Title: Ascorbic acid can promote the generation and expansion of neuroepithelial-like stem cells derived from hiPS/ES cells under chemically defined conditions through promoting collagen synthesis

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Abstract

Introduction: Spinal cord injury (SCI) is a neurological, medically incurable disorder. Human pluripotent stem cells (hPSCs) have the potential to generate neural stem/progenitor cells (NS/PCs) which hold promise in therapy for SCI by transplantation. In our study, we aimed to establish a chemically defined culture system by using serum-free medium and ascorbic acid (AA) to generation and expansion of long-term self-renewing neuroepithelial-like stem cells (lt-NES cells) differentiated from hPSCs effectively and stably.

Methods: We induce hESC/iPSC to neurospheres by using a newly established induction system in vitro in our study. And lt-NES cells derived from hESCs/iPSCs-neurospheres using two induction systems, including conventional N2 medium with gelatin-coated (coated) and N2+AA medium without pre-coated (AA) were characterized by reverse transcription-polymerase chain reaction (RT-PCR) analysis and immunocytochemistry staining. Subsequently, lt-NES cells were induced to neurons and the microelectrode array (MEA) recording system was used to evaluate the functionality of neurons differentiated from lt-NES cells.

Moreover, the mechanism of AA-induced lt-NES cells was explored through RNA-seq and the use of inhibitors.

Results: HESCs/iPSCs were efficiently induced to neurospheres by using a newly established induction system in vitro. And lt-NES cells derived from hESCs/iPSCs-neurospheres using two induction system (coated vs AA) both expressed neural pluripotency-associated genes PAX6, NESTIN, SOX1, SOX2. After long-term cultivation, we found that they both can maintain the long-term expansion for more than a dozen generations while maintaining neuropluripotency. Moreover, the lt-NES cells retain the ability to differentiate into general functional neurons that highly express β-tubulin. We also demonstrated that AA promotes the generation and long-term expansion of lt-NES cells by promoting collagen synthesis via the MEK-ERK1/2 pathways.

Conclusions: Taken together, this new chemically defined culture system is stable and effective to generate and culture the lt-NES cells induced by hESCs/iPSCs using serum-free medium combined with ascorbic acid (AA). The lt-NES cells under this culture system can maintain the long-term expansion and neural pluripotency, with the potential to differentiate into functional neurons.

Keywords: Spinal cord injury, Neurospheres, Ascorbic acid, It-NES cells, Human pluripotent stem cells.

Introduction

Spinal cord injury (SCI) is a destructive neuro-degenerative disorder with life-long consequences, and it often leads to irreversible changes due to the loss of neurons and glial cells [1,2]. However, the quality of life for SCI patients has been improved by the findings of past research, but there is still no effective therapy available. In recent years, there are many experimental animal models simulating SCI in humans and the transplantation of neural stem/progenitor cells (NS/PCs) has been shown to be an effective treatment to cure the neurological disorders and repair damaged brain tissue due to their ability to differentiate into neurons and glial cells and the competence to secrete neurotropic factors [3,4].

In the research field of SCI, NS/PCs derived from mouse embryonic stem cells (mESCs) [5] and rat embryonic spinal cord[3] have been transplanted into the injured spinal cord of mice and rats, promotes functional recovery in animal SCI models. Also, the NS/PCs, derived from the human fetal brain [6,7] have been transplanted into the injured spinal cord model of mice or nonhuman primates [6,8], greatly promoted the

development of stem cell-based transplantation therapy for human patients. However, the collection of NS/PCs from brains of aborted fetuses or surplus embryos is not allowed on clinical applications in Japan. This fact has been a major hinder to the clinical use of human NS/PCs[9].

Under this circumstance, human embryonic stem cells (hESCs) [10,11] and induced pluripotent stem cells (iPSCs) established by Yamanaka et al. [12,13] have the ability to differentiate into NS/PCs, providing a rich source of neurons and glial cells [14], therefore, they provided a new approach for studying human neurodevelopment, simulating neurological disease, and regenerative medicine. Moreover, iPSCs reprogrammed from somatic cells provide the new prospect of generating patient-specific cells for disease modeling and regenerative medicine[12]. The ability of proliferation and differentiation of iPSCs is almost equal to that of ES cells, and the use of iPSCs could circumvent the ethical issues and rejection in NS/PCs transplantation related to the use of embryos and aborted tissues.

Neurosphere is a well-known classic culture system for NS/PCs extension [15] and the present neurospheres induction protocols of for human pluripotent stem cells (hPSCs) is depended upon the formation of embryoid body [16–18], but this method has many considerable disadvantages, such as unclear culture conditions, prolonged differentiation and low differentiation efficiency. In addition to these methods, human

115 long-term self-renewing neuroepithelial-like stem cells (lt-NES cells) [18]
116 have reached the standard defined as NS/PCs, providing a more
117 homogeneous and robust cell generation in ways of monolayer adherent
118 cultures [19]. And all lt-NES cells derived from different hESC/hiPSCs
119 exhibit characteristics like continuous expandability, stable neuronal and
120 glial differentiation competence and the capacity to generate functionally
121 mature human neurons [14,20,21]. However, these lt-NES cells culture
122 systems are inseparable from the plates coated with adhesive materials, so
123 they are not chemically clear culture systems. Therefore, it is very crucial to
124 develop a culture system of lt-NES cells under chemically defined
125 conditions.

126 In our research, we describe for the first time a serum-free culture
127 system to generate lt-NES cells from different hPSCs under chemically
128 defined conditions without the need for pre-coated and avoiding the use of
129 basement membrane preparation such as Matrigel matrix,
130 poly-L-lysine/laminin-coated or inactivated MEF-feeder plates [22,23] with
131 unclear chemical composition, which limit the clinical application of NS/PCs.
132 In this system, we applied ascorbic acid (AA) which promoting collagen
133 synthesis through the MEK-ERK1/2 pathway [24] to induce the generation
134 and long-term expansion of lt-NES cells. This method is expected to
135 facilitate the clinical application of NS/PCs derived from hPSCs for the

regenerative medicine of neurological disorders and injuries, like spinal cord injury [25].

Materials and Methods

1 Human PSCs culture

Human PSCs including hESCs-H9 and urine-derived iPSCs (UiPSCs, generated in our laboratory by standard Sendai Reprogramming (CytoTune^{TM}-iPS 2.0 Sendai Reprogramming Kit, Invitrogen, USA)) were cultured in PSCeasy medium (Cellapy, China) on six-well plates (Corning, US) coated with a 1:500 dilution of hESC-matrigel (Corning). Medium was changed every day. PSCs were passaged every 3–4 days at 70-80% confluence with 0.5 mM EDTA (Cellapy). All cells were maintained at 37 °C, 5% CO_2 in incubator (Thermo Fisher Scientific, USA).

2 Neurosphere differentiation of PSCs

The protocol of neurosphere differentiation was shown in Figure 1A. When hPSCs were grown for 4 days on human recombinant laminin fragment iMatrix-511 (Nippi , Japan) - coated (0.5 μg/cm^2) 6-well plate, at which time they reached 100% confluence, namely at day 0 of induction of differentiation, cells were washed with PBS (Hyclone, USA), and then added the 2ml NeuroEasy Human Neural Stem Cell Induction Culture medium (Cellapy, DMEM/F12 containing 500 μg/mL recombinant human

albumin (A0237, Sigma-Aldrich)+2 uM Chir99021(Selleck, China)) per well, and change the medium every two days until the cells exhibited a rosette morphology (5-6d). And then, cells were dissociated with Accutase (Innovative Cell Technologies, USA) for 10min and resuspended by NeuroEasy Human Neural Stem Cell Culture medium (Cellapy, DMEM/F12 containing 2% NeuroEasy Human Neural Stem Cell supplement (xeno-free version of B27, Cellapy), supplemented with 20 ng/ml FGF2, 25ng/ml EGF and 25ng/ml heprin) on low cell-adhesion plates. A ROCK inhibitor, Y27632 (Selleck, China) at a concentration of 10uM, was used only at the time of plating. Next day, cell aggregated to form neurospheres, and the medium was changed to Neural stem cell culture medium without Y27632. Neurospheres were passaged every 3-4 days when they grow to 20um in size with accutase. Medium was changed every 2-3days. All cells were maintained at 37 °C, 5% CO_2 in incubator.

3 lt-NES cells differentiation of neurosphere

When the neurospheres grow to 20um in size, we perform PBS washing and dissociated neurospheres with Accutase for 10min and resuspended by lt-NES cells culture medium (DMEM/F12 containing 2% N2 (xeno-free version, Cellapy), supplemented with 20 ng/ml FGF2, 25ng/ml EGF and 25ng/ml heprin) on gelatin-coated plate. When we identified the effect of AA in the generation and expansion of lt-NES, we use

uncoated-plate and we add the 50 μg/ml L-ascorbic acid-2-phosphate (Selleck) in the lt-NES cells culture medium during resuspension, long-term culture and other experimental procedures.

4 Cell viability and proliferation assays

Cell viability and proliferation was measured by the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan). The lt-NES cells under different condition were plated in 96-well plates (3000 cells/well) and cell viability was detected after 12h according to the manufacturer's protocol. Cell proliferation was detected every 24 h. At every point in time, 10 μl of CCK-8 reagent was added to each well，and the plate was incubated for 2 h at 37 °C. And then, each sample was measured absorbance at 450 nm using an automatic microplate reader (BioTek Synergn 4, USA). All experiments had three technical replicates each.

5 Differentiation of lt-NES cells into generic neurons and glia

lt-NES cells were dissociated by for 10mins and plated on human recombinant laminin fragment iMatrix-511-coated 12-well plate with glass slides at a low density of 2×10^4 cells/cm2 in Neuron maintenance medium (DMEM/F12 containing 2% NeuroEasy Human Neural Stem Cell supplement (xeno-free version of B27, Cellapy)), and half of the medium was changed every 2 or 3 days.

6 RNA extraction and quantitative real-time PCR

Cells were lysed and harvested using TRIzol™ Reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA was quantified with NANO drop 2000 (Thermo Fisher Scientific), and 1ug RNA was reverse transcribed into cDNA using the PrimeScript™ RT Master Mix reverse transcription System (TaKaRa, Japan). The levels of relative gene expression were analyzed by quantitative reverse transcriptase PCR (qRT-PCR) with TB Green™ Premix Ex Taq™ II (TaKaRa) using the iCycler iQ5 (Bio-Rad, USA). The housekeeping gene GAPDH was used for internal normalization and the relative quantification of gene expression was calculated according to the △CT method. The qRT-PCR primers are listed in Supplementary Table S1.

7 RNASeq Processing and Biological Information Analysis

Clustering and sequencing were executed by Novogene Corporation (Tianjing, China). The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) . After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform .Raw data (raw reads) of fastq format were processed through in-house perl scripts. All the downstream analyses were based on the clean data with high quality.

Differential expression analysis was performed using the DESeq2 R package (1.16.1). The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate . Genes with an adjusted P-value <0.05 found by DESeq2 were assigned as differentially expressed. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package, in which gene length bias wascorrected. GO terms with corrected Pvalue less than 0.05 were considered significantly enriched by differential expressed genes. KEGG is a database resource for understanding high-level functions and utilities of the biological system from molecular-level information. We used clusterProfiler R package to test the statistical enrichment of differential expression genes in KEGG pathways.

8 Immunofluorescence staining

Cells were fixed with 4% PFA (Solarbio, China) for 10-15min at room temperature, permeabilized with 0.3% Triton X-100 (Sigma) for 10-15 min at room temperature, blocked with 3% bovine serum albumin (Solarbio) for 45-60 min at room temperature, and then incubated with primary antibodies against OCT4 (1:100; Santa cruz Biotechnology), SSEA4 (1:100; Santa cruz Biotechnology), SOX2 (1:100; Santa cruz Biotechnology), NESTIN (1:100; Sigma), β3-tubulin (1:100; Abcam), and GFAP (1:100; Abcam) overnight at 4 °C . And then cells were incubated with secondary antibodies:

Goat anti-Rabbit IgG Alexa Fluor 488 (1:200; Invitrogen) and Goat anti-Mouse IgG Alexa Fluor 594 (1:200; Invitrogen) for 1hour at 37 °C. Wash with PBS three times before each step. Nuclei were stained with DAPI (300 nM, Invitrogen) for 15 min at room temperature. Fluorescence images were captured by Leica DMI 4000B fluorescence microscope and Leica TCS SP5 MP confocal laser scanning microscope (Leica, Germany).

9 Western blotting

Cells were lysed using tissue protein extraction reagent (Thermo, USA) containing phosphatase inhibitor cocktail (1:100,Thermo), protease inhibitor cocktail (1:100,Thermo), and 5mM EDTA (Thermo), lysates were oscillated and centrifuged (13 000 × g, 15 min, 4 °C). The supernatant was collected and stored at −80 °C. The protein concentrations were determined using the BCA protein assay kit (Thermo). The protein was mixed with 5X SDS-PAGE protein loading buffer (Beyotime, China) and denatured by 100 °C water bath. Then, the samples denatured were electrophoresed in 10% SDS-PAGE and transferred to PVDF membranes using transfer device (Bio-Rad). The membranes were blocked with 5% non-fat milk prepared in TBST for 1hour at 37 °C and then incubated at 4 °C overnight with the primary antibodies: ERK1/2(1:1000, Cell Signaling Technology, p44/42 MAPK Rabbit mAb, 4695), p-ERK1/2(1:1000, Cell Signaling Technology, Phospho-p44/42 MAPK (Erk1/2) XP®, Rabbit mAb, 4370) and

the internal normalization mouse anti-GAPDH (1:1000, Santa cruz Biotechnology). Next, the membranes were washed in TBST, incubated with secondary antibody: Goat anti-Rabbit IgG (H + L) IRDye 800CW or Goat anti-Rabbit IgG (H + L) IRDye 800CW (1:20 000; LI-COR) for 1 h at 37 °C. The images were observed with a UVA Bio Imaging System and analyzed with ImageJ software.

10 Data analysis and statistics

All data are analyzed by means ±standard errors of the means (S.E.M.). Statistical significance was evaluated using two-sided t-test for two groups, and using one-way ANOVA test for statistical differences of multiple groups. Significant differences were considered when *P < 0.05, **P < 0.01.

Results

Generation of neurosphere from hESCs/iPSCs.

To evaluate the availability of our approach, studies were performed in parallel in two cell lines, including hESC-H9 and hiPSC produced from urine [26] cells by standard Sendai Reprogramming. Urine-iPSCs (UiPSs) are morphologically identical from hESC-H9 and express representative pluripotent markers, including SSEA4 and OCT4 (Figure.1B a,b,f,g).

Neurospheres for culturing NS/PCs in vitro have been reported and are widely used [16], the protocol of neurospheres induction from

hESC/hiPSCs is shown in Figure 1A. HESC/hiPSCs can be efficiently differentiated to neurospheres with the rosette formation using the neural stem cell induction medium and rosette dissociation using the neural stem cell medium. In the process of differentiation, the morphology of hPSCs transition to neurospheres phenotypes is shown in Figure 1B. There is a significant change in cell morphology along with the cell proliferation. At the stages of hPSCs, cells expanded and displayed a round, colony morphology (Figure.1B a,b,f,g). As the duration of neural induction to day 5, cells exhibited a rosette morphology (Figure.1B c,h). At day 6, cells were digested and resuspended to form neurospheres (Figure.1B d,i). By starting with 100% confluence pluripotent stem cells, it is possible to get 20–40-fold increases in cell number of NS/PCs in 6 days, so we can get enough NSCs to meet the needs of clinical applications.

Gene relative expression analysis shown in Figure 1C confirmed the identity of neurospheres at day7 through the progressive loss of hPSCs pluripotency markers such as *NANOG* and *OCT4*, and a significantly increase in the expression of neural progenitor markers *SOX1*, *PAX6, NESTIN* and *SOX2* which defining early neural fate. Neurospheres at day7 of neural induction were plated for immunostaining with pluripotent NS/PCs markers and most of the cells were positive for NS/PCs markers SOX2 and NESTIN (Figure.1B e,j). Neurospheres generation and gene expression

was evaluated using two cell lines H9 and UiPS, and no significant difference was observed (Figure.1C&Figure.1D).

These results suggest that neurospheres derived from hESC/hiPSCs by neural induction medium possess the NS/PCs phenotype.

AA promoted the generation of lt-NES cells from neurospheres under chemically defined conditions.

Whereas neurospheres exhibit heterogeneous character and tent to reproduce the neural development process, monolayer adherent cultures provide a more robust and homogeneous cell generation [19].

We have tried many ways to get lt-NES cells (data not shown), and we found that ascorbic acid (AA) can promote the attachment of lt-NES cells. To evaluate the efficiency of using AA to induce lt-NES cells, we subsequently performed paralleled derivation of lt-NES cells from H9/UiPS-neurospheres using conventional N2 medium with gelatin-coated and N2+AA medium without gelatin-coated. The lt-NES cell derivation protocol from neurospheres is shown in Figure 2A. Neurospheres were dissociated into single cells and plated on gelatin-coated/uncoated plates in the N2 medium with FGF2 and EGF (Coated/Uncoated) or plated on uncoated plates in the N2+AA medium with FGF2 and EGF (AA). The morphologies of the lt-NES cells derived from H9/UiPS-neurospheres in N2 medium (coated) and N2+AA medium (AA) were comparable with each

other (Figure.2B a,c vs. d,f), and there were few lt-NES cells in uncoated plate (Figure.2B b,e). The viability of lt-NES cells in different culture condition was evaluated by a CCK-8 assay (Figure.2C)，and there were no differences in the cell viability of the lt-NES cells in coated and AA conditions after plated for 12h. Growth kinetics showed no significant difference in proliferation of the lt-NES cells in coated and AA conditions (Figure.2D), and we can see from this result that lt-NES cells expanded in N2+AA medium (AA) generate large numbers of cells, almost two-fold expansion can be achieved upon each passage, and lt-NES cells were passaged once a week on average.

All cells cultured in two methods in passage one expressed NS/PCs markers *SOX1*, *PAX6, NESTIN* and *SOX2* (Figure.3A&3B), and had low expression of hPSCs pluripotency markers *NANOG* and *OCT4*. What deserves our attention is that N2+AA (AA) cultured cells have higher expression levels of NS/PCs markers than N2 cultured (coated) cells, which indicates that the AA maybe can promote the neural pluripotency of lt-NES cells. The immunocytochemical analysis indicated that there were no significant differences in the percentage of NS/PCs marker-positive cells (Figure.3C, 3D&3E), however, there are slightly more SOX2-positive cells in N2+AA culture, demonstrating the optimal culture using N2+AA.

These data suggest that N2+AA without gelatin-coated is suitable for the induction and growth of lt-NES cells.

AA can maintain the long-term expansion and neural pluripotency of lt-NES cells

To verify whether the N2+AA culture system supports excellent proliferation and preserves the neural pluripotency of lt-NES cells, we cultured the cells of N2 cultured (coated) and N2+AA cultured (AA) to 15 generations and identified the capacity of proliferative and the potential of neural differentiation by qPCR and immunochemistry. Gene relative expression analysis by qPCR shown in Figure 4A&4B displayed that cells cultured in N2 (coated) or N2+AA (AA) in passage 5 and passage 10 expressed NSC markers *SOX1*, *PAX6, NESTIN* and *SOX2*, and had almost no expression of pluripotency markers *NANOG* and *OCT4*. And analyses of expanded lt-NES cells by immunofluorescence staining showed consistent expression of NSC markers, including SOX2 (> 75%) and NESTIN (>95%) at passage 5 and passage 10 (Figure.4C&D). Furthermore, N2+AA can maintain normal karyotype over 15 passages (Figure.4E).

The above results reveal that N2+AA without gelatin-coated can maintain the long-term expansion and excellent proliferation, and preserve the neural pluripotency of lt-NES cells.

lt-NES cells cultured in N2+AA can differentiate into functional neurons

The neurons generated from NSCs provide a useful model for studying human disease, drug screening, toxicity testing, and cell therapy. To test the neuronal differentiation potential of lt-NES cells cultured by N2+AA medium, we assessed whether the lt-NES cells could differentiate into generic neurons. As shown in Figure 5A a,c, after two weeks of neuronal differentiation, H9-lt-NES cells in N2 cultured (coated) and N2 AA (AA) culture systems can differentiate into a large number of neurons. And this phenomenon can be repeated by UiPS-lt-NES cells, they could also differentiated into substantial numbers of neurons (Figure.5B).As demonstrated by the immunocytochemical of the neuronal marker class III β-tubulin and the glia marker glial fibrillary acidic protein (GFAP) (Figure.5A b,d) respectively, the neurons produced by our system are highly pure (≥ 95% class III β-tubulin-positive neurons; < 5% GFAP-positive glia) and can be maintained in long-term culture. Quantitative RT-PCR analysis shows that neurons generated by neuronal differentiation of N2 cultured (coated) and N2+AA (AA) cultured lt-NES cells exhibit similar gene expression patterns (Figure.5C): after 14 days in neuron culture medium, the expression of neuronal type markers *NeuN*, *Neurog2* and *β-tubulin*, and glutamatergic neuron markers *vGlut1* was up-regulated, and the expression

of the astrocyte markers *S100B-β* and *GFAP* was low. Subsequently, we investigated the electrophysiological activity of neurons differentiated from H9-lt-NES cells using a microelectrode array (MEA) recording system. We plated lt-NES cell-derived neurons on the MEA plate, then we cultured them in the Neuron maintenance medium and recorded the spontaneous activity (Figure 5D). The spontaneous activity of the lt-NES cell-derived neurons gradually increased and maintained a high level for 50 days after differentiation (Figure 5E). This indicate that N2+AA cultured lt-NES cell-derived neurons are functional and able to maintain long-term spontaneous neuronal activity.

These data indicate that N2+AA cultured lt-NES cells have the capacity to differentiate into generic functional neurons.

Transcriptome analysis of lt-NES cells

In order to explore the changes in gene expression, functional consequences, and the potential molecular mechanism caused by ascorbic acid, we performed RNA-seq analysis on N2 cultured (coated) and N2+AA (AA) cultured lt-NES cells. We found that 3050 genes were significantly upregulated and 1967 downregulated after AA cultured (Figure 6A).

In order to understand the biological mechanism of AA effects, we performed GO (Gene ontology) enrichment analysis on differentially

expressed genes (DEG). The 10 most significant categories were identified in the cell composition, molecular function and biological process groups respectively (Figure. 6B). Most remarkable categories are concentrated in regulation of extracellular matrix (ECM) assembly, collagen metabolic process within the biological process, and extracellular matrix component within the cell composition (Figure. 6B). Among them, cell adhesion molecule binding was the most different categories (Figure. 6C). The above results indicated that AA plays a critical role in cell adhesion, ECM deposition/remodeling and collagen synthesis, which is consistent with previous researches [27,28]. To understand the biological impact of these differentially expressed transcripts, we next performed KEGG (Kyoto Encyclopedia of Genes and Genomes) functional analysis (Figure. 6D). Among 20 significantly different pathways, these pathways were mainly related to cell adhesion and ECM-receptor interaction.

Overall, these transcriptional profiling results indicate that AA is involved in the regulation of cell adhesion and is closely related to the regulation of ECM deposition/remodeling, especially in collagen synthesis.

AA promotes the generation of lt-NES cells through the MEK-ERK1/2 pathway by promoting collagen synthesis.

Transcriptome analysis indicates that ascorbic acid plays a role by affecting cell adhesion, ECM deposition/remodeling and collagen synthesis. However, it is known that ascorbic acid is also an antioxidant, so we need to determine whether the generation of lt-NES cells by AA is due to its antioxidant properties. We found that treatment with other antioxidants such as vitamin B1 (Vb1) and reduced glutathione (GMEE) could not mimic the effect of AA on lt-NES cell adhesion (Figure 7A), indicating that the effect of AA on promoting adherence of lt-NES cells is not related to its antioxidant properties. Collagens are indispensable components of ECM, which play a vital role in cell development and function, and have been shown to affect cell proliferation and differentiation [29]. In addition, It has been pointed out that AA enhanced the proliferation of CPCs via the MEK-ERK1/2 pathway through manipulating collagen synthesis [24]. In order to verify whether the mechanism of AA promoting the generation and expansion of lt-NES cells is in the same way as the above article, we analyzed the effect of AA on collagen synthesis and found that the expression of collagen genes *Col1a1* and *Col4a1* of lt-NES cells cultured by N2+AA (AA) increased significantly (Figure.7B), which is consistent with the transcriptome results. In order to further explore the role of AA in the synthesis of collagen, we used a generic collagen synthesis inhibitor L-2-Azetidine carboxylic acid (AzC), and we found that the adhesion of lt-NES cells by AA were completely eliminated by AzC (50 μmol/l)

(Figure.7C). And then, to confirm whether AA promotes collagen synthesis via the MEK-ERK1/2 pathway, we performed the validation of AA-induced ERK1/2 activation and the effect of ERK1/2 inhibitors. Western blot analysis shown in Figure 7D&7E indicated that the level of p-ERK1/2 was higher in N2+AA (AA) cultured lt-NES cells than N2 cultured (coated). Moreover, the ERK1/2 inhibitor (LY3214996) completely abolished AA-induced generation and expansion of N2+AA (AA) cultured lt-NES cells (Figure.7F), whereas the N2 cultured remained unchanged when addition of LY3214996, suggesting that the MEK-ERK1/2 pathway is involved in the AA-dependent generation and expansion of lt-NES cells.

The above data demonstrate that the AA-induced collagens synthesis promotes the generation and expansion of lt-NES cells through the MEK-ERK1/2 pathway.

Discussion

In this study, a new system was established to induce lt-NES cells from hESC/hiPSCs-neurospheres under chemically defined conditions and this system designed to meet the requirements of quality in clinical settings. This system also demonstrated that N2+AA culture system is suitable for the induction and long-term expansion of lt-NES cell (Figures 2,3,4), and the differentiation of generic neurons (Figure 5). AA added in N2 medium

for generation and expansion of lt-NES cells avoids pre-coating and avoids the use of basement membrane preparations, therefore, the chemical composition of this culture system is more specific and this culture system is subject to the standards of serum-free system for clinical application in the future. Moreover, this system is suitable for various pluripotent stem cells, including ESCs and iPSCs, therefore, we can use the patient-specific iPSCs to avoid ethical issues and immune rejection.

Neural stem/progenitor cells (NS/PCs) can maintain long-term expansion, constantly self-renew, and have the ability to differentiate into a variety of neural cell lineages, including neurons and astrocytes, which illustrating the potential of neural stem cells in cell replacement therapies of neurological disorders and injuries caused by the loss of neurons and glial cells, such as spinal cord injury [30,31]. Neurospheres, a classic culture system of expanding NS/PCs, it's cultivation as a free-floating aggregate and it is considered as a more natural environment for cells due to the 3D niche-like structure [15]. Methods of neurospheres induction and expansion are slightly changed from the previous protocols. Neurospheres are usually derived from single-cell suspensions of neural stem and progenitor cells isolated from adult or fetal central nervous system (CNS), but neurospheres can also be established from ES cells [32]. In our research, we used neural stem cell induction medium to induce hESC/hiPSCs into rosettes, and after

485 the rosette was digested, they were spread on uncoated plate in the form of
486 a single cell suspension, then the single cells aggregate to form a spherical
487 shape and expand continuously. (Figure 1). The medium was
488 supplemented with B27 containing fibroblast growth factor (bFGF) and
489 epidermal growth factor (EGF) [33]. This neurosphere induction method is
490 proposed for the first time in this study, and it has a higher yield and a
491 higher efficiency than previous methods.

492 However, heterogeneity is one of the significant limitations of
493 neurospheres culture system [34]. This heterogeneity is because the size of
494 the neurospheres is difficult to control and usually not uniform, and cells
495 inside and outside the neurospheres are exposed to different environments.
496 The heterogeneity makes it difficult to maintain long-term expansion and
497 excellent differentiation potential. Moreover, after transplantation of
498 neurospheres into the nervous system, the neurogenic potential is gradual
499 loss and the yield of neurons decreases [34]. Therefore, there is a demand
500 to develop a new NS/PCs culture system that should be homogeneous,
501 robust, stable over time, and can constantly produce a large number of
502 neurons. Culture of NSCs in monolayer has been studied previously to
503 solve above mentioned needs, and human long-term self-renewing
504 neuroepithelial-like stem cells (lt-NES cells) are NS/PCs in a monolayer
505 culture system. They are a fairly homogeneous population of

undifferentiated cells with the ability to continuously expand and stably produce functional neurons and glial [21]. The most common method to generate lt-NES cells is to pre-coat the culture plates with basement membrane preparations rich in extracellular matrix (ECM), like laminin [35], fibronectin, gelatin [36], and Matrigel [37]. However, basement membrane preparations listed above all contain exogenous components, and these exogenous components hinder the progress of cell transplantation due to uncertain chemical composition. In our research, we have established a new system for inducing and maintaining lt-NES cells under chemically defined conditions by using ascorbic acid (AA) (Figure.2A). The lt-NES cells cultured under this new system can stably expand in large numbers and preserve the neural pluripotency (Figure.2-3), and can maintain long-term culture (Figure.4). Moreover, lt-NES cells cultured under our system can maintain the differentiation potential of functional neurons (Figure.5). Therefore, it is expected that this system will contribute to regenerative medicine for spinal cord injury and other central nervous system (CNS) diseases in the future, because this method can reliably provide a large number of functional cells for regenerative medicine.

Ascorbic acid (AA) is widely known as an essential nutrient for guinea pigs and primates [38,39]. Ascorbic acid has two major biological activities, it serves as an antioxidant, and a cofactor of collagen synthesis [40]. In our

study, other antioxidants were tested, such as vitamin B1 (Vb1) and reduced glutathione (GMEE), but none of them mimicked the effect of AA on lt-NES cells (Figure.7A), suggesting that antioxidant activity is not a critical factor in the generation and expansion of lt-NES cells. Therefore, we focus on the role of ascorbic acid in the synthesis of collagen, as the extracellular matrix (ECM) may affect cell adhesion. Ascorbic acid (AA) is essential for the biosynthesis of collagen. It is a cofactor of prolyl and lysyl hydroxylase and a stimulator of collagen gene expression. After addition of AA to the culture medium, many studies have evaluated the relationship between AA and collagen expression on the short- and long-term effects of cells [27]. This study evaluated the use of AA in the long-term culture of lt-NES cells, and from the RNAseq results (Figure.6), it can be seen that AA affects cell acquisition and long-term culture by affecting cell adhesion, ECM remodeling and collagen synthesis. Moreover, during the long-term culture, the expression of collagen genes *Col1a1* and *Col4a1* was enhanced (Figure.7B), and we used one general collagen synthesis inhibitor, L-2-Azetidine carboxylic acid (AzC) and found that the adhesion of lt-NES cells by AA were completely eliminated by AzC (50 μmol/l) (Figure.8C). These data indicate that the AA plays a promotive role in the generation and expansion of lt-NES cells is due to collagen synthesis rather than antioxidant properties. It has been reported that AA specifically enhances CPC proliferation by manipulating collagen synthesis through the

MEK-ERK1 / 2 pathway [24], consistent with our results, the MEK-ERK1/2 pathway is activated by AA and the adhesion of lt-NES cells by AA were completely abolished by ERK inhibitor (1 μM) (Figure 7D-F). Moreover, we found that lt-NES cells cultured in N2+AA (AA) have higher expression levels of NS/PCs markers than cells cultured in N2 (coated) (Figure 2-3). The above studies indicate that AA may play a critical role in development of NS/PCs, and may provide a more suitable culture environment for lt-NES cells and promote the neural pluripotency of lt-NES cells.

Conclusion

We have successfully established a new culture system for the long-term large-scale monolayer culture of NS/PCs from hESC/hiPSCs. AA promotes a homogenous population of lt-NES cells grown in an environment without foreign components. After 10-15 passages, the cells on the N2+AA can maintain good self-renewal ability, and the cells can maintain neural pluripotency and the ability to differentiate to generic functional neurons. In addition, we have demonstrated that AA specifically promotes the generation and expansion of neuroepithelial-like stem cells through the MEK-ERK1 / 2 pathway by increasing collagen synthesis. Findings from this study help promote the clinical application of hPSC-derived NS/PCs in regenerative medicine for spinal cord injury and neurological diseases.

Abbreviations

SCI: Spinal cord injury; hPSCs: Human pluripotent stem cells; NS/PCs: neural stem/progenitor cells; AA: ascorbic acid; lt-NES cells: long-term self-renewing neuroepithelial-like stem cells; RT-PCR: reverse transcription-polymerase chain reaction; MEA: microelectrode array; mESCs: mouse embryonic stem cells; hESCs: human embryonic stem cells; iPSCs: induced pluripotent stem cells; UiPSs: Urine-iPSCs; GFAP: glial fibrillary acidic protein; ECM: extracellular matrix; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; Vb1: vitamin B1; GMEE: reduced glutathione; AzC: L-2-Azetidine carboxylic acid; CNS: central nervous system; bFGF: fibroblast growth factor; EGF: epidermal growth factor.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LF and LWJ conceived the idea and designed the experiments, BR and CY performed cell experiments, data analysis. BR and Amina performed manuscript preparation. WFJ, TL, and ZSY are responsible for cell-culture experiments and the collection and assembly of data. LYN, MSH,DT contributed to molecular experiments. GTW and YY contributed to function analysis. All authors read and approved the final manuscript.

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Not applicable

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References

1. Lindvall O, Kokaia Z. Stem cells for the treatment of neurological disorders. Nature. 2006;441:1094–6.

2. Potter K, Saifuddin A. MRI of chronic spinal cord injury. Br J Radiol. 2003;76:347–52.

3. Ogawa Y, Sawamoto K, Miyata T, Miyao S, Watanabe M, Nakamura M, et al. Transplantation of in vitro-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats. J Neurosci Res. 2002;69:925–33.

4. Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, et al. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc Natl Acad Sci U S A. 1995;92:11879–83.

5. Mcdonald JW, Liu XZ, Qu Y, Liu S, Mickey SK, Turetsky D, et al. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat Med. 1999;5:1410–2.

6. Iwanami A, Kaneko S, Nakamura M, Kanemura Y, Mori H, Kobayashi S, et al. Transplantation of human neural stem cells for spinal cord injury in primates. J Neurosci Res. 2005;80:182–90.

7. Cummings BJ, Uchida N, Tamaki SJ, Salazar DL, Hooshmand M, Summers R, et al. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. Proc Natl Acad Sci U S A. 2005;102:14069–74.

8. Iwanami A, Yamane J, Katoh H, Nakamura M, Momoshima S, Ishii H, et al. Establishment of graded spinal cord injury model in a nonhuman primate: The common marmoset. J Neurosci Res. 2005;80:172–81.

9. Nakamura M, Okano H. Cell transplantation therapies for spinal cord injury focusing on induced pluripotent stem cells. Cell Res [Internet]. Nature Publishing Group; 2013;23:70–80. Available from:

http://dx.doi.org/10.1038/cr.2012.171

10. Keirstead HS, Nistor G, Bernal G, Totoiu M, Cloutier F, Sharp K, et al. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. J Neurosci. 2005;25:4694–705.

11. Sharp J, Frame J, Siegenthaler M, Nistor G, Keirstead HS. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants improve recovery after cervical spinal cord injury. Stem Cells. 2010;28:152–63.

12. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell. 2007;131:861–72.

13. Takahashi K, Yamanaka S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell. 2006;126:663–76.

14. 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. 2012;7:1–13.

15. Yang S, Cao Z, Zhu J, Zhang Z, Zhao H, Zhao L, et al. In Vitro Monolayer Culture of Dispersed Neural Stem Cells on the E-Cadherin-Based Substrate with Long-Term Stemness Maintenance. ACS Omega. 2019;4:18136–46.

16. Okada Y, Matsumoto A, Shimazaki T, Enoki R, Koizumi A, Ishii S, et al. Spatiotemporal Recapitulation of Central Nervous System Development by Murine Embryonic Stem Cell-Derived Neural Stem/Progenitor Cells. Stem Cells. 2008;26:3086–98.

17. Zhang SC, Wernig M, Duncan ID, Brüstle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol. 2001;19:1129–33.

18. Koch P, Opitz T, Steinbeck JA, Ladewig J, Brüstle O. A rosette-type, self-renewing human ES cell-derived neural stem cell with potential for in vitro instruction and synaptic integration. Proc Natl Acad Sci U S A. 2009;106:3225–30.

19. Pollard SM, Conti L, Sun Y, Goffredo D, Smith A. Adherent neural stem (NS) cells from fetal and adult forebrain. Cereb Cortex. 2006;16.

20. Fujimoto Y, Abematsu M, Falk A, Tsujimura K, Sanosaka T, Juliandi B, et al. Treatment of a mouse model of spinal cord injury by transplantation of human induced pluripotent stem cell-derived long-term self-renewing

685 neuroepithelial-like stem cells. Stem Cells. 2012;30:1163–73.

686 21. Isoda M, Kohyama J, Iwanami A, Sanosaka T, Sugai K, Yamaguchi R,
687 et al. Robust production of human neural cells by establishing
688 neuroepithelial-like stem cells from peripheral blood mononuclear
689 cell-derived feeder-free iPSCs under xeno-free conditions. Neurosci Res
690 [Internet]. Elsevier Ireland Ltd and Japan Neuroscience Society;
691 2016;110:18–28. Available from:
692 http://dx.doi.org/10.1016/j.neures.2016.04.003

693 22. Gerrard L, Rodgers L, Cui W. Differentiation of Human Embryonic Stem
694 Cells to Neural Lineages in Adherent Culture by Blocking Bone
695 Morphogenetic Protein Signaling. Stem Cells. 2005;23:1234–41.

696 23. Li W, Sun W, Zhang Y, Wei W, Ambasudhan R, Xia P, et al. Rapid
697 induction and long-term self-renewal of primitive neural precursors from
698 human embryonic stem cells by small molecule inhibitors. Proc Natl Acad
699 Sci U S A. 2011;108:8299–304.

700 24. Cao N, Liu Z, Chen Z, Wang J, Chen T, Zhao X, et al. Ascorbic acid
701 enhances the cardiac differentiation of induced pluripotent stem cells
702 through promoting the proliferation of cardiac progenitor cells. Cell Res.
703 2012;22:219–36.

704 25. Okano H, Nakamura M, Yoshida K, Okada Y, Tsuji O, Nori S, et al.

705 Steps toward safe cell therapy using induced pluripotent stem cells. Circ
706 Res. 2013;112:523–33.

707 26. Zhou T, Benda C, Duzinger S, Huang Y, Li X, Li Y, et al. Generation of
708 induced pluripotent stem cells from urine. J Am Soc Nephrol.
709 2011;22:1221–8.

710 27. Kishimoto Y, Saito N, Kurita K, Shimokado K, Maruyama N, Ishigami A.
711 Ascorbic acid enhances the expression of type 1 and type 4 collagen and
712 SVCT2 in cultured human skin fibroblasts. Biochem Biophys Res Commun
713 [Internet]. Elsevier Inc.; 2013;430:579–84. Available from:
714 http://dx.doi.org/10.1016/j.bbrc.2012.11.110

715 28. Tagler D, Makanji Y, Tu T, Bernabé BP, Lee R, Zhu J, et al. Promoting
716 extracellular matrix remodeling via ascorbic acid enhances the survival of
717 primary ovarian follicles encapsulated in alginate hydrogels. Biotechnol
718 Bioeng. 2014;111:1417–29.

719 29. Bowers SL, Banerjee I, Baudino TA. The extracellular matrix: at the
720 center of it all. J Mol Cell Cardiol. 2010;48(3):474-482.
721 doi:10.1016/j.yjmcc.2009.08.024.

722 30. Temple S. The development of neural stem cells. Nature.
723 2001;414:112–7.

724 31. Huang L, Zhang L. Neural stem cell therapies and hypoxic-ischemic
725 brain injury. Prog Neurobiol [Internet]. Elsevier; 2019;173:1–17. Available
726 from: http://dx.doi.org/10.1016/j.pneurobio.2018.05.004

727 32. Tropepe V, Hitoshi S, Sirard C, Mak TW, Rossant J, Van Der Kooy D.
728 Direct neural fate specification from embryonic stem cells: A primitive
729 mammalian neural stem cell stage acquired through a default mechanism.
730 Neuron. 2001;30:65–78.

731 33. Tropepe V, Sibilia M, Ciruna BG, Rossant J, Wagner EF, Van Der Kooy
732 D. Distinct neural stem cells proliferate in response to EGF and FGF in the
733 developing mouse telencephalon. Dev Biol. 1999;208:166–88.

734 34. Jensen JB, Parmar M. Strengths and limitations of the neurosphere
735 culture system - Jensen and Parmar Mol Neurobiol 2006 (1).pdf.
736 2006;34:153–4. Available from: file:///C:/Users/marrac/Downloads/Jensen
737 and Parmar Mol Neurobiol 2006 (1).pdf

738 35. Ray J, Gage FH. Differential properties of adult rat and mouse
739 brain-derived neural stem/progenitor cells. Mol Cell Neurosci.
740 2006;31:560–73.

741 36. Gong L, Cao L, Shen Z, Shao L, Gao S, Zhang C, et al. Materials for
742 Neural Differentiation, Trans-Differentiation, and Modeling of Neurological
743 Disease. Adv Mater. 2018;30:1–24.

744 37. D'Aiuto L, Zhi Y, Kumar Das D, Wilcox MR, Johnson JW, Mc Clain L, et
745 al. Large-scale generation of human ipsc-derived neural stem cells/early
746 neural progenitor cells and their neuronal differentiation. Organogenesis.
747 2014;10:365–77.

748 38. Ashino H, Shimamura M, Nakajima H, Dombou M, Kawanaka S,
749 Oikawa T, et al. Novel function of ascorbic acid as an angiostatic factor.
750 Angiogenesis. 2003;6:259–69.

751 39. Sato H, Takahashi M, Ise H, Yamada A, Hirose SI, Tagawa YI, et al.
752 Collagen synthesis is required for ascorbic acid-enhanced differentiation of
753 mouse embryonic stem cells into cardiomyocytes. Biochem Biophys Res
754 Commun. 2006;342:107–12.

755 40. Arrigoni O, De Tullio MC. Ascorbic acid: Much more than just an
756 antioxidant. Biochim Biophys Acta - Gen Subj. 2002;1569:1–9.

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759 **FIGURE LEGENDS**

760 **Figure 1. Generation of neurosphere from hiPS/ESCs**

761 (A)The protocol for deriving neurospheres from H9 and UiPS.(B) The
762 morphology and Immunostaining of H9 and UiPS at days -1 (a,b,f,g), day 5

(c,h), and day 7 (d,e,i,j) during neural induction. Immunostaining showing emergence of SSEA4+ (green), OCT4+(red) H9 and UiPS(b,g), and SOX2+ (red),NESTIN+ (green) neurospheres induced from H9 and UiPS at day 7(e,j). Scale bars = 100 μm. Nuclei were counterstained with DAPI (blue). (C) Quantitative RT-PCR analysis of the expression level of neural progenitor markers *SOX1, PAX6, NESTIN* and *SOX2*， and pluripotency markers *NANOG* and *OCT4* of H9 and H9-neurospheres. (D) Quantitative RT-PCR analysis of the expression level of neural progenitor markers *SOX1, PAX6, NESTIN* and *SOX2*， and pluripotency markers *NANOG* and *OCT4* of UiPS and UiPS-neurospheres. n = 3 each. Data are expressed as means ± SD.*P < 0.05, **P < 0.01 vs. control.

Figure 2. lt-NES cells can be generated from H9/UiPS-neurospheres under a chemically defined culture system using N2+AA medium.

(A)The lt-NES cells derivation protocol from neurospheres. (B) The morphologies of the lt-NES cells in N2 medium (coated/uncoated) and N2+AA (AA). Scale bars = 100 μm. (C) The viability of P0 lt-NES cells was assessed by a CCK-8 assay after plated for 12 h. (D) Expansion capacity of lt-NES cells investigated by continuous culture in N2 medium (coated/uncoated) and N2+AA (AA) in 8 days. n = 3 each.

Figure 3. N2+AA medium can maintain the neural pluripotency of lt-NES cells

(A) Quantitative RT-PCR analysis of the expression level of NSC markers *SOX1, PAX6, NESTIN* and *SOX2*, and pluripotency markers *NANOG* and *OCT4* of P0 H9 neurospheres-derived lt-NES cells. (B) Quantitative RT-PCR analysis of the expression level of NSC markers *SOX1, PAX6, NESTIN* and *SOX2*, and pluripotency markers *NANOG* and *OCT4* of P0 UiPS neurospheres-derived lt-NES cells. n = 3 each. Data are expressed as means ± SD.*P < 0.05, **P < 0.01 vs. control. (C) Immunofluorescence for NSC markers SOX2 (red) and NESTIN (green) of P0 H9 & UiPS neurospheres-derived lt-NES cells. Scale bars = 100 μm. Nuclei were counterstained with DAPI (blue). (D) Quantification of SOX2, NESTIN positive cells of Immunofluorescence of P0 H9 neurospheres-derived lt-NES cells. P, passage.(E) Quantification of SOX2, NESTIN positive cells of Immunofluorescence of P0 UiPS neurospheres-derived lt-NES cells.

Figure 4. lt-NES cells can maintain long-term expansion and neural pluripotency using N2+AA medium.

(A) Quantitative RT-PCR analysis of the expression level of NSC markers *SOX1, PAX6, NESTIN* and *SOX2*, and pluripotency markers *NANOG* and *OCT4* of P5 H9 neurospheres-derived lt-NES cells. (B) Quantitative RT-PCR analysis of the expression level of NSC markers *SOX1, PAX6,*

NESTIN and *SOX2*, and pluripotency markers *NANOG* and *OCT4* of P10 H9 neurospheres-derived lt-NES cells. n = 3 each. Data are expressed as means ± SD.*P < 0.05, **P < 0.01 vs. control. (C) Immunofluorescence for NSC markers SOX2 (red) and NESTIN (green) of P5&P10 H9 neurospheres-derived lt-NES cells. Scale bars = 100 μm. Nuclei were counterstained with DAPI (blue). (D) Quantification of SOX2+, NESTIN+ cells of Immunofluorescence. P, passage. (E) Representative karyotypes of H9 neurospheres-derived lt-NES cells at passage 15.

Figure 5. lt-NES cells differentiated into functional neurons.

(A) Neurons and glia differentiated from P10 H9 neurospheres-derived lt-NES cells (a, c). Immunofluorescence for neuronal marker class III β-tubulin (green) and glia marker GFAP (red) of Neurons and glia (b, d). (B) Neurons and glia differentiated of P10 UiPS neurospheres-derived lt-NES cells (a, c). Immunofluorescence for neurons marker class III β-tubulin (green) and glia marker GFAP (red) of Neurons and glia (b, d). Scale bars = 100 μm. Nuclei were counterstained with DAPI (blue).

(C) Quantitative RT-PCR analysis of the expression level of neurons markers NeuN, β-tubulin,vGlut1, and Neurog, and glia markers S100B-β and GFAP . Transcript levels were normalized to undifferentiated lt-NES cells ,n = 3 each. (D) Representative images of activity map of neurons differentiated from H9 neurospheres-derived lt-NES cells on the

24-electrode array. (E) Spikes of neurons differentiated from H9 neurospheres-derived lt-NES cells in 30s.

Figure 6. Transcriptome analysis of lt-NES cells cultured by coated or AA .

(A) Volcano plot of upregulated (red) or downregulated (green) transcripts in AA cultured lt-NES cells vs coated cultured. (B) Significantly enriched Gene Ontology (GO) terms in AA cultured lt-NES cells.

(C) GO terms categorization and distribution of differentially expressed genes. GO terms were processed and categorized under three main categories (biological process, cellular component, and molecular function).

(D) KEGG (Kyoto Encyclopedia Genes and Genomes) pathway rich detail.

Figure 7. AA activates ERK signaling in a collagen synthesis-dependent manner.

(A) The morphology of lt-NES cells treated with AA or alternative antioxidants vitamin B1(Vb1) and reduced glutathione(GMEE). (B) Quantitative RT-PCR analysis of the relative expression of collagen genes *Col1a1* and *Col4a1*. n = 3 each. Data are expressed as means ± SD.*P < 0.05, **P < 0.01 vs. control. (C) The morphology of lt-NES cells treated with AA and collagen synthesis inhibitor AzC. (D) The whole-cell extracts of N2 cultured (coated) and N2+AA cultured (AA) lt-NES cells were analyzed

by Western blot with phospho-specific antibodies against ERK1/2 (pERK1/2), and total ERK1/2. GAPDH was used as a control. (E) ERK activation is defined as the ratio of p-ERK/total ERK. n = 3 each. Data are expressed as means ± SD.*P < 0.05, **P < 0.01 vs. control. (F) The morphology of lt-NES cells when treated with or without ERK inhibitor. Scale bars = 100 μm.

Supplementary

Table S1. Primer sequences used for q-PCR

Gene	Forward 5'-3'	Reverse 3'-5'
PAX6	CTGAGGAATCAGAGAAGACAGGC	ATGGAGCCAGATGTGAAGGAGG
NESTIN	TCAAGATGTCCCTCAGCCTGGA	AAGCTGAGGGAAGTCTTGGAGC
SOX1	GAGTGGAAGGTCATGTCCGAGG	CCTTCTTGAGCAGCGTCTTGGT
SOX2	GCTACAGCATGATGCAGGACCA	TCTGCGAGCTGGTCATGGAGTT
OCT4	CCTGAAGCAGAAGAGGATCACC	AAAGCGGCAGATGGTCGTTTGG
NANOG	CTCCAACATCCTGAACCTCAGC	CGTCACACCATTGCTATTCTTCG
NeuN	TACGCAGCCTACAGATACGCTC	TGGTTCCAATGCTGTAGGTCGC

TUBB3	TCAGCGTCTACTACAACGAGGC	GCCTGAAGAGATGTCCAAAGGC
NEUROG	CAAGCTCACCAAGATCGAGACC	AGCAACACTGCCTCGGAGAAGA
vGlut1	GCAAGTACATCGAGGACGCCAT	GCCACGATGATGGCATAGACTG
GFAP	CTGGAGAGGAAGATTGAGTCGC	ACGTCAAGCTCCACATGGACCT
S100B	ACGTCAAGCTCCACATGGACCT	TCCTGGAAGTCACATTCGCCGT
Col1a1	GATTCCCTGGACCTAAAGGTGC	AGCCTCTCCATCTTTGCCAGCA
Col4a1	TGTTGACGGCTTACCTGGAGAC	GGTAGACCAACTCCAGGCTCTC
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

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