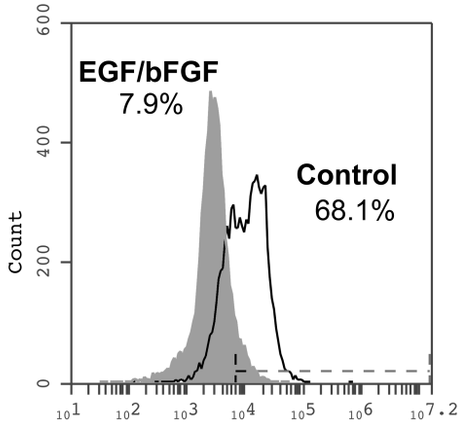


Figure 4

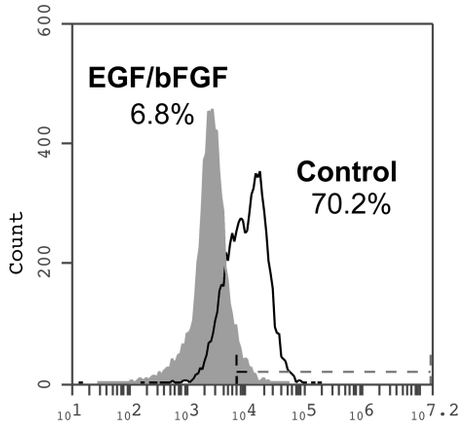
Click here to access/download;Figure;Figure 4.pdf



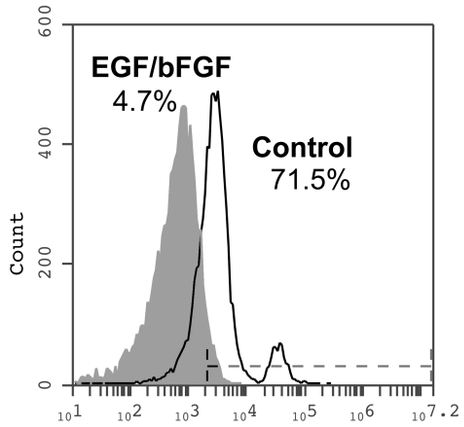
CD44-PE



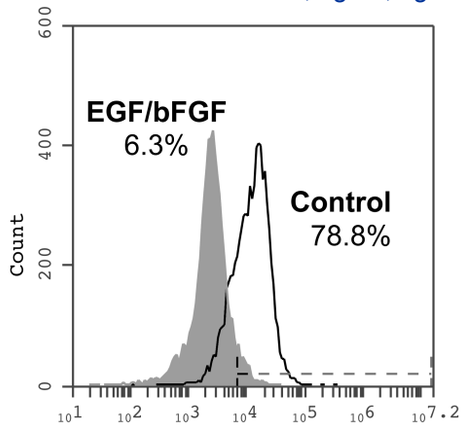
CD90-PE



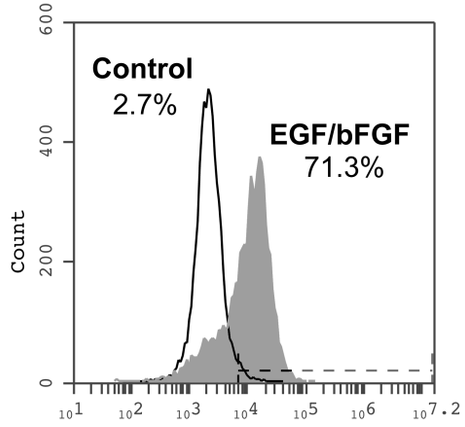
CD105-PE



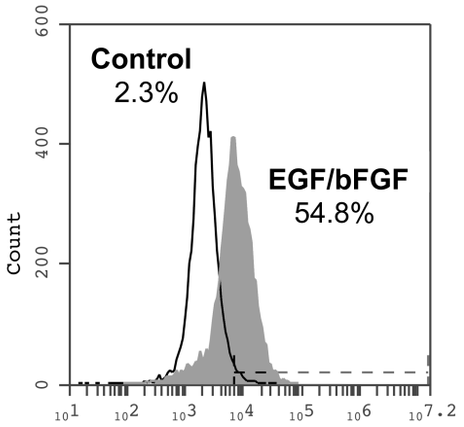
CD73-CY5



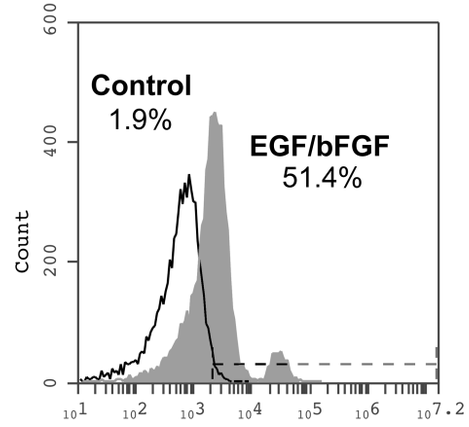
STRO-1-PE



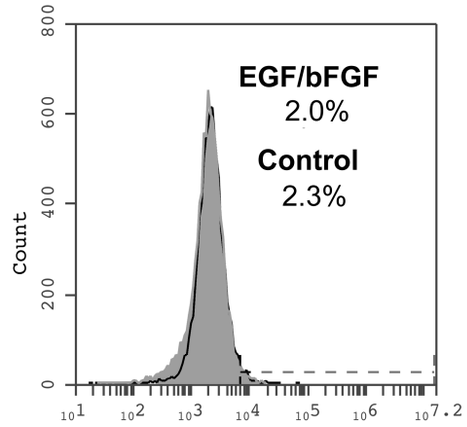
B3 TUBULIN-PE



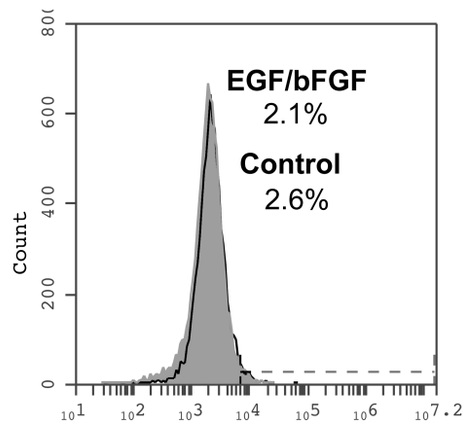
NFH-PE



GAP43-CY5



CD14-PE



CD19-PE

Figure 5

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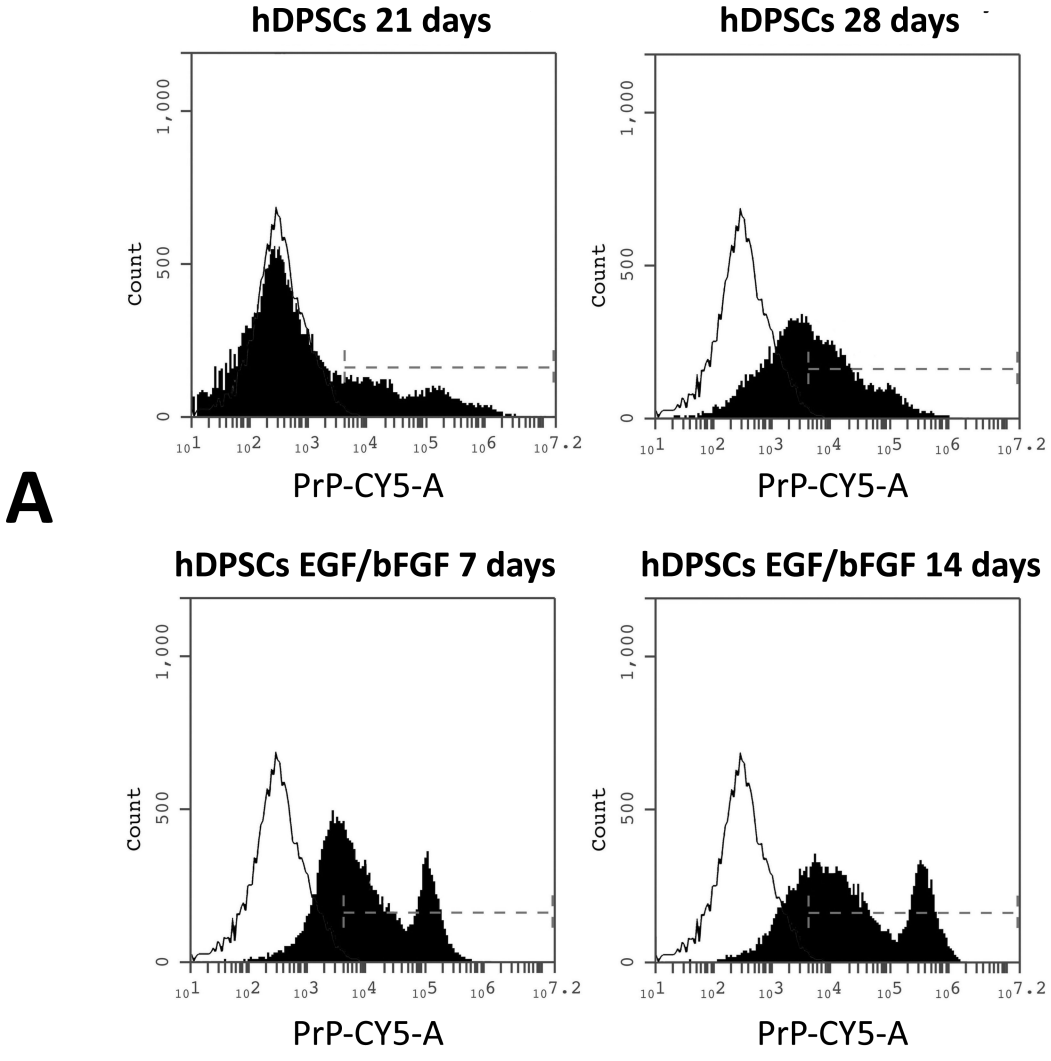

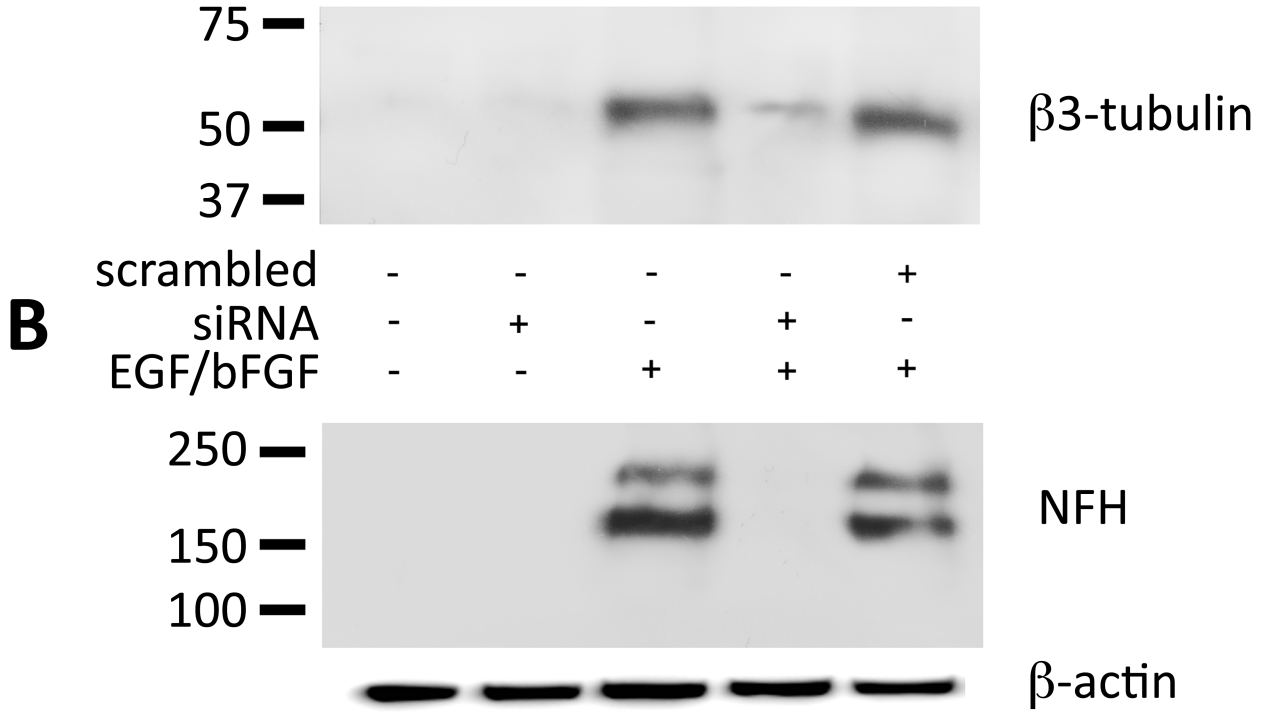

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Amphotericin B solution	Sigma-Aldrich	A2942
Anti-B3tubulin	Cell Signaling Technology	#4466
Anti-CD105	BD Biosciences	611314
Anti-CD44	Millipore	CBL154-20ul
Anti-CD73	Cell Signaling Technology	13160
Anti-CD90	Millipore	CBL415-20ul
Anti-GAP43	Cell Signaling Technology	#8945
Anti-mouse PE	Abcam	ab7003
Anti-NFH	Cell Signaling Technology	#2836
Anti-PrP mAb EP1802Y	Abcam	ab52604
Anti-rabbit CY5	Abcam	ab6564
Anti-STRO 1	Millipore	MAB4315-20ul
B27 Supp XF CTS	Gibco by life technologies	A14867-01
BD Accuri C6 flow cytometer	BD Biosciences	AC6531180187
BD Accuri C6 Software	BD Biosciences	
bFGF	PeproThec, DBA	100-18B
Centrifuge CL30R	Termo fisher Scientific	11210908
CO_2 Incubator 3541	Termo fisher Scientific	317527-185
Collagenase, type IV	Life Technologies	17104019
Disposable scalpel	Swann-Morton	501
DMEM-L	Euroclone	ECM0060L
EGF	PeproThec, DBA	AF-100-15
Fetal Bovine Serum	Gibco by life technologies	10270-106
Filtropur BT50 0.2,500ml Bottle top filter	Sarstedt	831,823,101
Flexitube GeneSolution for PRNP	Qiagen	GS5621
Hank's solution 1x	Gibco by life technologies	240200083

HiPerFect Transfection Reagent	Qiagen	301705
Neurobasal A	Gibco by life technologies	10888022
Paraformaldehyde	Sigma-Aldrich	30525-89-4
penicillin/streptomycin	Euroclone	ECB3001D
Phosphate buffered saline (PBS)	Euroclone	ECB4004LX10
TC-Platte 6 well, Cell+,F	Sarstedt	833,920,300
Tissue culture flask T-25,Cell+,Vented Cap	Sarstedt	833,910,302
Triton X-100	Sigma-Aldrich	9002-93-1
Trypsin-EDTA	Euroclone	ECB3052D
Tube	Sarstedt	62,554,502
VBH 36 C2 Compact	Steril	ST-003009000
ZEISS Axio Vert.A1 – Inverted Microscope	Zeiss	3849000962

Comments/Description

It is use to supplement cell culture media, it is a polyene antifungal antibiotic from Streptomyce
One of six B-tubulin isoform, it is expressed highly during fetal and postnatal development, remaining high in the peripheral nervous system
Endoglin (CD105), a major glycoprotein of human vascular endothelium, is a type I integral membrane protein with a large extracellular region, a hydroph
Positive cell markers antibodies directed against mesenchymal stem cells
CD73 is a 70 kDa glycosyl phosphatidylinositol-anchored, membrane-bound glycoprotein that catalyzes the hydrolysis of extracellular nucleoside monoph
Positive cell markers antibodies directed against mesenchymal stem cells
Is a nervous system specific, growth-associated protein in growth cones and areas of high plasticity
Is an antibody used in in flow cytometry or FACS analysis
Is an antibody that detects endogenous levels of total Neurofilament-H protein
Rabbit monoclonal [EP 1802Y] to Prion protein PrP
Is an antibody used in in flow cytometry or FACS analysis
Positive cell markers antibodies directed against mesenchymal stem cells

B-27 can be used to support induction of human neural stem cells (hNSCs) from pluripotent stem cells (PSCs), expansion of hNSCs, differentiation of hNSC
Flow cytometer equipped with a blue laser (488 nm) and a red laser (640 nm)
Controls the BD Accuri C6 flow cytometer system in order to acquire data, generate statistics, and analyze results
basic Fibroblast Growth Factor
it is a device that is used for the separation of fluids,gas or liquid, based on density
it ensures optimal and reproducible growth conditions for cell cultures
Collagenase is a protease that cleaves the bond between a neutral amino acid (X) and glycine in the sequence Pro-X-Glyc-Pro, which is found with high fre
It is use to cut tissues
Dulbecco's Modified Eagle's Medium Low Glucose with L-Glutamine with Sodium Pyruvate
Epidermal Growth Factor

FBS is a popular media supplement because it provides a wide array of functions in cell culture. FBS delivers nutrients, growth and attachment factors and

it is a device that is used for filtration of solutions
4 siRNAs for Entrez gene 5621. Target sequence N.1 TAGAGATTTCATAGCTATTTA N.2 CAGCAAATAACCATTGGTTAA N.3. CTGAATCGTTTCATGTAAGAA N.4

The essential function of Hanks' Balanced Salt solution is to maintain pH as well as osmotic balance. It also provides water and essential inorganic ions to c

HiPerFect Transfection Reagent is a unique blend of cationic and neutral lipids that enables effective siRNA uptake and efficient release of siRNA inside cel

Neurobasal-A Medium is a basal medium designed for long-term maintenance and maturation of pure post-natal and adult brain neurons
Paraformaldehyde has been used for fixing of cells and tissue sections during staining procedures
It is use to supplement cell culture media to control bacterial contamination
PBS is a balanced salt solution used for the handling and culturing of mammalian cells. PBS is used to to irrigate, wash, and dilute mammalian cells. Phospl
It is a growth surface for adherent cells

Tissue culture flask T-25, polystyrene, Cell+ growth surface for sensitive adherent cells, e.g. primary cells, canted neck, ventilation cap, yellow, sterile, Pyrc
Widely used non-ionic surfactant for recovery of membrane components under mild non-denaturing conditions
Trypsin will cleave peptides on the C-terminal side of lysine and arginine amino acid residues. Trypsin is used to remove adherent cells from a culture surfa
Tube 15ml, 120x17mm, PP
Offers totally protection for the enviroment and worker
ZEISS Axio Vert.A1 provides a unique entry level price and can provide all contrasting techniques, including brightfield, phase contrast, PlasDIC, VAREL, imp

obic transmembrane region, and a short cytoplasmic tail

osphates into bioactive nucleosides

:s, and maintenance of mature differentiated neurons in culture

quency in collagen

protects cells from oxidative damage and apoptosis by mechanisms that are difficult to reproduce in serum-free media (SFM) systems

CAGTGACTATGAGGACCGTTA

:ells

ls, resulting in high gene knockdown even when using low siRNA concentrations

ıate buffering maintains the pH in the physiological range

ɔgen-free, non-cytotoxic, 10 pcs./bag

ıce

ɔroved Hoffman Modulation Contrast (iHMC), DIC and fluorescence. Incorporate LED illumination for gentle imaging for fluorescently-labeled ce

lls. Axio Vert.A1 is ergonomically designed for routine work and compact enough to sit inside tissue culture hoods.

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Title of Article: hDPSC: ISOLATION, PROPAGATION AND PRION PROTEIN EXPRESSION DURING NEURONAL DIFFERENTIATION

Author(s): STEFANO MARTELLUCCI, COSTANTINO SANTACROCE, VALERIA MANGANELLI, FRANCESCA SANTILLI, LUCA PICCOLI, MICHELE CASSETTA, ROBERTA MISASI, MAURIZIO SORICE, VINCENZO MATTEI

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Point 1.1.1) comment A1.
Is this correct
Answer: yes, it is correct

Point 3.5) comment A2.
Why only these two time points and not other? It will be nice if you provide the passage number as well.
Answer: we propagated the cells up to 21 or 28 days (approximately 6-8 passages) to avoid the presence of not stem cells in the culture.

Point 3.7) comment A3, A4, A5
Please provide the step number for both instead
Answer: we provided the step number

Point 4.1.1) comment A6
Is there a specific reason to have 444.7 and not 445 ml?
Answer: Yes, it is a standard preparation to have 500 mL of final neuronal culture medium (now point 5.1.1.)

Point 4.2)
A7. So the neuronal induction is performed only after 28 days of culture?
Answer: Yes. The neuronal induction is performed only after 28 days of dental pulp separation (now point 5.2)

A8. Do you remove the medium and then add the new one? Do you perform a wash in between? Volume?
Answer: We improved the point 4 (now point 5)

Point 4.3) A9
Is there a specific reason to work only after 7 and 14 days? Do you check for neuronal morphology/biomarker at this stage? Please include a step stating the same.

Answer: yes, generally after 14 days is possible to observe morphological and biochemical changes. Moreover, we decided to investigate at 7 days to check a middle step.
Yes, we checked the neuronal morphology and neuronal biomarkers at 14 days (Figure 3 and 4). Moreover we tested the presence of prion protein at 7 and 14 days with neuronal culture medium.

Point 5.1) A10, A11
Is this after neuronal induction? Please provide the step number. If this is induced, then please refer to as induced hDPSCs. If not then please provide the step number which comes before this step. Volume?
Answer. No, it is before the neuronal induction. So, we moved the step before neuronal induction process of hDPSCs. We improved the point with missed things.

Point 5.2) A12
If 5.2. is highlighted, the steps describing the procedure should also be highlighted. Please either highlight the substeps or convert this to a table and reference the table in step 5.2.
The table should be uploaded separately as a .xls/.xlsx file in your editorial manager account.
Answer: we unmarked the point 5.2 (now 4.2)

Point 5.2.4) A13
Do you perform the vortexing for 10 min or after vortexing you incubate for 10 min. please check.
Answer: we improved the point 5.2.4 (now 4.2.4)

Point 5.4) A14
Please provide the volume as well. Do you remove the transfection mix before adding the complete medium?
Answer: we improved the point 5.4 (now 4.4)

Point 5.5.1) A15
Needs clarity. Please reword.
Answer: we reworded the point 5 (now 4)

Point 6.2) A16
Is there a specific reason to use these time points? Do you add medium to stop the trypsin action?
Answer: Yes. We propagated the cells up to 28 days (approximately 8-10 passages) to avoid the presence of not stem cells in the culture. Yes, we added the trypsin stop solution.

Point 6.3) A17
Volume and concentration?
Before this step, there are few missing steps. Do you stop the trypsin action, do you plate the cells for treatment with growth factors, do you leave it for incubation or 14 days? Please include all specific details with respect to your protocol.
Are these treatment performed on coverslips coated with cells, slide, plate? Please specify.
Answer: we improved the point 6

Point 7) A18, A19
Is this cytofluorometric analysis or flow cytometry? Please highlight the difference between step 6 and 7 apart from the antibody.
These detail needs to be provided earlier.
Answer: It is a flow cytometry. The most important differences are: the time of induction with neuronal culture medium, the time of culture of hDPSCs (21 and 28 from the dental pulp separation). The experiment showed in point 7 (Fig. 5A) was modified from ““Role of Prion protein-EGFR multimolecular complex during neuronal differentiation of human dental pulp-derived stem cells”. Prions

Point 7.2) A20
Please include the step number or include all necessary information.
Answer. We included the step number.

Point 7.4) A21
The cells in this case are fixed in the
Answer: PBS

Point 7.5) A22
0.1% in PBS?
Answer: yes

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Our Ref: JB/KPRN/P18/1712

30 November 18

Dear Vincenzo Mattei on behalf of JoVE,

Material requested: Figures 2a & 6bc 'Role of Prion protein-EGFR multimolecular complex during neuronal differentiation of human dental pulp-derived stem cells' by Stefano Martellucci, Valeria Manganelli, Costantino Santacroce, Francesca Santilli, Luca Piccoli, Maurizio Sorice & Vincenzo Mattei *Prion* Vol 12:2 pp. 117-126 (2018).

Thank you for your email requesting permission to include the above material in your forthcoming publication entitled 'hDPSCs: isolation, propagation and prion protein expression during neuronal differentiation' to be published by Journal of visualized experiments (JoVE).

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Yours sincerely

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