Optimization of surface-immobilized extracellular matrices for the proliferation of neural progenitor cells derived from induced pluripotent stem cells

Running title: Culture substrates for proliferation of NPCs

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Abstract

Neural progenitor cells derived from induced pluripotent stem cells have been considered as a potential source for cell-transplantation therapy of central nervous disorders. However, efficient methods to expand neural progenitor cells are further required for their clinical applications. In this study, a protein array was fabricated with nine extracellular matrices and used to screen substrates suitable for the expansion of neural progenitor cells derived from mouse induced pluripotent stem cells. The results showed that neural progenitor cells efficiently proliferated on substrates with immobilized laminin-1, laminin-5, or Matrigel. Based on this result, further attempts were made to develop clinically compliant substrates with immobilized polypeptides that mimic laminin-1, one of the most effective extracellular matrices as identified in the array-based screening. We used here recombinant DNA technology to prepare polypeptide containing the globular domain 3 of laminin-1 and immobilized it onto glass-based substrates. Our results showed that neural progenitor cells selectively proliferated on substrate with the immobilized polypeptide while maintaining their differentiated state.

Key words: extracellular matrix, iPS cell, neural progenitor cell, laminin, regenerative medicine

Introduction

Neural progenitor cells (NPCs) undergo self-renewal and differentiation into neuronal and glial lineages and considered as a promising cell source for the treatment of central nervous system disorders such as Parkinson's disease (Dyson et al., 2011), spinal cord injury (Tetzlaff et al., 2011), and traumatic brain injury (Gögel et al., 2011). NPCs isolated from fetal human brain tissues have been transplanted for the treatment of a patient with Parkinson's disease (Freed et al., 2001). However, the limitation of donor cells and ethical concerns make this therapy difficult to clinically apply. Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are other candidate cell sources for stem cell-based transplantation therapies (Robinton et al., 2012). These cells can unlimitedly proliferate in vitro and differentiate into multiple types of functional cells. Recently, two clinical trials using human ES-derived cells were started (Solbakk et al., 2011; Schwartz et al., 2012). In addition, iPS-derived cells may be useful for studying the mechanisms underlying the progression of central nervous system disorders and for developing effective treatments for associated diseases (Bellin et al., 2012).

Various protocols have been reported for the differentiation of iPS cells into a neural lineage (Czepiel et al., 2011; Cai, et al., 2010; Selvaraj et al., 2012). It was also reported that neural cells derived from iPS cells had been successfully integrated into the animal brain and spinal cord (Nori et al., 2011; Rhee et al., 2011). iPS cell-derived neural cells improved the motor function of the animals to a certain degree. Although neural cells derived from pluripotent stem cells are considered to have utility in cell replacement therapy, various difficulties still remain to be overcome. First, graft rejection by the host

immune system is unavoidable in transplantation therapy and should be carefully controlled. When cells derived from ES cells are used, the graft is expected to be recognized as allogeneic tissue and thus will be rejected by the host immune system (Bradley et al., 2002; Drukker et al., 2004; Taylor et al., 2011). Even when iPS cells are derived from a patient, however, the reprogramming protocols may affect the immunogenicity of the grafts (Taylor et al., 2011). Second, tumor formation must be carefully considered in cell transplantation therapy using iPS-derived cells (Hentze et al., 2010; Fong et al., 2010). Undifferentiated pluripotent stem cells and progenitor cells contained in transplants may proliferate and overgrow in the host brain (Li et al., 2008).

Most of the applications mentioned above require an in vitro culture that allows the proliferation of NPCs without altering their properties. Extracellular matrices (ECMs) play pivotal roles in the maintenance of various stem and progenitor cells in native tissues (Kim et al., 2011) and in the behaviors of neural cells (Wojcik-Stanaszek et al., 2011). In the present study, protein arrays (Soen et al., 2006; Nakajima et al., 2007) were fabricated with various ECMs and used to screen for the proliferation of NPCs derived from mouse iPS (miPS) cells. Array-based assays enable high-throughput screening of substrates with immobilized proteins and synthetic polymers (Little et al., 2011; Derda et al., 2007; Derda et al., 2010). Based on the results of the ECM screening, globular domain 3 (LG3), a part of the laminin α1 chain, was synthesized as a fusion with a hexahistidine sequence (His-tag). LG3 is relatively small domain (16.5 kDa) and known to efficiently bind with $\alpha 6\beta 1$ integrin (Ido et al., 2004). The engineered protein was anchored through the coordination of

the His-tag with Ni^{2+} ions bound on a glass plate, and feasibility of the substrate with surface-immobilized LG3 was examined for the proliferation of NPCs.

Materials and Methods

Maintenance of miPS cells and their differentiation into NPCs

miPS cells (20D17) (Okita et al., 2007) were obtained from the Riken cell bank (Ibaraki, Japan). The cells had been transformed to express GFP driven by the promoter of Nanog. miPS cells maintained on a feeder layer of mouse embryonic fibroblasts in DMEM (Invitrogen Corp., Carlsbad, CA, USA) containing 15% fetal bovine serum, 0.1 mM MEM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 1000 U/mL leukemia inhibitory factor (Millipore, Billerica, MA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. For differentiation, miPS cells were collected as single cells by treatment with 0.05% trypsin solution containing 0.53 mM ethylenediamine- N,N,N',N'-tetraacetic acid (EDTA). The cells were seeded to a gelatin-coated dish and incubated for 40 min to allow for depleting feeder cells possibly present in a population, and then unattached miPS cells were recovered for the later experiments.

The neurosphere method was used to induce neural cells from miPS cells (Okada et al., 2008). miPS cells were suspended in DMEM/F12 (1:1) containing 2% (v/v) B27 supplement (Invitrogen), 5 μg/mL heparin, 100 U/mL penicillin, and 100 μg/mL

streptomycin (hereafter referred to as base medium) supplemented with 20 ng/mL epidermal growth factor (EGF, Wako Pure Chemical Industries, Osaka, Japan), 20 ng/mL basic fibroblast growth factor (bFGF, Wako), and 10 nM retinoic acid (Sigma, St. Louis, MO, USA), and then plated at a density of 8.5×10^3 cells/cm^2 onto a tissue culture dish previously coated with Pluronic F127 (Sigma). Every 5-7 days, floating cell aggregates were collected and dissociated into single cells by treatment with AccuMax (Innovative Cell Technologies, Inc., San Diego, CA, USA) containing 0.05% trypsin, and subcultured to form secondary aggregates. Dissociation and aggregation were repeated to obtain fourth-generation aggregates.

Preparation of ECM arrays

ECMs were covalently immobilized onto the glass surface. First, amine groups were introduced on the surface of glass plates (22 mm × 26 mm × 0.5 mm) by treating with aminopropyltriethoxysilane (APTES; Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) (Konagaya et al., 2011). These plates were immersed in 10% (v/v) aqueous glutaraldehyde solution for 1 h at room temperature to introduce aldehyde groups. After washing the plates with water, a silicone frame with multiple circular windows (2 mm in diameter, 5 × 4 windows) was mounted on top of each glass plate to make a microwell array. ECM solutions in phosphate-buffered saline (PBS) containing 50 μg/mL collagen I (Cellmatrix type I, porcine tendon, Nitta Gelatin, Inc., Osaka, Japan), 50 μg/mL collagen IV (Cellmatrix type IV, bovine crystalline lens, Nitta Gelatin, Inc.), 50 μg/mL gelatin (porcine

skin, Sigma), 50 μg/mL laminin-1 (mouse Engelbreth-Holm-Swarm sarcoma, Invitrogen), 50 μg/mL Matrigel (mouse Engelbreth-Holm-Swarm sarcoma, BD Biosciences, San Jose, CA, USA), 50 μg/mL fibronectin (human serum, BD Biosciences), 50 μg/mL vitronecitn (Sigma), 50 μg/mL ProNectin F (synthetic ECM, Sanyo Chemical Industries Ltd., Kyoto, Japan), or 2 μg/mL laminin-5 (human recombinant, Oriental Yeast Co., Ltd., Tokyo, Japan) were separately pipetted into different wells on a single array (15 μL for each well). Among the ECMs used, ProNectin F is a commercially available synthetic ECM consisting of cell-adhesive arginine-glycine-aspartic acid (RGD) residues connected with β-sheet-forming hydrophobic segments. All ECMs were immobilized on the glass surface through Schiff base formation between aldehydes and amines contained in proteins. After 1 h, the ECM array was washed with PBS to remove unbound proteins. As a control surface, PBS containing 1 M ethanolamine was used instead of protein solutions. Ethanolamine was used to block unreacted aldehyde groups of the glass surface. Summary of the preparation of ECM array was shown in supporting figure 1.

Amounts of immobilized ECM proteins

ECMs were separately immobilized onto the surfaces of glass plates (22 mm × 26 mm × 0.5 mm) through the method similar to that described above. The amounts of immobilized ECM proteins were determined using the Micro BCA Protein Assay Kit (Thermo, Waltham, MA, USA). A silicone frame with a square window (inner area: 4 cm^2) was placed on the ECM-immobilized surface. The Micro BCA reaction mixture (150 μL)

was pipetted into the window, and the temperature was maintained at 37 °C for 2 h to allow for the color reaction. The absorbance of the resulting solution was measured at 562 nm using a spectrophotometer (SpectraMax^{TM} 2Me, Molecular Devices, LLC, Sunnyvale, CA, USA). The amount of immobilized protein was determined using bovine serum albumin as a standard.

Array-based assays

NPC-containing cell aggregates of 4th generation were dissociated into single cells by treatment with AccuMax containing 0.05% trypsin. The cells were suspended in base medium supplemented with 20 ng/mL EGF and 20 ng/mL bFGF (Okada et al., 2008). The cells were applied to each spot of an ECM array at a density of 2.0×10^4 cells/cm^2 and incubated for 3 d at 37 °C under 5% CO_2 atmosphere. The expression of various marker proteins in these cells was analyzed by immunostaining as described below.

Immunostaining

Cells were fixed with PBS containing 4% paraformaldehyde and then permeabilized by treatment with 0.2% Triton X-100 solution for 15 min at room temperature. The cells were then treated with Blocking One reagent (Nacalai Tesque, Kyoto, Japan) for 90 min to inhibit nonspecific adsorption of antibodies, followed by binding of primary antibodies against octamer-binding transcription factor 3/4 (Oct-3/4; 1:50, mouse monoclonal, Santa

Cruz Biotechnology, Inc., Santa Cruz, CA, USA), green fluorescent protein (GFP; 1:1000, chicken polyclonal, Millipore), nestin (1:200, mouse monoclonal, BD Biosciences), or β-tubulin III (1:500, rabbit monoclonal, Covance, Princeton, NJ, USA) for 1 h at room temperature. After washing with PBS containing 0.05% Tween 20, cells were treated with a solution of Alexa Fluor 594 anti-mouse IgG, Alexa Fluor 488 anti-rabbit IgG, or Alexa Fluor 488 anti-chicken IgG (Invitrogen) at a dilution of 1:500 for 1 h at room temperature and washed with PBS containing 0.05% Tween 20. Then, cell nuclei were counterstained with 1 μg/mL Hoechst 33258 (Dojindo Laboratories, Kumamoto, Japan). The localization of secondary antibodies was analyzed with a fluorescence microscopy (BX51 TRF, Olympus Optical Co., Ltd., Tokyo, Japan). Cells reactive for antibodies to nestin were counted on the merged images with nuclei staining. The original images were recorded at a magnification of ×200, and individual cells were carefully identified on merged images.

Preparation of His-tagged human LG3

The preparation of human LG3 (MW = 16.5 kDa) carrying hexahistidine residues at the terminus (LG3-His) was previously reported (Nakaji et al., 2012). Briefly, complementary DNA (cDNA) for human LG3 was obtained by polymerase chain reaction using human brain cDNA libraries (Takara Bio Inc., Shiga, Japan) as templates and specific primers. The resulting DNA fragments were extended with an N-terminal linker and a His-tag sequence, and then inserted into the multiple cloning site of the pET22 vector (Millipore). The protein was expressed in Escherichia coli (BL21 codonplus strain). LG3-

His, obtained as inclusion bodies, was extracted under denaturing conditions with 8 M urea, purified using a Ni^{2+}-chelated affinity column (His Trap HP; GE Healthcare Bio-Science Corp., Piscataway, NJ, USA), and refolded by dialyzing against solutions in the presence of reduced and oxidized glutathione. The purity of LG3-His was checked by sodium dodecylsulfate-polyacrylamide gel electrophoresis. The concentration of LG3-His was determined using the Micro BCA Protein Assay Kit (Thermo). The solution of purified LG3-His was stored at –80 °C until use.

Immobilization of LG3-His on glass surfaces

LG3-His was immobilized on glass surfaces via chelation of Ni^{2+} with the His-tag, as previously described (Konagaya et al., 2011). Briefly, cleaned glass plates (22 mm × 26 mm × 0.5 mm; Matsunami Glass Ind. Ltd., Osaka, Japan) were immersed in 5% (v/v) APTES in toluene for 1 h at room temperature, then washed with ethanol and water. The plates were placed in a vacuum oven and heated to 80 °C for 3 h to obtain glass plates surface-modified with APTES, which carries an amino group at its terminus. The APTES-modified glass plates were immersed in 10% (v/v) aqueous glutaraldehyde (Nacalai Tesque) for 30 min at room temperature to introduce aldehyde groups. The plates were then washed with water to remove unreacted glutaraldehyde. Then, a 10 mM aqueous solution of N-(5-amino-1-carboxypentyl) iminodiacetic acid (Dojindo Laboratories) was applied onto the activated surface, the plates were kept at room temperature for 2 h, and then washed with water to remove unreacted N-(5-amino-1-carboxypentyl) iminodiacetic acid.

The glass plates were immersed in 40 mM $NiSO_4$ for 30 min at room temperature to form Ni^{2+} chelates, and then washed with water. The solution of LG3-His in PBS (3 μM) was plated onto the Ni^{2+}-chelated surface and kept for 1 h at room temperature to allow for the immobilization of LG-His. The amount of immobilized LG3-His was determined using the Micro BCA Protein Assay Kit (Thermo). As a control, substrates with immobilized laminin-1 or ethanolamine were prepared.

NPC culture on the LG3-immobilized substrate

NPC-containing cell aggregates of 4th generation were dissociated into single cells by treatment with AccuMax containing 0.05% trypsin. The cells were suspended in base medium supplemented with 20 ng/mL EGF and 20 ng/mL bFGF. The cells were seeded onto the LG3- or laminin-1-immobilized substrate at a density of 2.0×10^4 cells/cm^2 and incubated for 3 d at 37 °C under 5% CO_2 atmosphere.

miPS cell culture on LG3-immobilized substrate

Undifferentiated miPS cells were collected as single cells by treatment with 0.05% trypsin solution containing 0.53 mM EDTA. The cells were suspended in DMEM/F12 containing 15% fetal bovine serum, 0.1 mM MEM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 1000 U/mL leukemia inhibitory factor, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were seeded onto the LG3- or laminin-1-immobilized

substrate at a density of 2.0×10^4 cells/cm^2 and cultured for 3 d at 37 °C under 5% CO_2 atmosphere. After 24 h and 72 h, phase-contrast images were recorded at a magnification of ×200, and the number of cells that adhered to the substrate was counted on the images by carefully identifying individual cells.

Statistical analysis

The data were compared by means of one-way analysis of variance (ANOVA), followed by Tukey's honestly significance difference (HSD) test. All statistical analyses were performed using the software JMP (SAS Institute Inc., NC).

Results

Induction of miPS cells into a neural linage

miPS cells used in this study had been transformed to express GFP driven by the promoter of Nanog, known as a key transcription factor to maintain cells in the undifferentiated state. miPS cells were detached from a feeder cell layer and cultured as neurosphere-like aggregates in the presence of a low concentration (10 nM) of retinoic acid to differentiate miPS cells into a neural lineage. The time schedule for differentiation is shown in Fig. 1a. Although the GFP expression level of the aggregated cells gradually

decreased during the first 5 d of culture, most cells still expressed GFP and an undifferentiated marker Oct-3/4 in primary aggregates (Fig. 1 b and c). To induce differentiation into NPCs, aggregates were dissociated into single cells and further cultured in suspension in the presence of retinoic acid. In contrast to the primary aggregates, the majority of cells in the 4th-generation aggregates expressed neither GFP nor Oct-3/4, but expressed nestin and β-tubulin III, markers of NPCs and of neurons, respectively. During repeated subcultures, the NPC population was enriched, and nestin-positive cells reached more than 70% of the total cells at the end of the 4th aggregate culture (Fig. 1d). Cells obtained from the 4th-generation aggregates were used for the following array-based assays.

Immobilization of ECM proteins

Nine ECMs (collagen I, collagen IV, gelatin, laminin-1, laminin-5, Matrigel, fibronectin, vitronectin, and ProNectin F) were separately immobilized on glass surfaces. As shown in Fig. 2, the amounts of immobilized ECMs after extensive washing with PBS were determined to be 0.05–0.5 μg/cm^2. The observed low density of laminin-5 was probably caused by the low concentration (2 μg/mL) of the laminin-5 solution used, compare to the other proteins (50 μg/mL). This was simply because laminin-5 of higher concentration was not commercially available.

Array-based screening of ECMs for NPC proliferation

Fourth-generation aggregates were dissociated into single cells and seeded to the ECM array to examine the effect of ECMs on NPC proliferation. As a negative control, ethanolamine was used instead of ECMs. Figure 3 shows the number of cells adhering to various substrates with immobilized ECMs 24 h after cell seeding. As is seen, laminin-1, laminin-5, Matrigel, and fibronectin were effective for cell adhesion, but vitronectin, collagen I, collagen IV, gelatin, and ProNectin F were relatively ineffective. Fluorescence micrographs of cells cultured on ECM arrays and then immunologically stained for nestin and β-tubulin III are shown in Fig. 4a. The majority of cells were positive only for nestin, but small numbers of β-tubulin III-positive cells were found on all ECM-immobilized substrates. After 3 d of culture, cell numbers increased approximately 10-fold on the most substrates. Among the substrates with various ECMs, nestin-positive cells most efficiently proliferated on the substrate with laminin-1, laminin-5, and Matrigel (Fig. 4b), yielding populations rich in nestin-positive cells (more than 95% of cells). Although the amount of immobilized laminin-5 was at least 3-fold or so lower than the levels of the other ECMs (Fig. 2), the laminin-5-immobilized surface effectively mediated adhesion and facilitated expansion of NPCs derived from miPS cells (Figs. 3 and 4).

Expansion of NPCs on LG3-immobilized substrate

The surface with immobilized LG3 domain of a laminin α1 chain (an α chain contained in laminin-1) was examined as a culture substrate for the proliferation of NPCs

because a chemically defined and xeno-free cell culture substrate will be required for future clinical applications. LG3 was fused with a His-tag through recombinant DNA technology and immobilized onto the Ni^{2+}-bound glass surface through coordination as previously reported (Konagaya et al., 2011). The amounts of immobilized LG3-His was determined to be 0.50 ± 0.11 μg/cm^2. Figure 5a shows a phase-contrast micrograph of 4th generation aggregate cells that were seeded to and cultured on the LG3-immobilized substrate for 3 d. NPCs adhered and proliferated on the LG3-immobilized substrate as well as on the laminin-1-immobilized substrate. On the LG3-immobilized substrate, most of cells expressed nestin after 3 d of culture, while a few cells expressed β-tubulin III (Fig. 5b). Overall, 98.3 ± 1.6% and 98.0 ± 0.1% of cells expressed nestin when cultured on the LG3- and laminin-1-immobilized substrates, respectively. Cell numbers increased 6-fold and 10-fold on LG3- and laminin-1-immobilized surfaces, respectively (Fig. 5c). These increases were significantly higher than those determined for the control surface reacted with ethanolamine (no protein on the surface).

It was striking to observe that highly proliferative colonies occasionally formed on the laminin-1-immobilized substrate after 6 d of NPC culture (Fig. 6a). Most cells in these colonies expressed GFP driven by the Nanog promoter (Fig. 6b), indicating that these cells are in the undifferentiated state. In contrast, these colonies were not found on the LG3-immobilized substrate (Fig. 6 c and d). This observation suggests that trace of undifferentiated miPS cells present in the 4th-generation aggregates were attached to and proliferated on the laminin-1-immobilized substrate, but not on the LG3-immobilized substrate. We examined this possibility by culturing undifferentiated miPS cells on the

LG3- and laminin-1-immobilized substrates. As shown in Fig. 6e, miPS cells adhered on the laminin-1-immobilized substrate and expanded approximately 5-fold within 3 d (Fig. 6g). On the other hand, only a few miPS cells adhered on the LG3-immobilized substrate (Fig. 6f), and the number of cells hardly increased on the LG3-immobilized substrate (Fig. 6gG).

Discussion

Many protocols have been reported for the induction of NPCs from ES and iPS cells (Chambers et al., 2009; Cai, et al., 2007). However, methods to efficiently expand NPCs derived from ES and iPS still remain to be developed for future clinical applications. In the present study, we differentiated miPS cells into NPCs in suspension (Okada. et al., 2008). During repeated subcultures, nestin-positive cells reached more than 70% of the total cells at the end of the 4th aggregate culture (Fig. 1). However, there are still limitations associated with the suspension culture. The most critical problems may be heterogeneity of cell populations in an aggregates. The content of the rest of cells are more differentiated cells. In addition, cell death occur in a core of a large aggregate due to limited supplies of oxygen and nutrients.

To date, several research groups have examined various ECMs, such as Matrigel (Colleoni et al., 2010), gelatin (Axell et al., 2009), fibronectin (Hong et al., 2008), and laminin (Koch et al., 2009) as culture substrates for NPCs. In the present study, nine ECM proteins were systematically examined using an array-based method. Except for laminin-5

the amounts of ECM proteins immobilized on an array were 0.3-0.5 μg/cm^2. Although the amount of immobilized laminin-5 was at least 3-fold or so lower than the amounts of the other ECMs (Fig. 2), we think that the amount of immobilized laminin-5 was sufficient for NPC proliferation. A density of closely packed laminin molecules in a two-dimensional lattice on a flat surface was estimated at 1×10^{-12} mol/cm^2 (Silva et al., 2004). In addition, Massia et al. reported that 1×10^{-14} mol /cm^2 of RGD ligands were sufficient for cell speeding (Massia et al., 1991). In the present study, the density of immobilized laminin-5 was approximately 2×10^{-13} mol/cm^2. The laminin-5-immobilized surface is expected to effectively mediate adhesion and facilitated expansion of NPCs. It was demonstrated in Figs. 3 and 4. We did not examine the concentration dependence of other ECMs either on NPC adhesion and proliferation. Comparing functions of ECMs at similar surface molar concentration may be more relevant, because cell receptors interact with individual molecules. However, it is quite difficult to adjust surface molar concentration for different ECMs. As mentioned related to laminin-5, we expect sufficient amount of ECMs were immobilized on an array for NPC adhesion and proliferation. Using this analytical platform, we successfully found that NPCs derived from miPS cells proliferated most efficiently on substrates with laminin-1, laminin-5, or Matrigel. In contrast, NPC adhesion was poor on substrates with collagen I, collagen IV, or gelatin. These differences may be due to dissimilarities in the expression levels of integrins on the cell surface. It has been reported that NPCs derived from fetal brain tissue express integrin β1, α3, α6, and α7 and integrin complexes such as α3β1, α6β1, and α7β1, all of which constitute specific receptors for the laminins (Flanagan et al., 2006; Prowse et al., 2010). On the other hand, the NPCs poorly

expressed collagen-associated integrin α1, α2, α10, and α11 (Prowse et al., 2010). In our previous study, neural stem cells derived from rat fetal brain also did not adhere on substrate with collagen I, collagen IV, and gelatin (Nakajima et al., 2007).

From the clinical point of view, functional substrates used for cell preparation should be chemically defined, xeno-free, and low-cost. For these reasons, further investigations were conducted using laminin-derived small domains or short peptides that can partially replace the function of full-length laminin-1 (Ido et al., 2004; Tashiro et al., 1989). In our previous study (Nakaji et al., 2012), LG3 fused with a collagen-binding sequence was synthesized, and the engineered protein was incorporated in a collagen hydrogel to design a novel carrier for effective cell transplantation. The survival of rat neural stem cells was significantly improved in the LG3-incorporated collagen hydrogel due to effective integrin ligation, compared to a pure collagen hydrogel (Nakaji et al., 2012). In the present study, we used LG3 fused with a His-tag and immobilized this polypeptide on a nickel-chelated glass plate to obtain a culture substrate. Although laminin-5 did well in NPC adhesion and proliferation at low density, we used LG3 derived from α chain of laminin-1. It is because laminin-1 has been widely studied and we can easily obtain information about three-dimensional structure and physiological properties of the protein. NPCs adhered well and expanded rapidly on the LG3-immobilized substrate (Fig. 5), although the rate of NPC proliferation on the LG3-immobilized substrate was slightly slower than that on the laminin-1-immobilized substrate. Approximately 98% of cells that expanded on the LG3-immobilized substrate expressed an NPC marker.

For the clinical use of stem cells, the risk of tumor formation should be minimized as much as possible (Fong et al., 2010). Undifferentiated pluripotent stem cells contaminating transplants may proliferate and overgrow in the host tissue (Li et al., 2008). In the present study, the results of array-based screening indicated that laminin-1 served as a preferable substrate for the expansion of NPCs. However, undifferentiated miPS cells cells also proliferated to form colonies on the laminin-1-immobilized substrate (Fig. 6). In contrast, undifferentiated miPS cells hardly attached and proliferated on the LG3-immobilized substrate (Fig. 6 f and g). The observed difference is probably due to distinct specificity of LG3 and laminin-1 toward integrins. It is reported that LG3 domain binds most efficiently to $\alpha_6\beta_1$ integrin (Belkin et al., 2000), whereas laminin-1 has an affinity for a variety of integrin complexes (Durbeej 2010). It is therefore likely that not only NPCs but also undifferentiated miPS cells are able to adhere to the substrate with immobilized laminin-1.

Accordingly, we demonstrated here that the LG3-immobilized substrate is feasible for the selective proliferation of NPCs derived from miPS cells. However, several properties differ between human iPS cells and miPS cells (Yu et al., 2008). Therefore, we are currently conducting further study to verify the effectiveness of the LG3-immobilized substrate for the selective expansion of NPCs derived from human iPS cells. Human NPCs highly express integrin α_6 and β_1 integrin (Prowse et al., 2010). We think the LG3-immobilized substrate can be used for human NPC culture.

Conclusions

As demonstrated by the array-based assays, laminin-1, laminin-5, and Matrigel are effective ECMs for the proliferation and maintenance of NPCs derived from miPS cells. When immobilized to a substrate, the LG3 fragment of laminin-1 provides a suitable substrate for the selective proliferation of NPCs derived from miPS cells.

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Figure Captions

Figure 1. Differentiation of miPS cells into NPCs. (a) Experimental protocol of differentiation culture. (b) Expression of a pluripotent stem cell maker. miPS cells used in

this study expressed GFP driven by the Nanog promoter. Phase-contrast and fluorescent micrographs are shown in the upper and lower panels, respectively. (c) Representative fluorescence micrographs of primary (left) and 4th-generation (right) aggregates. Cells were stained using antibodies against Oct-3/4, GFP, nestin, and β-tubulin III. Scale bar: 100 μm. (d) Quantification of Oct-3/4$^+$, Nanog$^+$, nestin$^+$, and β-tubulin III$^+$ cells. The data are expressed as the mean ± standard deviation for n = 3. *and ** indicates statistical significance (Tukey's HSD test, * $p < 0.05$, ** $p < 0.01$). Gene expression of 4th aggregates was compared with all other aggregates.

Figure 2. The surface density of immobilized ECMs. The data are expressed as the mean ± standard deviation (n = 3). An asterisk indicates statistical significance (Tukey's HSD test, $p < 0.05$). The laminin-1 immobilized substrate was compared with all other substrates.

Figure 3. The number of cells on various substrates with immobilized ECMs determined 1 day after cell seeding. The data are expressed as the mean ± standard deviation (n = 6). An asterisk indicates statistical significance (Tukey's HSD test, $p < 0.05$).

Figure 4. The effects of various ECMs on NPC proliferation. (a) Phase-contrast (upper) and fluorescent (lower) micrographs of NPCs derived from miPS cells cultured for 3 d on

the ECM array. The cells were immunologically stained for nestin (red) and β-tubulin III (green) in the fluorescent images. Identification of immobilized ECMs is shown above the micrographs. Scale bar: 100 μm. (b) The number of cells on surfaces with immobilized ECMs determined 3 d after seeding. The data are expressed as the mean ± standard deviation for n = 3. Closed and open boxes indicate the fractions of nestin$^-$ and nestin$^+$ cells, respectively. An asterisk indicates statistical significance (Tukey's HSD test, $p < 0.05$).

Figure 5. Proliferation of NPCs on the LG3-immobilized substrate. (a) Phase-contrast micrograph of cells cultured for 3 d. (b) Fluorescence micrograph of cells stained for nestin (red), β-tubulin III (green), and cell nuclei (blue). Scale bar: 100 μm. (c) The number of cells on the LG3- and laminin-1-immobilized substrates. The data are expressed as the mean ± standard deviation for n = 3. Closed and hatched boxes indicate the number of cells after 1 d and 3 d of culture, respectively. *and ** indicates statistical significance (Tukey's HSD test, * $p < 0.05$, ** $p < 0.01$).

Figure 6. The proliferation of undifferentiated miPS cells on the laminin-1-immobilized substrate. Phase-contrast micrographs of miPS cell-derived NPCs cultured for 6 d on the substrate with immobilized laminin-1 (a, c) or LG3 (b, d). A circle indicates a highly proliferative colony formed on the laminin-1-immobilized substrate. Scale bar: 200 μm. (c, d) Fluorescence micrographs of cells stained for GFP (green) and cell nuclei (blue). Scale

bar: 100 μm. (e, f) Phase-contrast micrographs of undifferentiated miPS cells (miPSC) cultured for 3 d on the substrate with immobilized laminin-1 (e) or LG3 (f). (g) The number of cells on the substrate with immobilized LG3 and laminin-1. The data are expressed as the mean ± standard deviation for n = 3. Closed and hatched boxes indicate the number of cells after 1 d and 3 d of culture, respectively. Cells were seeded onto the substrate with immobilized LG3 and laminin-1 at a density of 2.0×10^4 cells/cm^2. An asterisk indicates statistical significance (Student's-t test, $p < 0.01$).

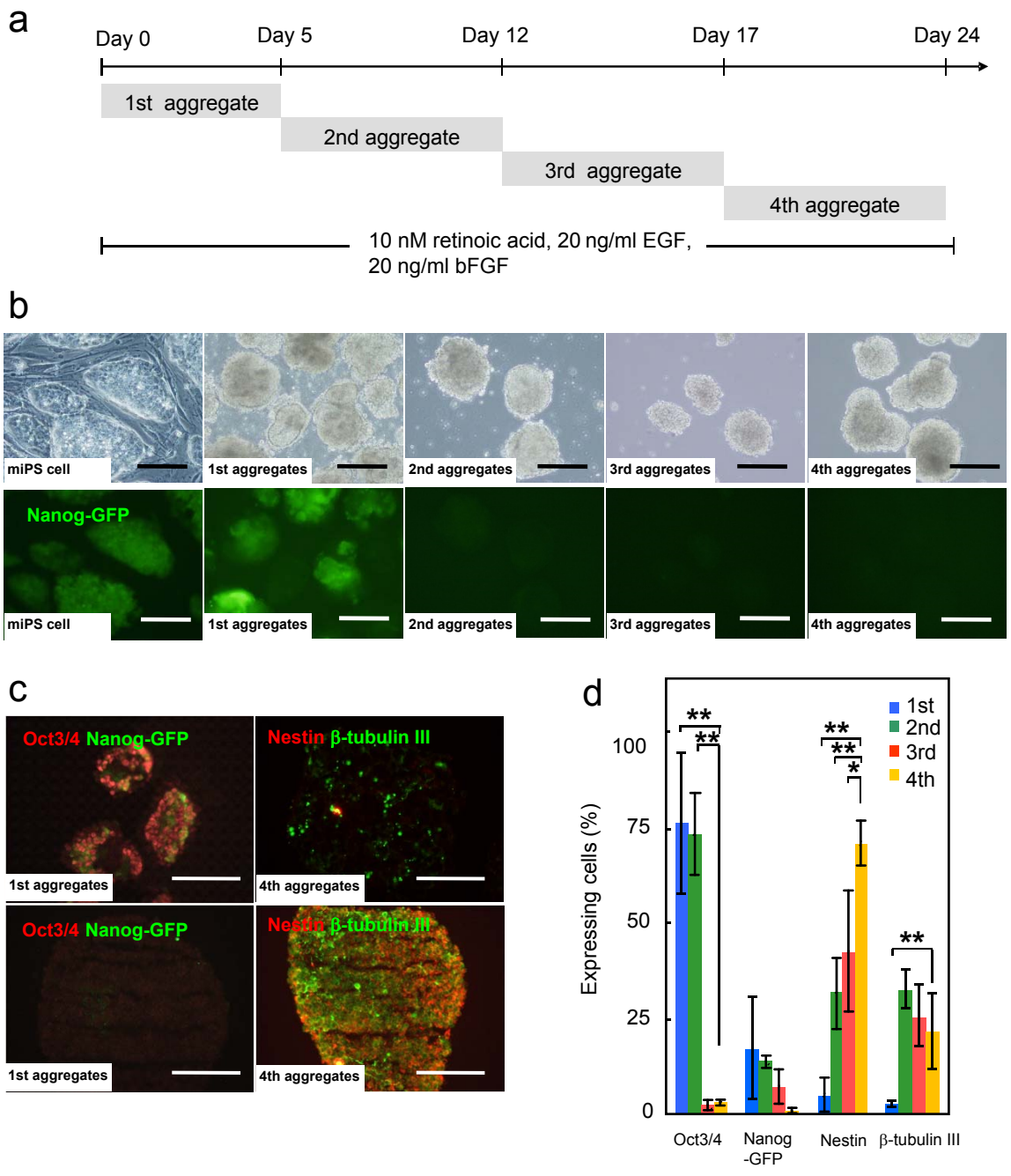

a
Day 0
Day 5
Day 12
Day 17
Day 24
1st aggregate
2nd aggregate
3rd aggregate
4th aggregate
10 nM retinoic acid, 20 ng/ml EGF,
20 ng/ml bFGF
b
miPS cell
1st aggregates
2nd aggregates
3rd aggregates
4th aggregates
Nanog-GFP
c
Oct3/4 Nanog-GFP
Nestin β-tubulin III
d
Expressing cells (%)
100
75
50
25
0
1st
2nd
3rd
4th
**
*
Oct3/4
Nanog -GFP
Nestin
β-tubulin III

Figure 1

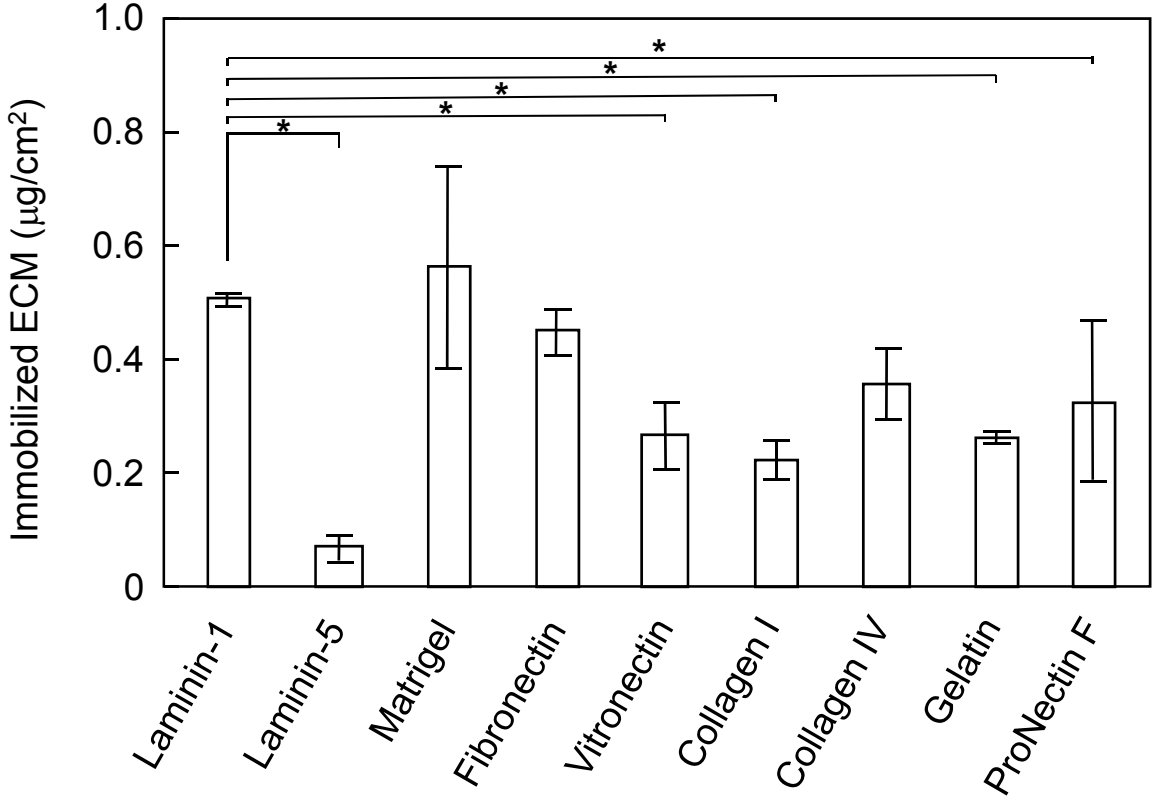

Immobilized ECM (μg/cm²)
1.0
0.8
0.6
0.4
0.2
0
*
*
*
*
*
Laminin-1
Laminin-5
Matrigel
Fibronectin
Vitronectin
Collagen I
Collagen IV
Gelatin
ProNectin F

Figure 2

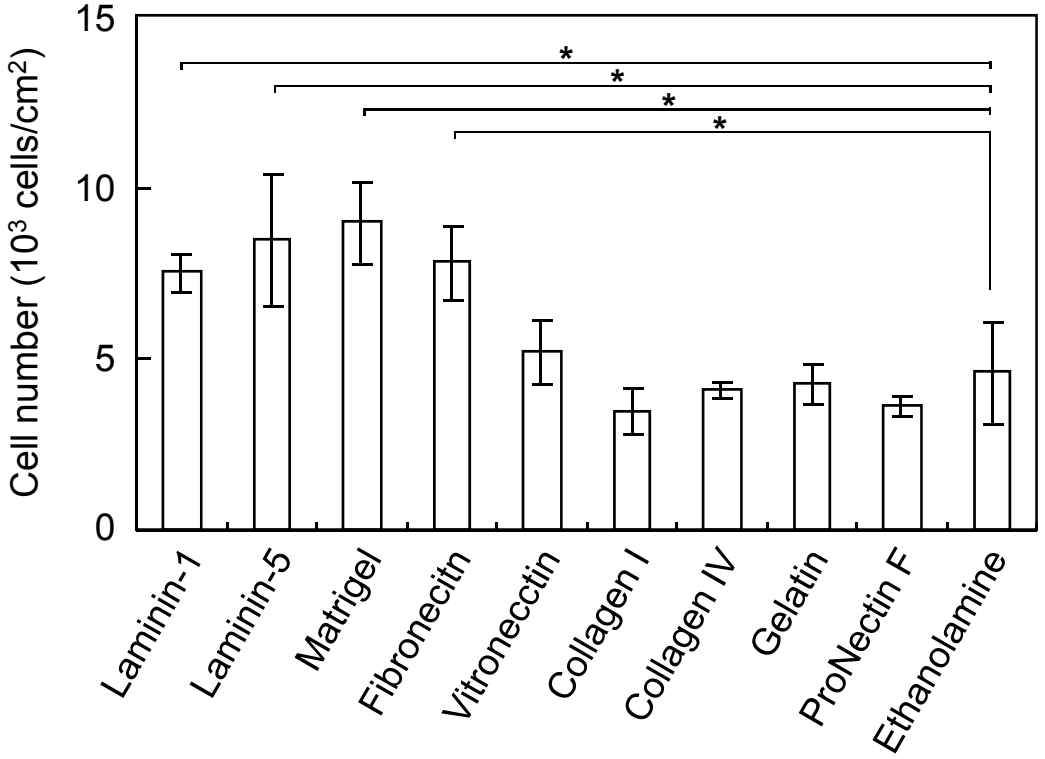
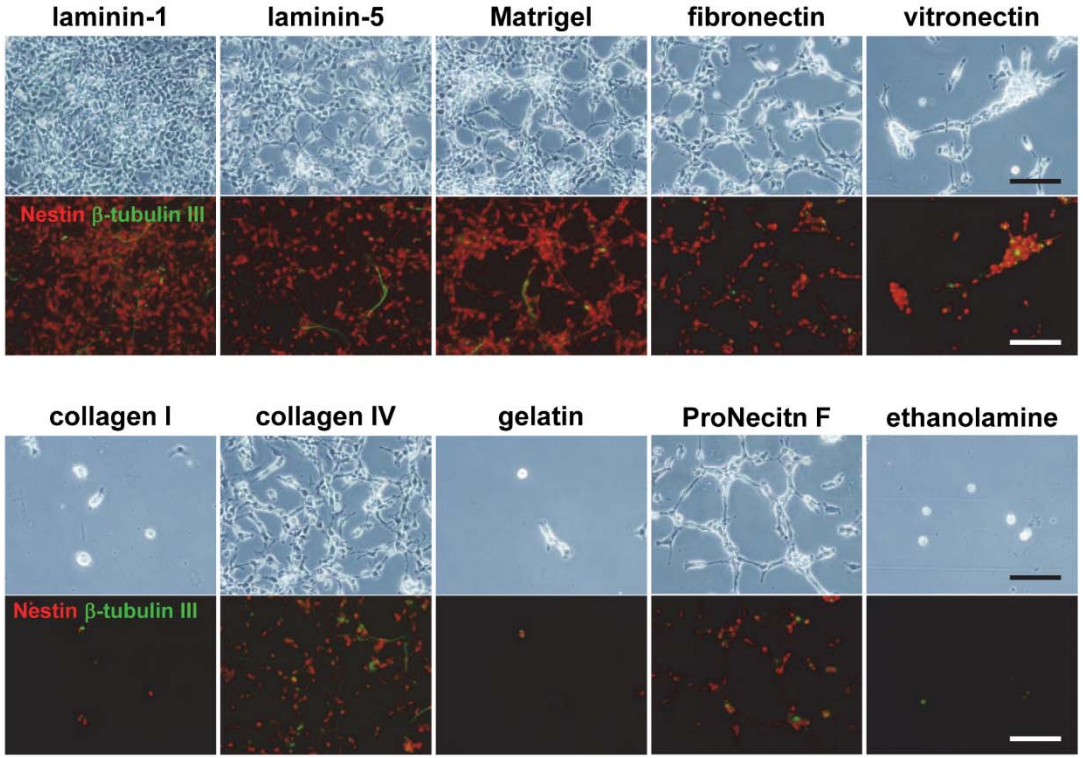

15
10
5
0
Cell number (10³ cells/cm²)
*
*
*
*
Laminin-1
Laminin-5
Matrigel
Fibronecith
Vitronecctin
Collagen I
Collagen IV
Gelatin
ProNectin F
Ethanolamine

Figure 3

a


laminin-1
laminin-5
Matrigel
fibronectin
vitronectin
Nestin β-tubulin III
collagen I
collagen IV
gelatin
ProNecitn F
ethanolamine
Nestin β-tubulin III

b

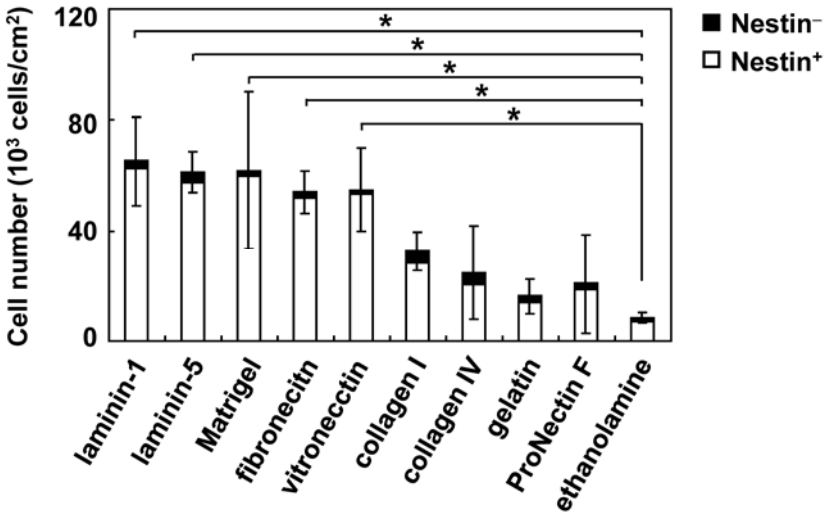
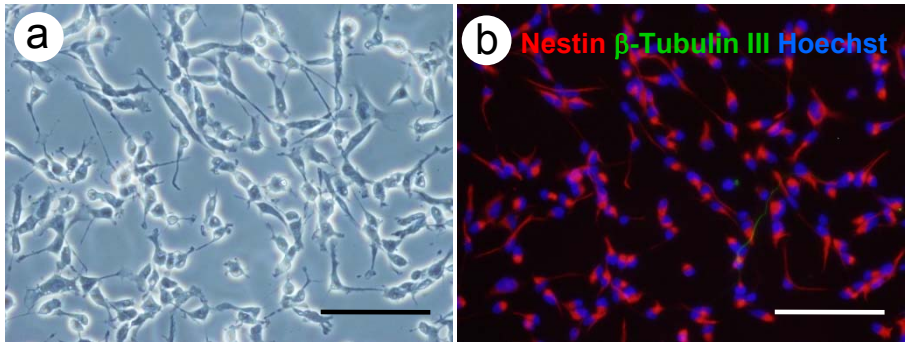

Cell number (10³ cells/cm²)
120
80
40
0
Nestin⁻
Nestin⁺
*
*
*
*
*
laminin-1
laminin-5
Matrigel
fibronectin
vitronecctin
collagen I
collagen IV
gelatin
ProNectin F
ethanolamine

Figure 4


a
b
Nestin β-Tubulin III Hoechst

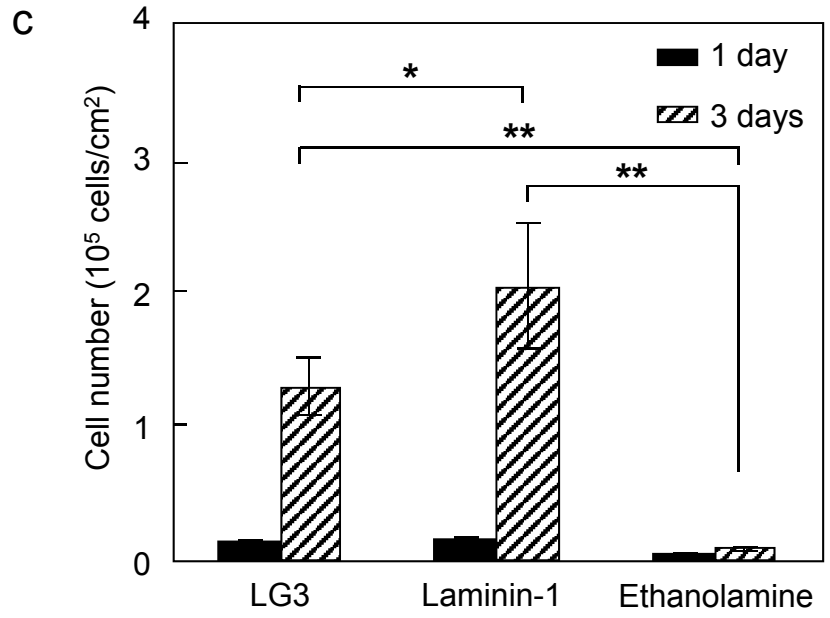

c
Cell number (10^5 cells/cm^2)
4
3
2
1
0
1 day
3 days
*
**
**
LG3
Laminin-1
Ethanolamine

Figure 5

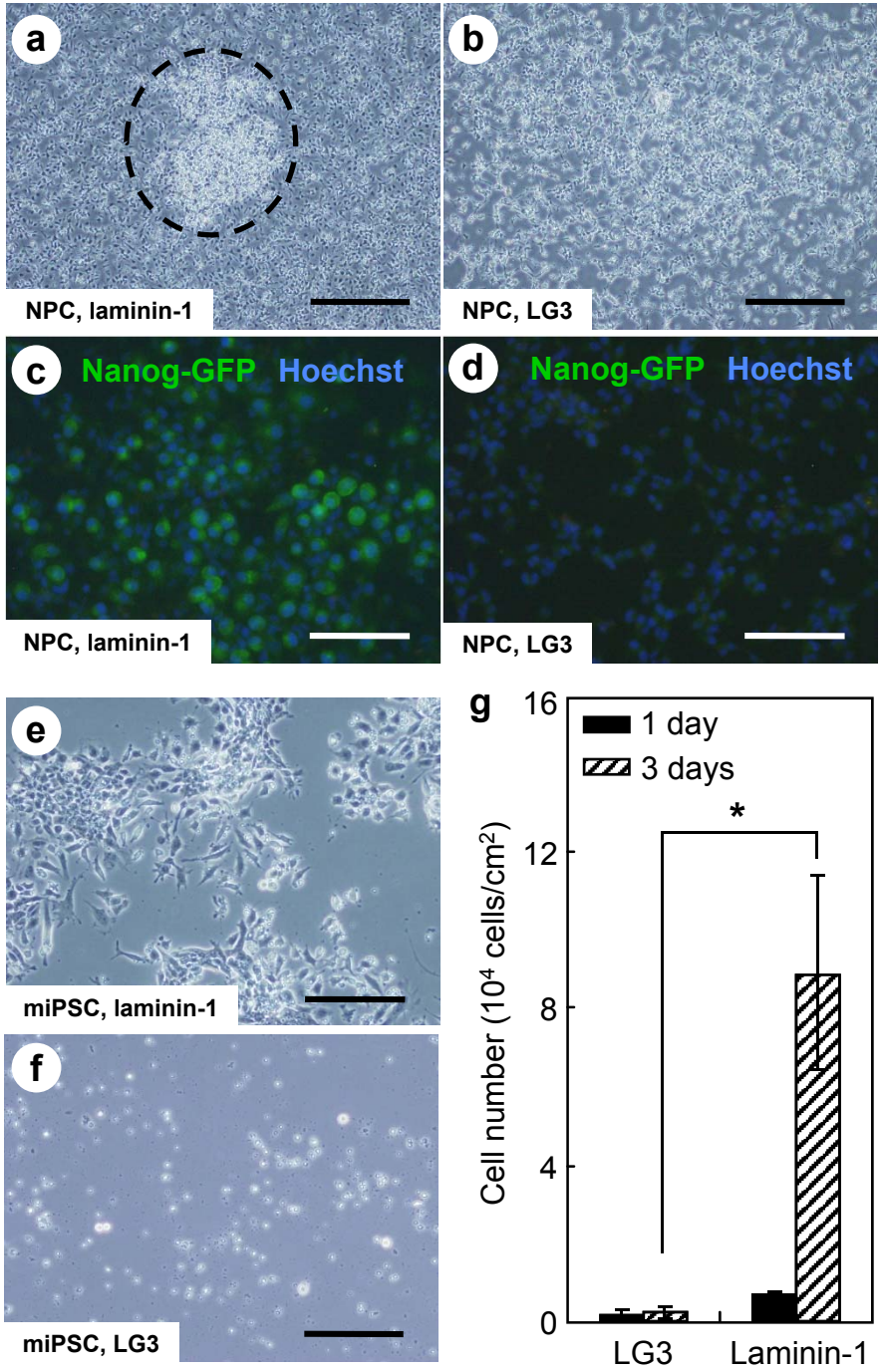

a
NPC, laminin-1
b
NPC, LG3
c
Nanog-GFP Hoechst
NPC, laminin-1
d
Nanog-GFP Hoechst
NPC, LG3
e
miPSC, laminin-1
f
miPSC, LG3
g
1 day
3 days
*
Cell number (10^4 cells/cm^2)
16
12
8
4
0
LG3
Laminin-1

Figure 6