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Gelatin Endorses the Pluripotent Stem Cells from the Reprogrammed Fibroblasts of Adult Mice Differentiation into Subtypes of Neurons in the Feeder-Free Condition for a Long-Term

Gele Liu*

Department of Neuroscience, Carleton University, Canada

Abstract

Stem cell research and application in the basic and clinical fields have been leading a prevalent in the past 20 years since these present significantly promising meanings. The relevant technologies have momentously advanced, including the cellular matrix to offer stem cells growing healthily environment. Stem cells can originate from several resources such as embryonic and induced pluripotent stem cells (iPSCs) but they all are dependent on a monolayer of feeder-cells, which are, in the original and most time, on the primary mouse embryonic fibroblasts (MEF feeder cells) to sustain their self-renewal growing. However, even though MEF application is still widely applied in the research laboratory and commercial supplies, it meets inordinate challenging since it appears some noteworthy problems such as zoonosis, contaminations, and inconsistence outcomes. Importantly, the hindmost of those halts the downstream applications of stem cells, in particular, in the clinical application. Thus, scientists have hunted around the replacements either biomaterials or non-biomaterials and their mixtures. Recently, we magnificently employed the gelatin-alone-coated dishes in feeder-free condition with the opposite medium for the proliferation of iPSCs derived from the reprogrammed fibroblasts of the adult mouse to overcome these drawbacks of MEF. Moreover, such dishes sponsor the differentiation of iPSCs into succeeding neural progenitor cells, neurons, and as the ultimate product subtype of neurons - dopaminergic neurons up to 6 months. This system is simple, modest to accomplish with lower expenses

Keywords: Gelatin; Stem cells; Neurons; Feeder-free; Long-term

Introduction

Stem cell lines have been widely applied for the human developmental biology [1], drug discovery [2], and transplantation medicine [3,4] since the first report by Thomson in 1998 [5] with the derivation of human embryonic stem cells (hESCs) lines.

The embryonic and induced pluripotent stem cells (iPSCs) from various resources such as mouse or human should, in general, bank on a monolayer of feeder-cells such as the primary mouse embryonic fibroblasts (MEF feeder cells) to hold out the self-renewal of hPSCs. The primary derivation of hESCs lines was used by mitotically inactivated MEF feeder layer to grow hESC successively in an undifferentiated phase [5]. This protocol was thought the gold standard technique for hESCs culture. MEF cells achieve these important functions in stem cell culture: they secrete several vital growth factors such as fibroblast growth factors (FGFs), cytokines, and extracellular matrices (ECM) such as TGFβ, activin A, laminin-511 and vitronectin into the medium [6-8], which support pluripotency and offer a cellular matrix for stem cells to grow. Fundamentally, feeder cells comprise in a layer of cells being incompetent to divide, which delivers extracellular secretions to promote the growth of target cells, which is dissimilar to a co-culture system because only one targeted-cell is proficient to proliferate. To avoid feeder cells to proliferate, they need to be treated by either one: the classical treatments as mitomycin or γ-irradiation, or the common conduct as electric pulses or chemical fixation [9].

However, several issues restrict MEF feeder cells to apply in the basic and clinical fields. For example, the result from the combined stem cells and feeder cells retort to various inducements and take a risk of zoonosis and contamination such as viruses or unknown proteins [9,10]. There are also existing the discrepancies in expression and secretion of these factors by different feeder- cells [6,11] make it problematic to define which components are crucial for the provision of hPSCs in an undifferentiated state. Moreover, the treatments such as γ-irradiation for feeder-cells can induce apoptosis and afterward modifies the secretions, which may harmfully disturb the self-renewal hPSCs [7,8].

Based on these matters above, recently, we established an effective protocol for the proliferation of iPSCs derived from the reprogrammed fibroblasts of the adult mouse by using the gelatin-alone-coated dishes with the appropriate Medium (see details in the MATERIALS AND METHODS). This model avoids the drawbacks of iPSCs culturing on feeder cell layers. Furthermore, the gelatin-coated dishes promote the differentiation of iPSCs into subsequent neural progenitor cells, neurons, and as the ending product subtype of neurons - dopaminergic neurons up to 6 months, which was relatively long-term.

Materials and Methods

Coating plates, wells or slides

As the regular coating for the primary cells cultures, a matrix of 100 x 60 mm plates, 6-well plates or 4-chambered slides were respectively equipped with Poly-D-Lysine (1 μg/cm^2 of surface area, Millipore A-003-E) and laminin (1 μg/cm^2 of surface area, Invitrogen 23017-015).

***Corresponding author:** Gele Liu, Department of Neuroscience, 327 Life Sciences Research Building, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada, Tel: +16135202600; E-mail: geleliu@gmail.com

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Specifically, Gelatin, as the sole attachment factor solution (Invitrogen S-006-100) at 0.1% concentration, was applied to coat all plates or wells in freshly daily use, in particular from iPSC stage to the subsequent multiple developing stages of neural cells, including the differentials to the neural progenitor cells, neural cells, mature neurons, and finally specific subtype of neurons - dopaminergic neurons. These procedures were kept a long-term period of 6 months.

Culturing the primary cells

The fibroblast cells from two-month-old adult CD1 mice were collected by micro-segmentation of tail-tips. The primary hippocampal cells of CD1 mouse were prepared from 2-month old adult animals. Tissues were composed with DMEM/F12 (Invitrogen 10565-018) and then were plated with 4 mL of DMEM/F12 for fibroblasts or of the Hibernate®-E Medium without Ca2+ (BrainBits LLC, HE-Ca) for brain tissues. Following tissues chipped, small pieces were enzymatically consumed by papain (filter-sterilized 2 mg/mL, Worthington, Cat. no. LS003119) for 45 minutes at 37°C. After tissues being centrifugated, the cell-containing supernatant was re-suspended in 5 mL of Knockout DMEM (Invitrogen 10829-018) for fibroblast cells or of complete Hibernate®-E medium for brain cells. After centrifuged for 4 minutes at 200×g, supernatants were removed and then cell pellets were re-suspended in 41.5 mL DMEM/F-12 (Invitrogen10565-018) for fibroblast cells or 41.5 mL Neurobasal® medium (Invitrogen 21103-049) for brain cells. A 3-day used stock 50 mL of Neurobasal® medium or DMEM/F-12 was fabricated by mingling 50 µl of 10 µg/mL (final 10 ng/mL) of b-fibroblast growth factors (bFGF, Invtrogen 13256-029), 7.5 mL of Knockout™ serum replacement (Invitrogen 10828-028), 0.5 mL of MEM Non-Essential Amino Acids Solution (NEAA) (Invitrogen11140-050), 0.5 mL of L-glutamine (Invitrogen 25030-081), and 91 µl (final 0.1 mM) of β- mercaptoethanol (Invitrogen 21985-023). All cells were incubated at 37°C with 5 % CO2 humidified atmosphere and the fresh media were nourished every third day.

Reprogramming factors inducing the formation of Induced Pluripotent Stem Cells (iPSCs)

The non-viral vector with four reprogramming genes (c-Myc, Klf4, Oct4, and Sox2), labelled pCAG2LMKOSimO (Addgene, 20866), was exploited for reprogramming somatic fibroblasts into iPSCs [12]. This vector has the benefit creating virus-free, factor-removable iPSCs. These reprogramming genes are transcribing from the universally expressed synthetic CAG enhancer/promoter. The mOrange frame of the vector as a biomarker was in the same reading frame corresponding with these reprogramming transcription factors.

Cells with 85-90% confluence were transfected with the vector 20866 containing four reprogramming genes by using lipofectamine® 2000 (Invitrogen 11668-027). With the fresh proper media, cells were uninterruptedly cultured to sustain reprogramming hooked to pluripotency. The colonies of stem-cell-like with expressing the mOrange-positive marker were distinguished at Days 5-6 after transfection. The iPSCs were confirmed by means of the gene expressions of stem cell stage-specific antigens - Oct-4, SSEA-1, SSEA-4, and GF141 (Millipore SCR002 and Millipore GF141). The iPSCs with the mOrange-positive marker were additionally recognized during Days 17-20. Then, these were relocated to the Neural Induction Medium in freshly coated-plates or wells comprising a 0.1% gelatin alone.

The transfection day was designated as Day 0. Through Days 1-14, the medium was newly altered with complete N2B27 Medium, containing with DMEM/F-12 (Invitrogen 11330-057) plus N-2 Supplement (Invitrogen 17502-048), B-27® Supplement (Invitrogen 17504-044), Knockout™ serum replacement, L-glutamine, and NEAA, β-Mercaptoethanol. To promote the competence of the reprogramming, the medium was enhanced with a CHALP molecule cocktail

The CHALP cocktail is comprised with CHIR99021 (GSK3β inhibitor, 3 µM, Stemgent 04-0004), PD0325901 (MEK inhibitor, 0.5 µM, Stemgent 04-0006), hLIF (human leukemia inhibitory factor, 10 ng/mL, Millipore LIF1005), A-83-01 (TGF-β/Activin/Nodal receptor inhibitor, 0.5 µM, Stemgent, 04-0014), bFGF (100 ng/mL), and HA-100 (ROCK inhibitor, 10 µM, Santa Cruz, sc-203072). The medium was provided with the Essential 8™ Medium (Prototype), confining DMEM/F-12 (HAM) 1:1, Essential 8™ Supplement (50X) (Invtrogen A14666SA), N-2 Supplement, B-27® Supplement, Knockout™ serum, L-glutamine, NEAA, and β-Mercaptoethanol from Day 15 to 20. All media were rehabilitated every three days.

Differentiation of neural cells and succeeding dopaminergic neurons

Neural progenitor cells were differentiated from the undifferentiated iPSCs by using a Neural Induction Medium from Days 21 to 28. This definite medium was produced with 50 mL Neurobasal® medium plus bFGF, heparin solution (Sigma H3149, 50 µl of 2-mg/mL), N-2 supplement, glutaMAX™-I supplement, non-essential amino acids solution, Knockout™ serum replacement and β-Mercaptoethanol. During Days 29-35, a Dopaminergic Neuronal Progenitor Medium was applied to lead the neural progenitor cells for further differentiation into dopaminergic neural progenitors. This medium consisted of FGF-8b (100 ng/mL, Invitrogen PHG0271) and sonic hedgehog (200 ng/mL, SHH, R&D systems 1314-SH-025), being liquefied into the Neurobasal® medium with other nutrition contents - heparin solution, N-2 supplement without vitamin A (Invitrogen 12587-010), and NEAA. Lastly, the dopaminergic neural progenitor cells were supplementary differentiated into the matured dopaminergic neurons under using a Dopaminergic Neuronal Differentiation Medium from Days 36 to 50. This medium contained the recombinant human BDNF (50 µl of 25-µg/mL, Invitrogen PHC7074), recombinant human GDNF (50 µl of 20-µg/mL, Invitrogen PHC7045), ascorbic acid (50 µl of 200 mM, Sigma A4403), dcAMP (Dibutyryl cyclic-AMP) (50 µl of 1-mM, Sigma D0627) in 50 ml of the Neurobasal® medium accompanying with heparin, N-2 supplement without vitamin A (Invitrogen 12587-010) and NEAA. All media were freshened every three days.

Immunocytochemistry

To spot the introduction of reprogrammed stem cells, the biomarker mOrange (excitation and emission maxima are 549 and 565 nm by using the appropriate fluorescence filter), flagged to the 20866 vector that comprises the four re-programming genes.

Indicators of stem cell were distinguished by stem cell stage-specific antigens - Oct-4, SSEA-1, SSEA-4, and GF141. Undeveloped neuronal cells were perceived using anti-doublecortin (DCX) antisera (Invitrogen 48-1200), whereas mature neuronal cells were recognized by anti-MAP2 antibodies (Abcam ab32454), and to end, dopaminergic neuronal cells were evaluated using anti-tyrosine hydroxylase (TH) antisera (ImmunoStar 22941). All tests were performed according to the manufacturers' standardization protocols.

Cells biomarkers were harmoniously imagined with a Zeiss Axio Imager M2 microscope with Hamamatsu Orca R^2 camera. Images were taken by applying Stereo Investigator and were adjusted for brightness/

contrast using ImageJ (National Institute of Health). Pictures were captured at 40X magnification where positive immunoreactivity.

Results

Gelatin advocates reprogramming fibroblast to form iPSCs in the feeder-free circumstances

The fibroblast cells of adult mice (Figure 1A) were composed from tail-tips for cell culturing. Cells were transfected with vector 20866 comprising four reprogramming genes and transformed into iPSCs from Days 17 to 20. The positive colonies with biomarker mOrange (Figure 1B) of vector 20866 represented a stem-cell-like morphology at Days 5–6. The iPSCs were further confirmed by the expressions of stem cell stage-specific antigens: Oct-4 (Figure 1C), SSEA-1 (Figure 1D), SSEA-4 (Figure 1E), and GF141 (Figure 1F). Importantly, all colonies were thriven on gelatin-coated plates (gelatin is an attachment factor) with the feeder-free, suggesting successful reprogramming, and then selected during Days 17-20.

Gelatin promotes the differential of iPSCs into subsequent and multiple staged neural cells

Specifically, continuous using Gelatin, as an attachment factor, encourages the transformation of iPSCs into the distinctive various neural cells in the succeeding multiple footsteps. The iPSCs were initially transformed into neural progenitor cells with the network formation from Days 20-35 (Figure 2A-2I) and afterward (Days 36-50) the latter was guided to discriminate into mature neurons (Figure 2D), which were verified by Microtubule-associated protein 2 (MAP2) (Figure 2E). Then, the neurons were differentiated into dopaminergic phenotype (Figure 2G), being articulated the dopaminergic rate-limiting enzyme, Tyrosine Hydroxylase (TH) (Figure 2H). The dopaminergic-like neurons have been continually monitored up to 6 months (183 days) under supporting of gelatin as the matric. These subsequent developing stages were scrutinized by the control groups of non-programmed tails in the parallel comparing in the same culturing conditions (Figure 2 C, 2F, and 2I).

Gelatin sponsors the formation of neurons from embryonic brain cells as a control

As the control experiments, to prove gelatin being capable as an appropriate matric coating material supporting the establishment of neurons from embryonic brain cells under the feeder-free surroundings, the hippocampal cells of the 2-month-old adult mouse were furnished for the primary culture. These neural cells were constantly tracked and monitored for the development. The cell living images of Days 25, 36, 40 and 183 were respectively documented (Figure 3A-3D). The results suggested that gelatin played the critical function to maintain the neuro cells up to 6 months at least.

Discussion

The first application of feeder cells in cell culture was described by Puck and Marcus in 1955. Despite the fact that some cell types are entirely hooked on the physical interaction with a feeder sheet for survival and expansion, several other feeder-dependent cells can be grown up on the feeder-free on condition. The latter is that cells are growing on the dishes coated with extracellular matrix proteins such as laminin, collagen, fibronectin, or Matrigel (extracellular matrix components) [9]. Recently, a simple and efficient protocol for the propagation of hPSCs using on a gelatin-coated dish in placenta-conditioned media has been developed [10]. However, we are still unclear whether or not iPSCs,

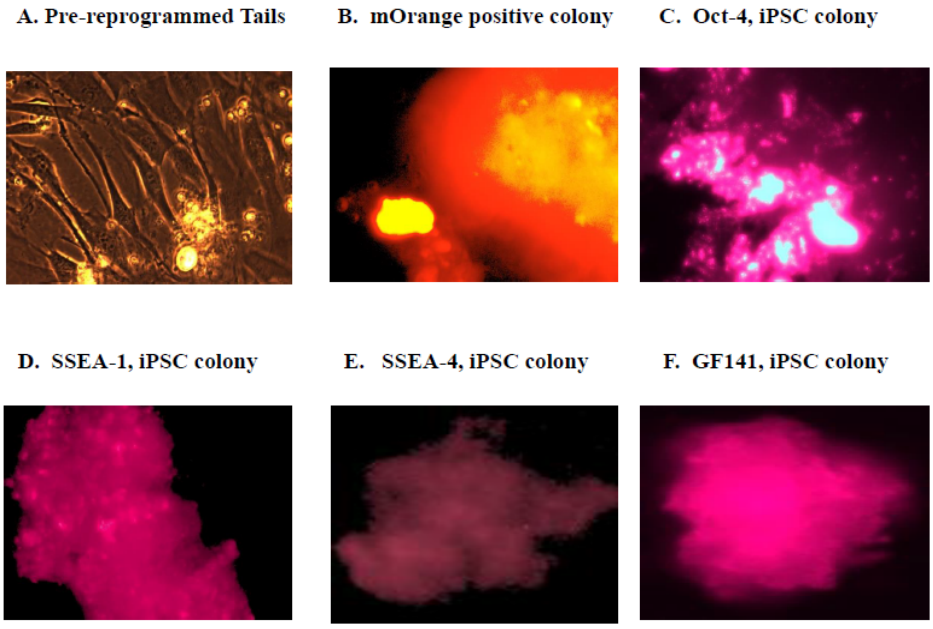

Figure 1: Gelatin-alone-coating plates support iPSCs derived from reprogrammed fibroblasts of the adult mouse in feeder-free surroundings. Gelatin, as the sole attachment factor (Invitrogen S-006-100) at 0.1% concentration, was practical to coat all plates on the base of the freshly daily use to replace the use of MEF seeds for the formation of iPSCs. The tail-tip fibroblast cells of adult CD1 mouse (A) were transfected using lipofectamine 2000 reagent with vector 20866 integrating the same proofreading frame with the four reprogramming transcription factors Oct4/Sox2/Klf4/c-Myc under culturing 17-20 days. Evidently, cells were altered into iPSCs as the biomarker expression of mOrange for vector 20866 (B) and the expressions of stem cell stagespecific antigens: Oct-4 (C), SSEA-1 (D), SSEA-4 (E), and GF141 (F).

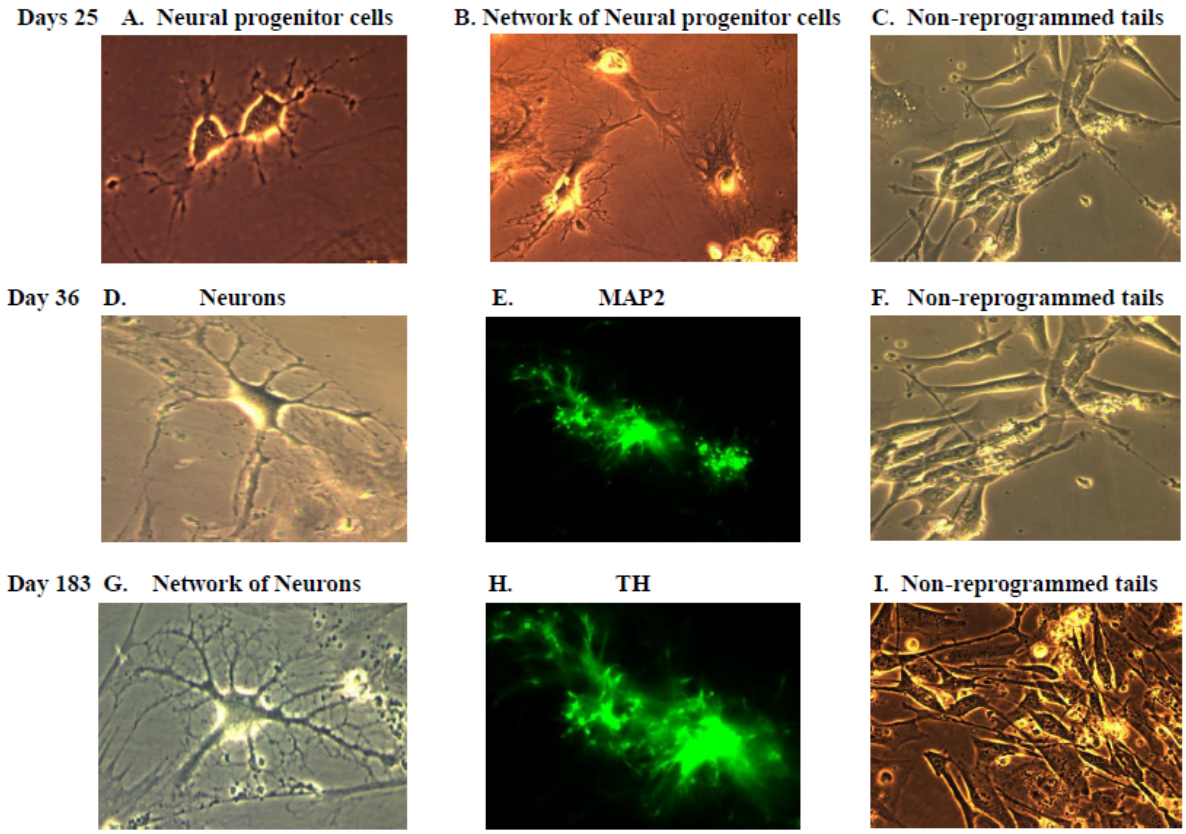

Figure 2: Gelatin endorses the iPSCs to differentiate into consequent several phased neural cells. As only one attachment factor, gelatin (0.1% concentration) was applied to coat all plates to withstand successive neural cells and neurons. The iPSCs were primarily transformed into the neural progenitor cells and their network formation (A and B) and subsequently led to mature neurons (D), which were validated by MAP2 (E). Finally, these neurons were discerned into subtype dopaminergic neurons (G), evidenced by the dopaminergic rate-limiting enzyme, Tyrosine Hydroxylase (TH) (H), indicating definitely these were mature neurons with the potential to produce dopamine. Prominently, the dopaminergic neurons were constantly living healthily up to 6 months (183 days) with gelatin as single supporting matric. All these were parallelly examined by non-programmed tails as the internal control groups, suggesting that these cells were maintained the same cell morphology (C, F, and I).

derived from the reprogrammed fibroblasts of the adult mouse, on an alternative option gelatin-alone-coated dish with other conventional resource media such as DMEM/F-12 (see details in the MATERIALS AND METHODS), are growing healthily; and whether or not such gelatin-coated dishes are able to encourage the differentiation of iPSCs into subsequent subtypes of neurons in the relatively long-term.

With the prodigious interests in the past 20 years, scientists have been looking for the substitute options for the MEF feeder layer in order to avoid its drawbacks, as described in the Introduction section. However, there are still some issues to be addressed at present. For example, the exposures of hESC to xenopathogens are immense risks since the human feeder layers such as fetal muscle, fetal skin, foreskin fibroblasts, and adult fallopian tubal epithelial feeder cells, amniotic mesenchymal cells, have been applied [13-17]. Although these some studies to avoid the ethical problems, an additional blockade still exists with immunological incompatibility causing tissue rejection and requiring the use of immune-suppressing agents [18]. Fortunately, the advanced technology eliminates the ethical concerns and immunological issues by using iPSCs from autologous patient-specific stem cells, which is reprogrammed and induced by transcription factors, modified mRNA, chemical agents, direct protein introduction, or gene transfer [19-21]. However, unfortunately, now many laboratory and commercial biotechnological companies are still maintaining hiPSCs on animal-derived feeder cells such as MEF since it has the significant security of the nutrition to supply hiPSCs growing with glowing. Thus, the replacement of allogeneic or xenogeneic feeder layers is still challenging for the downstream clinical application [22]. The extracellular matrix (ECM) proteins, such as laminin [23], vitronectin [24], collagen [25,26], fibronectin [10,27], or their mixed products such as Matrigel [9,28], with the synthetic polymer surfaces [29-33] support undifferentiated hPSCs proliferation in the feeder-dependent or feeder-free cultures. As one of the most commonly used substrates, Matrigel plays an important starting point to outline the prerequisites for hPSCs growth and differentiation. However, Matrigel derived from Engelbreth-Holm-Swarm mouse sarcomas [34] may display lot-to-lot inconsistency and may introduce xenogeneic impurities. Thus, Matrigel is not a supreme substrate for the feeder-free culture of hPSCs for ultimate human therapy. The recombinant human (rh) laminin-511 [35], rh vitronectin [36], and rh E-cadherin [37] were considered as the noteworthy landmarks when at the first time they were applied in the culture of hPSCs as the xenogeneic-free substrates. In addition, the recombinant protein fragments recombinant fibronectin fragment (FN III) and FBNI fragment (PF8) supported the human embryonic stem cell chondrogenesis [38]. Furthermore, the mixtures of fibronectin,

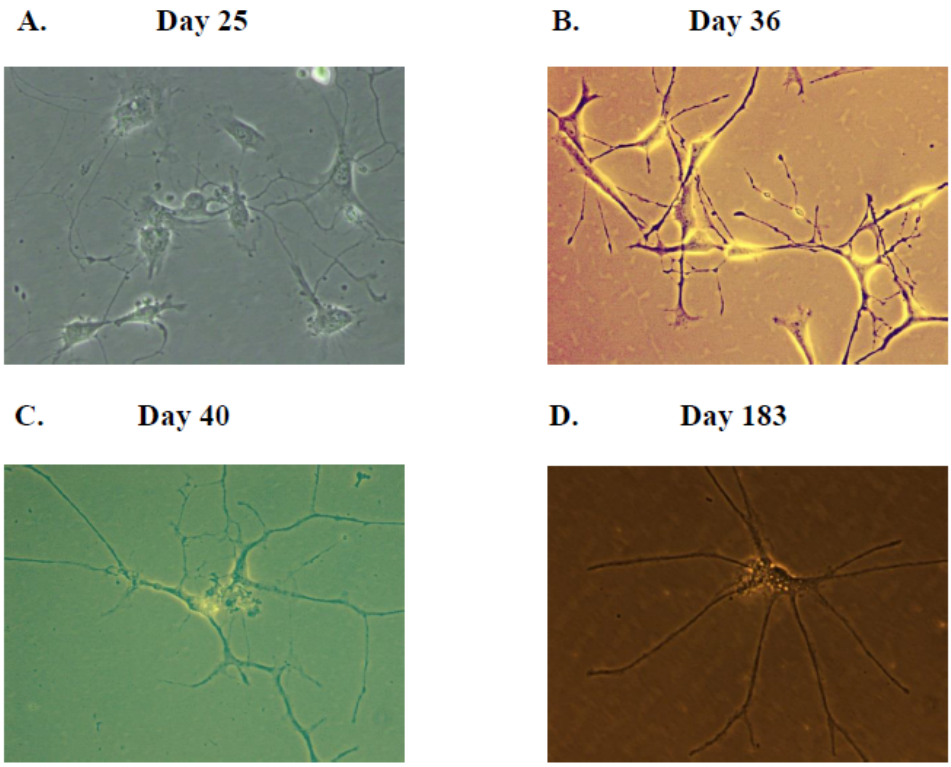

Figure 3: Gelatin is a guarantor for the development of neurons from embryonic brain cells as the external control. Gelatin, as the single attachment factor to coat the plates at 0.1% concentration, supports culturing embryonic brain cells during a 6-month period. As the external control, hippocampal cells of the 2-month-old adult mouse were sustained by gelatin-alone-coating plates. The images of cells on Days 25, 36, 40 and 183 were correspondingly shot (A-D).

laminin, and gelatin have been added into the suspension settings of the culture medium [39].

Based on these subjects above, we wonder whether or not gelatin, as the sole biomaterial to coat dishes or wells, is proficient to use in the feed-free culturing milieu for iPSCs derived from the reprogrammed fibroblasts of the adult mouse. Gelatin is a mixture of peptides and proteins formed by hydrolysis of collagen extracted from the skin, bones, and connective tissues. The promotive effect of gelatin-based hydrogels on chondrogenic differentiation of mesenchymal stem cells in vitro has been found [40]. The gelatin-coating of porous poly(ε-caprolactone) scaffolds supported growth and osteogenesis of human mesenchymal stem cells (hMSCs) in vitro [41]. Furthermore, according to our results, the gelatin-alone-coating cell culture plates are efficacy for iPSCs reprogramming and living on the feed-free setting with the appropriate mediums to replace the MEF environment with evading its downsides, described the above (Figure 1). Moreover, it's surprising to us that such coating plates can uphold the subsequent neural progenitor cells, neural cells, and dopaminergic neurons in feeder-free conditions in the relative long-term up to 6 months (Figure 2). This function of such plates has been validated by the control group - embryotic brain cells were well-growing on the same condition up to the same period (Figure 3). Gelatin-alone- application is an alternative to others such as laminin, collagen, fibronectin, or their mixtures Matrigel. In addition, this method facilitates simple to handle and decreases the costs.

In the meaning time, we need to point out that the most critical role which the gelatin-alone-coating plates/wells play is the physical contact and support iPSCs and subtypes of neurons but not secrete the growth factors and ECM, as MEF does. Thus, the suitable medium must be used with it together in order to provide enough nutrients to these cells. Therefore, the nourishing components have been added to the proper mediums, respectively, up to different stages (see details in the MATERIALS AND METHODS). The feeder layers such as MEF sustain the growth of target cells by releasing growth factors such as FGFs, as a critical nutritious factor to the culture media, and as the attachment factor to support and to detoxify the culture medium or synthesizing extracellular matrix proteins [15,42]. FGFs are compelling controllers of cell proliferation, differentiation, and function and are critically domineering in normal development, tissue maintenance and wound restoration [10]. Thus, the gelatin-coating for the physical contacting and supporting function, along with other growth factors (as the standardization uses) such as b-fibroblast growth factors (bFGF) [11,43-45], to replace the MEF were employed during our experiments. Another one leukemia inhibitory factor (LIF), a cytokine required for maintaining both the proliferation and developmental potential of stem cells, was also applied as a conventional factor [46]. In addition, HA-100 (ROCK inhibitor) was used as survival signals, including inhibition of apoptosis and cell detachment, mediated by remodeling of the actin cytoskeleton as well [47-49].

In summary, in order to optimize the applications of stem cell, in particular, iPSCs, in the human developmental biology, drug, and transplantation medicine, one of the most important techniques is how to overcome the disadvantages of the MEF feeder cells such as zoonosis, contaminations, and inconsistency results, etc. The gelatin-alone-

coating cell culture plates have been studied. We have found that such plates/wells with the suitable culturing mediums are the efficiencies for iPSCs derived from the reprogrammed fibroblasts of adult mice and they are differential into subtypes of neurons in the feeder-free condition for the relative long-term up to 6 months. Managing this system is humble with lower costs.

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Disclosure of Potential Conflicts of Interest

The author declares no conflict of interest.

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