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# Comparative Scale-Up Culture of Human Neural Progenitor Cells Derived from Human Embryonic Stem Cell and Transdifferentiated Human Foreskin Fibroblast under Defined Condition in Stirred Bioreactor

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## Abstract

**Introduction**: Generation of clinical grade human neural progenitor population is one of the great promises in stem cell biology which offers a unique opportunity in cell-based therapeutic product. Based on the large number of cells that needed in cell therapy studies, developing a robust bioprocess for large scale expansion of neural progenitors under defined conditions towards achieving clinical goals is a mandatory part before starting any trials.

**Objective**: Here, we have developed a robust culture system for large scale expansion of human neural progenitor cells derived from human embryonic stem cells and transdifferentiated human foreskin fibroblast in defined medium.

**Material and Methods**: Initially, a chemically defined medium have been developed for expansion of aforementioned cells under static suspension culture. Then, cells were successfully expanded in dynamic system by optimizing conditions such as medium volume, cell inoculation density, dissociation kinetics, aggregate formation and size growth. Results were assessed by immunofluorescent staining, Real-time PCR and karyotype analysis.

**Result**: Cell aggregates were expanded up to ten passages and fold increase reached to 4.2 over a period of 5 days. Finally, after serial passaging of cells in suspension bioreactor, characterization of them in gene and protein expression levels were done in comparison with adherent expanded cells.

**Conclusion**: The result revealed that both expansion systems had same cellular quality and differentiation potencies. Therefore, we could expect to reach a large number of human neural progenitor cells within a few days and this would be a great promise for cell therapy purposes in neurodegenerative diseases.

**Keyword**: Human neural progenitor cells; Scale up culture; Stirred suspension bioreactor; Chemically define medium

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## 1. Introduction

Neurodegenerative diseases (e.g., Parkinson, Multiple sclerosis and spinal cord injuries) are characterized by loss of neurons in the brain or spinal cord which afflict tens of millions of people worldwide [1]. These kinds of neuronal disorders can cause a sudden or gradual loss of sensory, motor, and autonomic functions. And still no certain cure has been introduced in neurological recovery. So, most of the patients suffering from neurodegenerative diseases still face substantial dysfunctions and lifelong disabilities with no available curative treatment option to halt their problem's progression [2]. Recent advances in neural stem cell therapy have paved new hopes for treatment of neurological disorders. Therapeutic strategies like employing damaged cells replacement, re-myelination, neuronal restoration, bridging of lesion cavities, releasing of neurotrophic factors and anti-inflammatory cytokines [3] have been used for this purpose. Over the last two decades, several stem cell types such as pluripotent stem cells [4, 5]; non-CNS tissue-specific stem cells [6]; genetically manipulated neural stem or progenitor cells induced to adopt a specialized neuronal fate (e.g., dopaminergic fate) [7, 8]. They have been used for treating the different types of neurodegenerative diseases [9, 10]. More recently, induced transdifferentiation technology offers a powerful strategy for producing patient-specific therapies [11]. However, previous studies have been reported the generation of induced neural stem cells (hiNSCs) from fibroblasts with subsequent differentiation to neurons [12-14]. Unlike iPS cell or transient dedifferentiation, no overt oncogenes, such as c-Myc, or any other specific pluripotency factors are needed to produce hiNSCs. Although, many obstacles remain to be overcome before the assessments of the therapeutic potential of hiNSCs [12]. The employment of neural stem cells and their progenies in applications such as clinical utility, drug development, feasibility or safety assessment will require the development of robust culture systems for consistent, high-quality uniform cell production [15]. To date, significant research has been conducted on the development of cell growth medium and passaging protocols for the in vitro expansion and differentiation of human neural progenitor cells (hNPCs).

Furthermore, most of the reported studies have been exclusively performed in static suspension culture system which has various drawbacks such as heterogeneity, limitation in the production of higher cell fold expansions and lack of control over important physiological conditions [10, 16]. To overcome these limitations, the necessity of using a more efficient and dynamic system strategy seems to be negligible. Thus, the best candidate for bridge clinical utility of hNPCs would be bioreactor system [16, 17]. However, there are some important bioprocess parameters such as medium volume, cell inoculation density, aggregate formation, size growth, and dissociation kinetics, for generating homogenous aggregates have been introduced to address these challenges [16, 17]. Here we developed a robust, scalable culture system for long-term maintenance and expansion of hiNSCs, and Human Embryonic Stem Cells derived neural progenitor cells (hESC-NPCs) in a micro carrier-free and defined static and dynamic suspension culture in stirred suspension bioreactors. This could be a significant step toward a robust bioprocess design for the production of clinically relevant numbers of hNPCs under GMP conditions.

## 2. Materials and Methods

### 2.1. Proliferation of hESCs-NPCs and hiNSCs

hiNSCs generated from transdifferentiated human foreskin fibroblast [14], and hESC-NPCs derived from hESC lines (Royan H6) were used in this study [18]. The cell lines were passaged and maintained in an adherent single cell system before applying them in defined suspension culture which is the aim of our study [19] and will discuss later in this paper.

### 2.2. Static suspension culture medium screening

hNPCs were inoculated ($2\times10^5$ cells/ml) into a serum-free expansion medium enriched by growth factors combinations in bacterial dishes. Totally, 5 defined media were screened for their propagation support efficacy and the base medium including

DMEM/F12 in the presence of 2 mM L-Glutamine (Gibco-Invitrogen; 31331-028), 1% Non-essential amino acid (Gibco-Invitrogen;11140-050), 1% N2 supplement (Gibco-Invitrogen; 17502-048), 0.001% B27 supplement (Gibco-Invitrogen;17504044), 1% Insulin-Transferrin-Selenium (Gibco-Invitrogen; 41400-045) (Table 1). Culture medium was renewed every other day, and both cell populations were passaged weekly. The effectiveness of each group was compared by cell counts and viability measurements after a minimum of five passages.

### 2.3. Fold increase and viability assessment

Cells Fold Increase (FI) was calculated in each passage and defined as the ratio XMAX/X0, where XMAX is the peak cell density (cells/ml) and X0 is the inoculation cell density (cells/ml). Cell viability was assessed by the trypan blue exclusion method.

### 2.4. Dynamic suspension culture in stirred bioreactor

The best static expansion medium group was selected for transfer to dynamic suspension culture in a 100 mL siliconized (Sigmacote; Sigma-Aldrich, SL2) stirred bioreactor (Cellspin; Integra Biosciences). The most important bioprocess parameters for expansion of hiNSCs and hESC-NPCs were identified and addressed through stepwise optimization of the key parameters for each bioprocess step and including:

1. Enzymatic dissociation of aggregates and passaging as single cells by different dissociation media (TrypLE, Acutase, and collagenase) (Figure 1F).
2. Cell inoculation density (2×105, 5×105 and 1×106 cells/mL) (Table 2).
3. Hydrodynamic culture conditions of bioreactor using different agitation rates (30–60 rpm) (Figure1 A, B and Supplementary Figure 2).
4. Aggregation kinetics under dynamic conditions (Figure 1C).

Then, obtained cells after a minimum of 10 passages in optimized conditions were tested for their differentiation potencies.

### 2.5. Characterization of hiNSCs and hESC-NPCs and their Progenies

Both studied cell lines passaged in optimized dynamic culture conditions were evaluated morphologically. We characterized passaged cell lines for their multipotency and differentiation potential after a minimum of 10 passages in the bioreactor.

Immunofluorescence staining was done for undifferentiated and spontaneously differentiated (after 14 days of culture) stage for both groups as previously described [9, 18]. To perform staining, aggregates of hNPCs were harvested from bioreactors and after enzymatic dissociation were re-plated on the poly-L-ornithin/laminin pre-coated tissue culture plate. Adherent single cells were then fixed using 4% paraformaldehyde (Mallinckrodt, Phillipsburg, NJ, P0154), and permeabilized with 0.1% Triton X-100 (Sigma, Cas No. 9002-93-1) for 15 min at ambient temperature. The cells were incubated with primary antibody for 1 h at room temperature (RT), washed, and incubated with secondary antibodies, as appropriate, for 1h at RT. (supplementary Table1). The nuclei were counterstained with 4,6-diamidino-2-phenylindole (0.1 mg/mL; Sigma, D8417) or propidium iodide (Sigma-Aldrich, P4170). The cells were analyzed with a fluorescent microscope (Olympus).

### 2.6. Real-time analysis

After sample collection, total RNA was extracted using TRIzol reagent (Invitrogen, 15596-018). DNA was degraded with the use of the DNaseI (Fermentas, EN0521). Then cDNA was synthesized using a RevertAidTM H minus First Strand cDNA Synthesis kit (Fermentas, K1632). Relative gene expression was analyzed by comparative Ct method and normalized to reference gene GAPDH and calibrated for each sample against their originate cells (hESCs, HDF) (Supplementary Table 2).

### 2.7. Karyotype analysis

Karyotype analysis was performed as described before [20] for hiNSCs and hESC-NPCs at passage 10 in static and suspension bioreactor culture, respectively. Twenty metaphase spreads were screened, of which 10 were evaluated for chromosomal rearrangement.

**Table 1.** Serum-free defined media screened for static suspension culture of hNSCs

| Group | Name | Basal medium | Serum | bFGF (20 ng/ml) | hEGF (20 ng/ml) | hLIF (10 ng/ml) | DHEA (1 µM/ml) | CHIR (3 µg/ml) |
|---|---|---|---|---|---|---|---|---|
| **1** | NM1 | NM* | - | + | - | - | - | - |
| **2** | NM2 | NM* | - | + | + | - | - | - |
| **3** | PPRFh1 | PPRF-m4** | - | + | + | + | - | - |
| **4** | PPRFh2 | PPRF-m4** | - | + | + | + | + | - |
| **5** | NM3 | NM* | - | + | + | - | - | + |

*NM: DMEM/F12(1:1), 6%glucose, 1.73g/lL Sodium bicarbonate, 2.0mM glutamine ,2.0mM nonesential amino acid, 1%ITS, 1%N2, 0.001% B27

**PPRF-m4: DMEM/F12(1:1), 6%glucose, 5mM HEPES, 1.73g/lL Sodium bicarbonate, 2.0mM glutamine , 0.023 g/L insulin , 20 nM progesterone , 9.0mg/L putrescine , 0.025 g/L transferrin , 30 nM sodium selenite , 20µg/L EGF and 10µg/L bFGF

**Table 2.** Impact of different cell inoculation densities on fold increase rate of hNPCs in stirred bioreactor

| | Inclusion density(cells/ml) | Fold increase | $X_{max}$(×$10^6$ cells/ml) |
|---|---|---|---|
| **hESC-NPC** | $2\times10^5$ | 4.2 | 0.84 |
| | $5\times10^5$ | 3.5 | 1.75 |
| | $1\times10^6$ | 2 | 2 |
| **hiNSC** | $2\times10^5$ | 4 | 0.8 |
| | $5\times10^5$ | 3 | 1.5 |
| | $1\times10^6$ | 2.19 | 2.19 |

### 2.8. Cryopreservation

Both groups of the study were frozen as previously described [9]. The cryo-vials were transferred to -70°C overnight and then transferred to liquid nitrogen tank at the next day for long-term storage (Figure 1G).

### 2.9. Statistical analysis

Results were expressed as mean–standard deviation of the mean from three biological replicates. The mean difference was significant at $p<0.05$ level.

## 3. Results

### 3.1. Medium screening in static suspension culture

Here, we design a defined serum-free expansion medium which can support the long-term proliferation of hNPCs in culture. To reach this goal, five different expansion media with the combination of various growth factors were evaluated to the culture of two different hNPCs lines. As shown in (Supplementary Figure 1A), cell proliferation rates after five passages were significantly higher in NM3 group for both hESC-NPCs and hiNSCs cell lines that reached $2.3 \times 10^6$ cells/ml and $1.8\times10^6$ cells/ml, respectively ($p<0.01$). In addition, there was no significant difference between PPRF-h1 ($1.2\times10^6$ for hESC-NPCs and $1.4\times10^6$ for hiNSCs) and PPRF-h2 ($1.4\times10^6$ for hESC-NPCs and $1.5\times10^6$ for hiNSCs) groups and the lowest proliferation rates were

obtained in NM1 and NM2. Therefore, NM3 was selected as the best group for further experiments.

Furthermore, we monitored the cumulative cell numbers, aggregate growth kinetic and diameters, in our selected group for both studied cell lines (supplementary figure 1B-D). As shown in the figure both tested cell lines successfully formed cell aggregates after 48 hours. Aggregate diameters gradually increased up to 7 days of culture and reached around 125µm in both cell lines. A kinetic study of the aggregate diameter size revealed that both studied cell lines aggregates had shown similar trends after 7 days of culture, with no significant differences in final size (Supplementary Figure 1B). Moreover, cell counts showed that both cell lines reached the peak of viable cell density after 7 days of culture (Supplementary Figure 1C). Therefore, cells were passaged every 7 days in static suspension cultures condition. The maximum fold increase observed in both tested cell lines was approximately a 1.2-1.5 fold in static culture conditions.

## 3.2. Dynamic suspension culture of hNPCs in the bioreactor

### 3.2.1. Inoculation density and its impact on the aggregate formation

Dynamic suspension cultures were performed in 50mL siliconized stirred bioreactors using hESC-NPC and hiNSC (without any adaptation in stationary culture system) in NM3 medium. To reach the optimum single-cell inoculation density in dynamic culture, bioreactors containing 50mL of NM3 medium were inoculated with three cell inoculum densities: $2\times10^5$, $5\times10^5$, and $1\times10^6$ cells/ml. The result revealed that the $2\times10^5$ cells/ml inoculation density resulted in higher cell yield for both cell lines (Table 2). Results were probably related to the efficient cell aggregation due to a suitable ratio of initial cell numbers to the culture medium volume. The maximum cell density obtained for hESC-NPCs and hiNSC was $0.84\times10^6$ cells/ml ($2\times10^5$ cells/ml inoculation) and $0.8\times10^6$, respectively. These results revealed that cell proliferation rates of hNSC lines in dynamic suspension 2-2.5 fold higher than static suspension culture system.

Besides, developing efficient protocols for dissociation of cell aggregates to achieve highly viable single cells is crucial for large scale expansion cultures. Enzymatic treatment of aggregates with Trypsin for both cell lines resulted in higher single cell viability (~85-90%) and proliferation rate (~ FI 4) compared to collagenase (~ 35% viability rate and ~ FI 0.5-1) (Figure 1, F, $P<0.05$). However, the optimum incubation time in the enzyme solution differed for different cell types of similar aggregate sizes, which indicated that different cell lines showed different resistance to enzymatic dissociation.

### 3.2.2. Size-control of cell aggregates

Here we evaluated various agitation rates (30–60 rpm) to reach the size-controlled homogenous cell aggregates under high throughput proliferation condition. Various conditions were compared based on cell proliferation, viability, and aggregate size kinetics.

Proliferation rate and viability of both cell lines significantly declined at the agitation rates of higher than 50 rpm ($p<0.05$; Supplementary Figure 2A-B). This may probably relate to excessive shear stress and subsequent cell death during and after aggregation at higher mixing rates. The maximum resulted fold increase reached to 4 for hESC-NPCs and 3.8 for hiNSC at 45 rpm agitation rate.

Afterward, we monitored the kinetics of hNSCs aggregate sizes and cell count in different mixing rates (Figure 1A-B). According to our evaluation, different aggregate sizes (50-500 µm) were generated by using different agitation rates. Our aim was to reach more homogenous aggregates without affecting proliferation rate and increase the risk of differentiation.

Moreover, we found that 45 rpm agitation rate resulted in more homogeneous and size-controlled

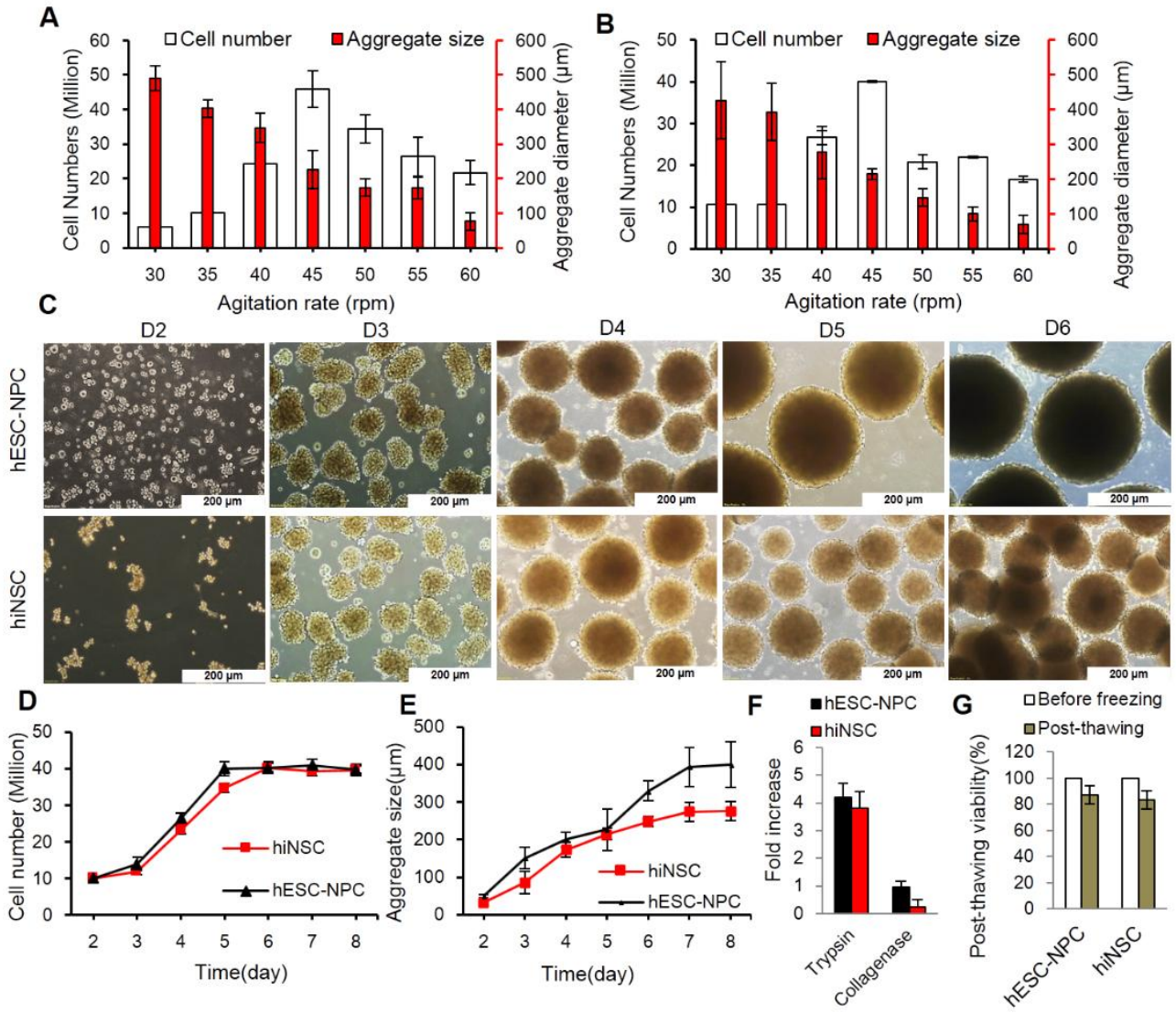



**Figure 1.** Kinetics of hNPCs aggregate formation and growth. A,B) Size controlled hNPCs aggregates formation by different agitation rates (A: hESC-NPCs, B: hiNPCs) (C) kinetics of hNPC aggregate growth morphology within 6 days, (D) cell number and (E) size within 8 days of culture in the bioreactor.(F) The impact of different types of enzymes on hNPCs folds increase after dissociation. (G) The impact of Freeze-thawing procedure on hNPCs viability.

aggregates (210±20µm) without a significant negative effect on cell proliferation for both cell lines (Figure 1C).

Accordingly, the optimum time of passaging time was determined after studying of proliferation rate and aggregate size growth kinetics in both studied cell lines during 8 days after passaging. The results revealed that the maximum cell numbers reached its peak after five days of culture with no increase up to 8 days (Figure 1D). However, aggregate growth kinetics have revealed that the aggregate size distribution was increased until seven days, although in the fifth day we had a homogenous aggregates size distribution which was the best point of viability and proliferation rates in both studied groups (Figure 1D). In the lower mixing rates (30-40 rpm) the proliferation rates had been gradually decreased due to the rise in the diameter size of aggregates. Besides

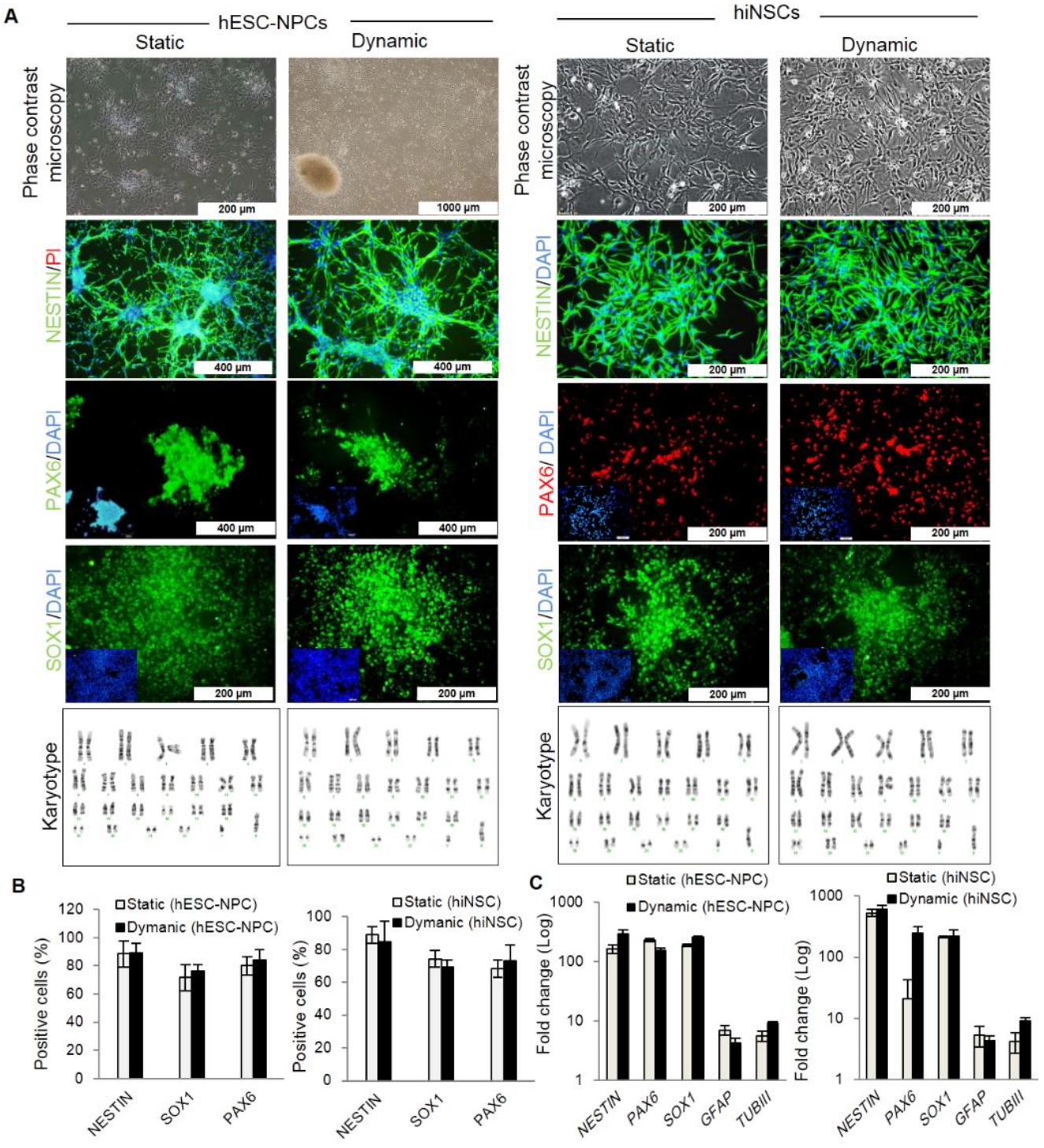



**Figure 2.** Characterization of hNPCs before and after expansion in bioreactor. (A) Characterization of hESC-NPCs and hiNSCs in static (before) and dynamic (after) expansion in the bioreactor with Phase contrast microscopy which shows the morphology of the hNPCs. Immunofluorescence staining for neural stem cell markers such as NESTIN, PAX6, SOX1. And karyotype analysis that shows the chromosomal stability after expansion in the bioreactor. (B) Flow cytometry analysis for NESTIN, PAX6 and SOX1 as neural stem cell markers in static and dynamic culture system for both cell lines. (C) Neural stem cell gene expression analysis for both hNPCs in static and dynamic culture system.

in the large aggregates size (< 300 µm), the diffusion rates of nutrients and metabolites inside the aggregates are largely limited [19, 21]. Therefore, the center of aggregates looks change to the dark parts which are related to the necrosis. And the average aggregate size declined at higher mixing rates.

After two passages in stirred bioreactor, all aggregates were harvested and cryopreserved by the freezing protocol which is introduced before (each vial contains ($1\times10^7$)) [9]. Then, the one vial of the frozen cells has been directly transferred to the bioreactor filled with 50 ml NM3 medium.

### 3.2.3. Characterizations of hNPCs

To ascertain the stability of neural stem/progenitor properties in both tested cell lines, they have been characterized after a minimum of ten passages in the stirred bioreactor [9].

The re-plated hNPCs as single cells were undifferentiated hNPCs in adherent culture (Figure 2A). Moreover, adherent hNPCs obtained from dynamic culture after 5 passages were positive for neural stem/progenitor cell markers such as Nestin, Sox1, and Pax6 for both studied cell lines (Figure 2A) determined by quantification of Immunofluorescent staining by flow cytometry analysis and QPCR (Figure 2B-C). Following karyotype analysis had been conducted for the cells obtained from optimum culture conditions. G-band karyotyping of hNPCs demonstrated a normal karyotype before and after expansion in stirred suspension bioreactor.

Then, to assess the in vitro differentiation potential of both studied cell lines which cultured before and after dynamic suspension system have been spontaneously differentiated in NM3 expansion medium by removing all growth factors during 35-day. We quantified the positive cell numbers for specific neural markers such as TUJ1, NF, GFAP following immunofluorescence staining of the differentiate cell aggregates (Figure 3A-B).

## 4. Discussion and Conclusion

In this study, a robust culture system has been developed for mass production of homogeneous size-controlled hESC-NPCs and hiNSCs in dynamic suspension culture conditions. This has been done by exploring a defined medium for expansion of both hNPCs from different sources and identifying key parameters in the stepwise optimization of suspension culture system [22].

Beside substantial efforts in dynamic culturing of hNPCs [16, 17, 23-25] to develop a robust culture system, we have addressed the most important challenges in previously reported studies which are described below.

Lack of more efficient introduced culture medium which could support proliferation while maintaining self-renewal properties is the first issue. Besides many efforts for finding the best expansion medium up to now [16, 17, 25, 26], the more efficient ones in dynamic culture system are PPRFh1 and PPRFh2 [16]. Here, we screened five different groups of defined hNPCs expansion media (supplemented with growth factors) in suspension culture. Results revealed that NM3 medium could support higher fold expansions compared to other media that introduced in previous studies [16] (Table 3). Our designed NM3 medium could support proliferation and maintenance of hNPCs in both static and dynamic suspension culture in the bioreactor by using bFGF (20 ng/ml), hEGF (20ng/ml) and CHIR99021 (3µg/ml).

Despite previous studies, we use a minimum amount of culture medium (50ml) for expansion of hNPCs in bioreactor system compared to others which use minimum 125ml culture medium [16, 17, 23].

Moreover, the significant cell death within passages when using the single inoculation strategy [27], is one of the important challenges here. So, in the second step, we could overcome this issue by a combination of trypsin enzyme and Rock inhibitor. The cell density is another key parameter [10, 16, 17, 25], that we addressed by largely improvement of expansion rates following the right selection of inoculation density for both studied cell lines. Efficient cell-cell interactions that inhibit significant cell death seem that resulted in suitable inoculation density of hNPCs. On the other hand, high cell density inoculation can cause sudden depletion of nutrients in the medium and rapid production of metabolic waste, which has noxious effects on cell growth and proliferation rates. Previous studies have shown that the formation of the heterogeneous and large size of aggregates (~ 500µm)

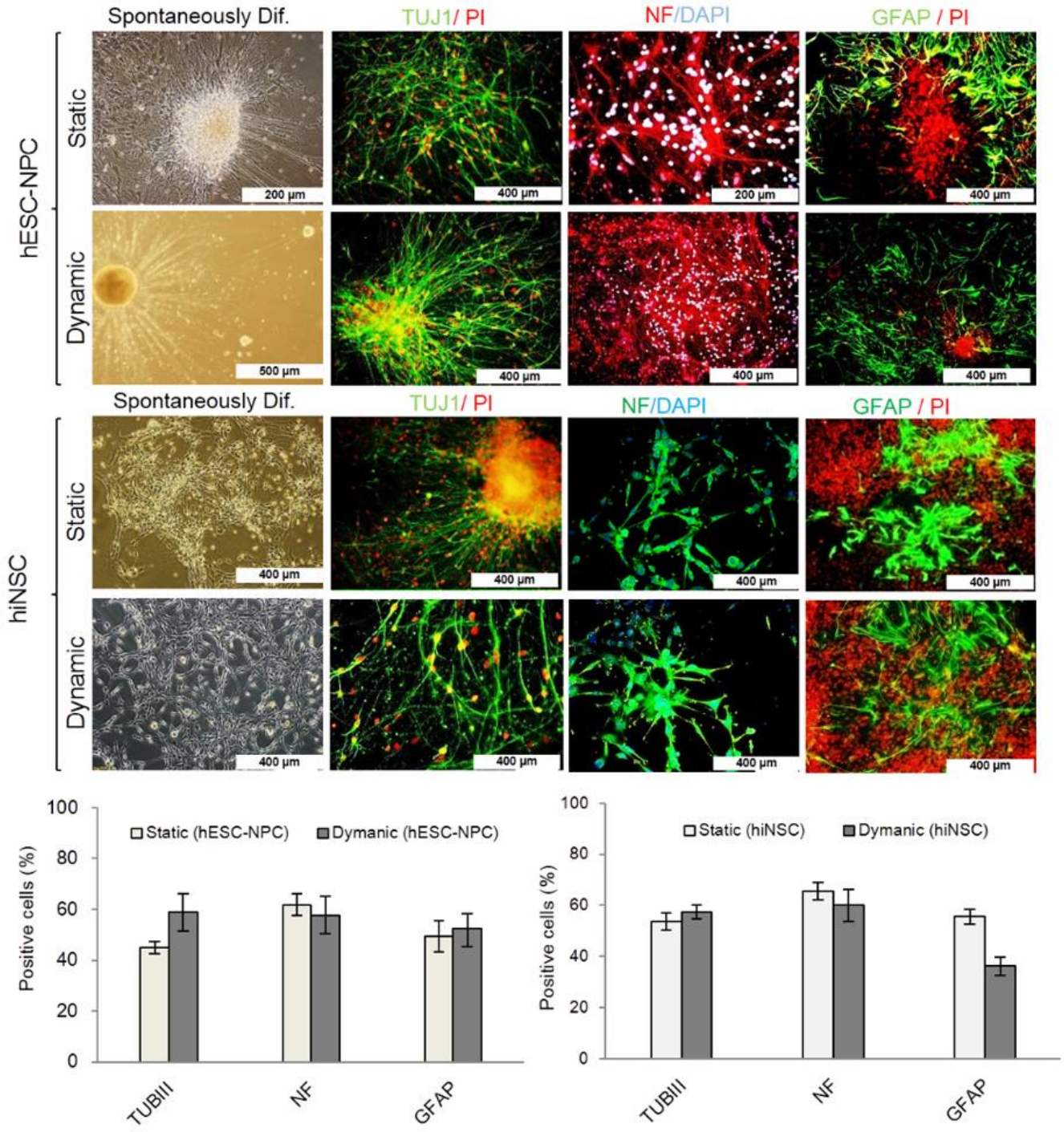



**Figure 3.** Characterization of spontaneously differentiation potential of hNPCs in static and dynamic culture in bioreactor. (A) Phase contrast image and immunofluroscence staining for neuronal markers such as TUJ1, NF and GFAP for spontaneously differentiation of hESC-NPCs and hiNSCs in static and dynamic culture. (B) Quantification of immunofluorescence staining for neuronal markers.

**Table 3.** Comparative evaluation of our study with other related works which is focused on the suspension culture of hNSCs in a spinner bioreactor.

| Bioprocess parameter | Michael S et al. 1998 | Kallos MS et al. 2006 | Behnam A. Baghberani et al. 2008 | Behnam A. Baghberani et al. 2010 | Current Study |
|---|---|---|---|---|---|
| Cell lines | Mouse NSCs | Mouse embryo (day14)NSCs | hNSCs (telencephalon) 10weeks embryo | ahNPC (M006) | hiNSCs & hESC-NPCs |
| Medium | DMED/F12+ (20 µg/ml)EGF | PPRF-m4* | PPRF-h2** | PPRF-h2** | NM3 |
| Passaging | Different time Mechanical | 7 days Not stated | 18-20 days Not stated | 14days Not stated | 5 days Trypsin |
| Bioreactor Type | Spinner flask | 4-blade & paddle bioreactor | computer-controlled suspension bioreactor | Not stated (suspension bioreactor) | Spinner flask |
| Inoculation density (Cell/ml) | 10000 (125-250ml) | 75000 (500ml) | 100000cell/ml (125-250ml) | 100000cell/ml (125ml) | 200000cell/ml (50ml) |
| Cell loss after inoculation | 20% | 20% | 10% | 20% | 10% |
| Agitation rate(rpm) | 100 | 40(first 3days) -60 | 70-130 | 100 | 45 |
| Aggregate size | 200µm | 80–150 µm | 500µm | Not stated | 250µm |
| Expansion rates in bioreactor | Not stated | $1.2\times 10^6$ cells | 36 fold over 18 days 3600000 | $7.8\times10^{13}$ Within 140 days | $1.024\times10^7$ cells Within 30days |
| Oxygen tension scale-up platform | 5 & 20% | Not stated | 20% | 20% | 20% |
| PH and viscosity | checked | Not stated | Not stated | Not stated | Not stated |

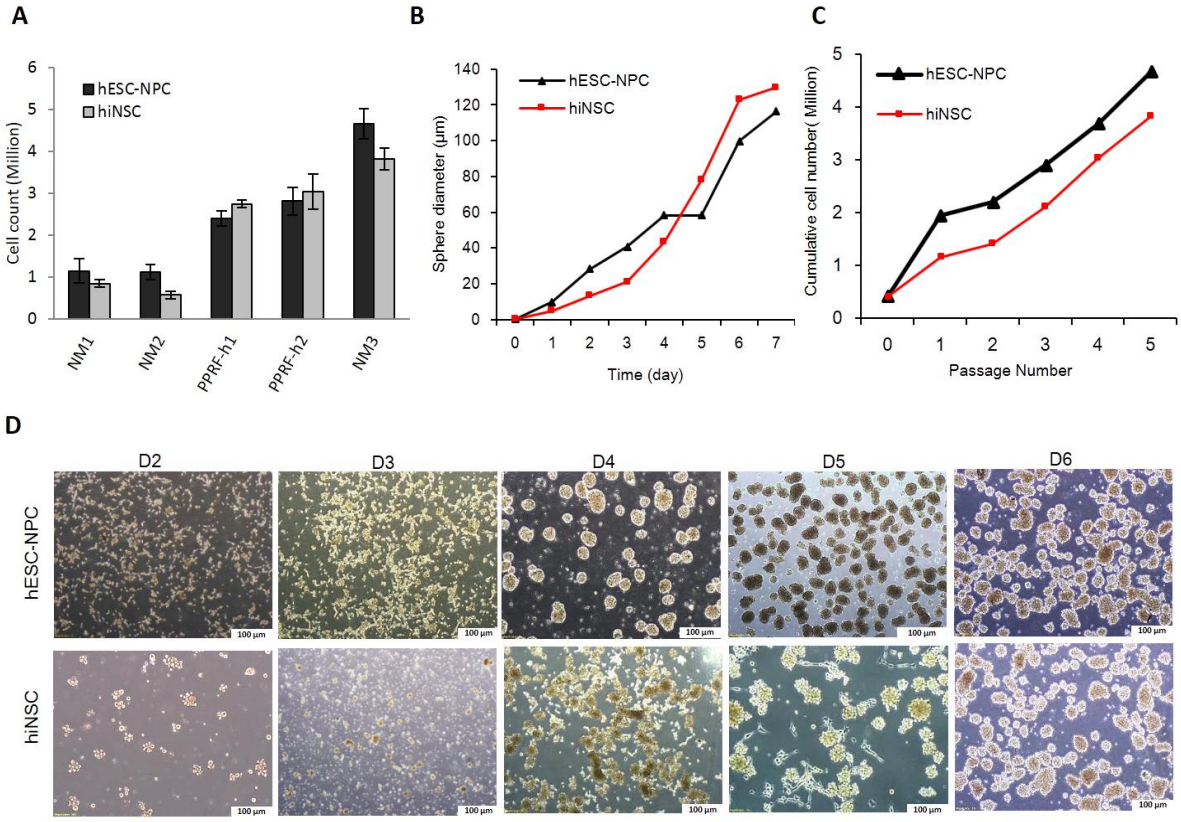
* PPRF-m4: DMEM/F12(1:1), 6%glucose, 5mM HEPES, 1.73g/lL Sodium bicarbonate, 2.0mM glutamine , 0.023 g/L insulin , 20 nM progesterone , 9.0mg/L putrescine , 0.025 g/L transferrin , 30 nM sodium selenite , 20µg/L EGF and 10µg/L bFGF

** PPRF-h2: PPRF-m4, 10µg/L hLIF, 20µg/L bFGF and 1 µM DHEA

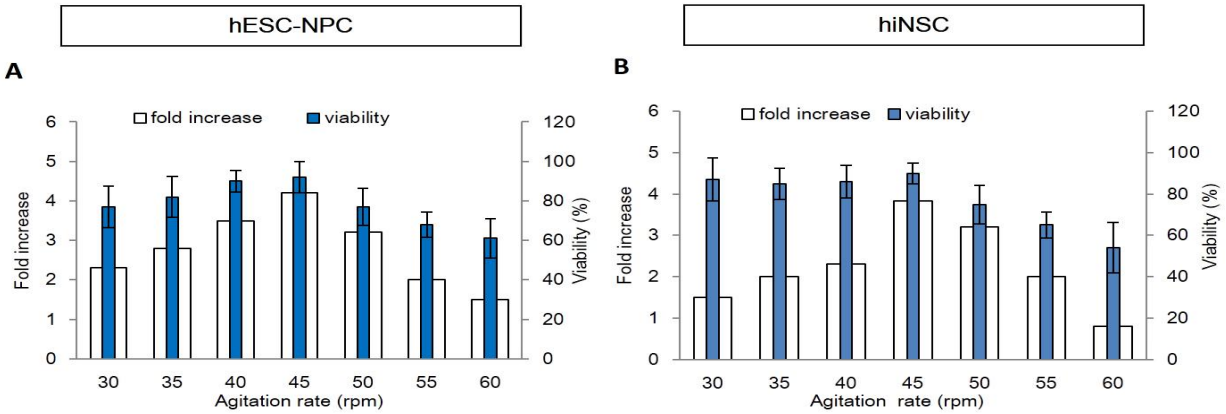
can cause the generation of heterogeneous cell populations with different potential fates [16, 17]. Furthermore, cell death in the center of big aggregates is the other point here [28, 29]. For addressing this issue, we demonstrated that reaching size-controlled aggregates (~250µm) could be possible by using precise control of the agitation rates of spinner flasks. And it seems more scalable than other technologies established for controlling the size of cell aggregates such as microwell-mediated control, hanging drops, and microprinting technologies [29]. Besides, higher mixing rates could improve nutrient distribution and providing a homogenous culture environment. Therefore,

aggregate formation should be well balanced in association with minimal shear stress in dynamic conditions to limit negative effect on cell proliferation and multipotency. Up to now, there have not been any reports into shear effects on growth kinetics in stem cell suspension cultures but the topic has been reviewed recently [30]. Here, we have shown that the hydrodynamic culture condition of a stirred suspension bioreactor is a crucial element for hNPCs culture scale-up culture due to the higher sensitivity of hNPCs to shear stress in higher agitation rates.

To sum up, present study demonstrates that the long-term expansion of hESC-NPCs and hiNSCs in suspension bioreactors is feasible and depends on appropriate growth factors, supplements and dynamic conditions in a serum-free medium. Cells expanded in serum-free NM3 medium retained their capacity to proliferate, and exhibited multipotency properties including the ability to produce large numbers of neurons. Moreover, $1.024\times10^{7}$ undifferentiated hNPC cells could be generated within 30 days in spinner flasks that contain 50 ml of expansion medium with the use of this culturing strategy and scale-up platform. This is a great privilege of our established suspension protocols which can simultaneously facilitate and expedite the production of a clinically relevant number of cells in a minimal culture volume and time. Therefore, hNPCs can be efficiently expanded in bioreactors, and will offer a viable and more attractive alternative to the fetal tissues that currently being used in experimental therapeutic modalities for treatment of neurodegenerative disorders.




**Supplementary Figure 1.** (A) Proliferation rates of hNPCs in stationary suspension culture by five different serum-free defined media. (B) Sphere size distribution of hNPCs cell lines in NM3 medium during 7 days of culture. (C) Cumulative viable cell counts of hNPCs in 7-day stationary suspension culture using NM3 medium after 5 passages, determined by the Trypan blue exclusion method. (D) Aggregate growth kinetics of hNPCs cell lines in NM3 medium during 6 days of culture.




**Supplementary Figure 2:** Expansion, viability, and aggregate diameter of hNPCs cultured in dynamic suspension at different agitation rates. (A, B) hESC-NPC and hiNPC cell lines were expanded at different agitation rates (35–60 rpm) and their viabilities under normal conditions in a bioreactor were evaluated sequentially.

**Supplementary Table 1.** Antibody used in this study.

| Antibody name | Species | Clonality | Dilution | Source |
|---|---|---|---|---|
| **Primary Ab** | | | | |
| **NESTIN** | Mouse | Monoclonal | 1:100 | Chemicon, MAB5326 |
| **PAX-6 (H-295)** | Rabbit | Polyclonal | 1:200 | Santa Cruz, CA, USA, 11357 |
| **SOX1** | Rabbit | Polyclonal | 1:1000 | Abcam, Cambridge, UK, 22572 |
| **β-TUBLIN III** | Mouse | Monoclonal | 1:250 | Sigma-Aldrich, T8660 |
| **GLIAL FIBRILLARY ACIDIC PROTEIN** | Mouse | Monoclonal | 1:200 | Sigma-Aldrich, G3893 |
| **NEUROFIBRILARY FIBROBLAST (NF-H)** | Mouse | Monoclonal | 1:200 | Sigma-Aldrich, N0142 |
| **Secondary Ab** | | | | |
| **FITC anti-mouse IgG** | | | 1:200 | Chemicon, AP308F |
| **FITC anti-rabbit IgG** | | | 1:200 | Sigma-Aldrich, F1262 |
| **Texas red anti-mouse IgG** | | | 1:100 | Jackson Immunoresearch Lab., 315-075-003, Hamburg, Germany |
| **FITC anti-mouse IgM** | | | 1:100 | Sigma-Aldrich, F9259 |

**Supplementary Table 2.** Primers sequence and information used in the current study.

| Genes | Primer sequences (5'-3') | Size (bp) | Annealing Temp. (°C) |
|---|---|---|---|
| **GAPDH** | F: 5' CTC ATT TCC TGG TAT GAC AAC GA 3'<br>R: 5' CTT CCT CTT GTG CTC TTG CT 3' | 121 | 60 |
| **NESTIN** | F: 5' CTCCAGAAACTCAAGCACC 3'<br>R: 5' TCCTGATTCTCCTCTTCCA 3' | 144 | 60 |
| **SOX1** | F: 5' GTG TAC CCT GGA GTT TCT G 3'<br>R: 5' TAG TCT GTG CCT CTA AGG TG 3' | 200 | 60 |
| **PAX6** | F: 5' CGGTTTCCTCCTTCACAT 3'<br>R: 5' ATCATAACTCCGCCCATT 3' | 196 | 60 |
| **GFAP** | F: 5' GAGATGCGGGATGGAGAG 3'<br>R: 5' TAGGGACAGAGGAGGGAG 3' | 201 | 60 |
| **TUBIII** | F: 5' GTATCCCGACCGCATCAT 3'<br>R: 5' TCTCATCCGTGTTCTCCA 3' | 142 | 60 |
| **OCT3/4** | F:TCT ATT TGG GAA GGT ATT CAG C<br>R:ATT GTT GTC AGC TTC CTC CA | 124 | 60 |
| **NANOG** | F:AAA GAA TCT TCA CCT ATG CC<br>R: GAA GGA AGA GGA GAG ACA GT | 110 | 60 |

## Conflict of interest

The authors declare that they have no conflict of interests.

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## References

[1] Chiu AY, Rao MS. Cell-based therapy for neural disorders-anticipating challenges. Neurotherapeutics. 8(4):744-52.

[2] Mothe AJ, Tator CH. Advances in stem cell therapy for spinal cord injury. J Clin Invest.122(11):3824-34.

[3] Lunn JS, Sakowski SA, Hur J, Feldman EL. Stem cell technology for neurodegenerative diseases. Ann Neurol.70(3):353-61.

[4] Fan Y, Wu J, Ashok P, Hsiung M, Tzanakakis ES. Production of Human Pluripotent Stem Cell Therapeutics under Defined Xeno-free Conditions: Progress and Challenges. Stem Cell Rev.

[5] Diecke S, Jung SM, Lee J, Ju JH. Recent technological updates and clinical applications of induced pluripotent stem cells. Korean J Intern Med.29(5):547-57.

[6] Ng TK, Fortino VR, Pelaez D, Cheung HS. Progress of mesenchymal stem cell therapy for neural and retinal diseases. World J Stem Cells.6(2):111-9.

[7] Torper O, Pfisterer U, Wolf DA, Pereira M, Lau S, Jakobsson J, et al. Generation of induced neurons via direct conversion in vivo. Proc Natl Acad Sci U S A.110(17):7038-43.

[8] Tian C, Ambroz RJ, Sun L, Wang Y, Ma K, Chen Q, et al. Direct conversion of dermal fibroblasts into neural progenitor cells by a novel cocktail of defined factors. Curr Mol Med.12(2):126-37.

[9] Nemati S, Hatami M, Kiani S, Hemmesi K, Gourabi H, Masoudi N, et al. Long-term self-renewable feeder-free human induced pluripotent stem cell-derived neural progenitors. Stem Cells Dev.20(3):503-14.

[10] Baghbaderani BA, Mukhida K, Hong M, Mendez I, Behie LA. A review of bioreactor protocols for human neural precursor cell expansion in preparation for clinical trials. Curr Stem Cell Res Ther.6(3):229-54.

[11] Pournasr B, Khaloughi K, Salekdeh GH, Totonchi M, Shahbazi E, Baharvand H. Concise review: alchemy of biology: generating desired cell types from abundant and accessible cells. Stem Cells.29(12):1933-41.

[12] Lujan E, Chanda S, Ahlenius H, Sudhof TC, Wernig M. Direct conversion of mouse fibroblasts to self-renewing, tripotent neural precursor cells. Proc Natl Acad Sci U S A.109(7):2527-32.

[13] Margariti A, Winkler B, Karamariti E, Zampetaki A, Tsai TN, Baban D, et al. Direct reprogramming of fibroblasts into endothelial cells capable of angiogenesis and reendothelialization in tissue-engineered vessels. Proc Natl Acad Sci U S A.109(34):13793-8.

[14] Shahbazi E, Moradi S, Nemati S, Satarian L, Basiri M, Gourabi H, et al. Conversion of Human Fibroblasts to Stably Self-Renewing Neural Stem Cells with a Single Zinc-Finger Transcription Factor. Stem cell reports. 2016;6(4):539-51.

[15] Hook L, Vives J, Fulton N, Leveridge M, Lingard S, Bootman MD, et al. Non-immortalized human neural stem (NS) cells as a scalable platform for cellular assays. Neurochem Int.59(3):432-44.

[16] Baghbaderani BA, Mukhida K, Sen A, Kallos MS, Hong M, Mendez I, et al. Bioreactor expansion of human neural precursor cells in serum-free media retains neurogenic potential. Biotechnol Bioeng.105(4):823-33.

[17] Baghbaderani BA, Behie LA, Sen A, Mukhida K, Hong M, Mendez I. Expansion of human neural precursor cells in large-scale bioreactors for the treatment of neurodegenerative disorders. Biotechnol Prog. 2008;24(4):859-70.

[18] Nemati S, Shahbazi E, Hajihosseini R. Generation and Maintenance of Neural Progenitor Cell Lines Derived from Human Embryonic Stem Cells by Small Molecules. Galen Medical Journal. 2017;6(2):145-56.

[19] Abbasalizadeh S, Larijani MR, Samadian A, Baharvand H. Bioprocess development for mass production of size-controlled human pluripotent stem cell aggregates in stirred suspension bioreactor. Tissue Eng Part C Methods.18(11):831-51.

[20] Mollamohammadi S, Taei A, Pakzad M, Totonchi M, Seifinejad A, Masoudi N, et al. A simple and efficient cryopreservation method for feeder-free dissociated human induced pluripotent stem cells and human embryonic stem cells. Hum Reprod. 2009;24(10):2468-76.

[21] Bauwens CL, Peerani R, Niebruegge S, Woodhouse KA, Kumacheva E, Husain M, et al. Control of human embryonic stem cell colony and aggregate size heterogeneity influences differentiation trajectories. Stem Cells. 2008;26(9):2300-10.

[22] Nemati S, Abbasalizadeh S, Baharvand H. Scalable Expansion of Human Pluripotent Stem Cell-Derived Neural Progenitors in Stirred Suspension Bioreactor Under Xeno-free Condition. Methods in molecular biology. 2016;1502:143-58.

[23] Carpenter MK, Cui X, Hu ZY, Jackson J, Sherman S, Seiger A, et al. In vitro expansion of a multipotent population of human neural progenitor cells. Exp Neurol. 1999;158(2):265-78.

[24] Kanemura Y, Mori H, Kobayashi S, Islam O, Kodama E, Yamamoto A, et al. Evaluation of in vitro proliferative activity of human fetal neural stem/progenitor cells using indirect measurements of viable cells based on cellular metabolic activity. J Neurosci Res. 2002;69(6):869-79.

[25] Baghbaderani BA, Behie LA, Mukhida K, Hong M, Mendez I. New bioengineering insights into human neural precursor cell expansion in culture. Biotechnol Prog.27(3):776-87.

[26] Falk A, Koch P, Kesavan J, Takashima Y, Ladewig J, Alexander M, et al. Capture of neuroepithelial-like stem cells from pluripotent stem cells provides a versatile system for in vitro production of human neurons. PLoS One.7(1):e29597.

[27] Sen A, Kallos MS, Behie LA. New tissue dissociation protocol for scaled-up production of neural stem cells in suspension bioreactors. Tissue Eng. 2004;10(5-6):904-13.

[28] Niebruegge S, Bauwens CL, Peerani R, Thavandiran N, Masse S, Sevaptisidis E, et al. Generation of human embryonic stem cell-derived mesoderm and cardiac cells using size-specified aggregates in an oxygen-controlled bioreactor. Biotechnol Bioeng. 2009;102(2):493-507.

[29] Bauwens CL, Song H, Thavandiran N, Ungrin M, Masse S, Nanthakumar K, et al. Geometric control of cardiomyogenic induction in human pluripotent stem cells. Tissue Eng Part A.17(15-16):1901-9.

[30] Kinney MA, Sargent CY, McDevitt TC. The multiparametric effects of hydrodynamic environments on stem cell culture. Tissue Eng Part B Rev.17(4):249-62.