Corning® rLaminin-521 (Human) for Expansion and Differentiation of Human Neural Stem Cells

Application Note

CORNING

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Introduction

Human neural stem cells (hNSCs) are self-renewing and multipotent cells that have the capacity to differentiate into multiple cell types found in the central nervous system (CNS), including neurons, oligodendrocytes, and astrocytes. Human NSCs have applications in basic understanding of the CNS for neuroscience research, as well as in tissue engineering and clinical applications[1]. Human NSCs have been expanded as adherent culture on a variety of surfaces, including extracellular matrix proteins such as mouse Laminin, to maintain cell attachment and prevent spontaneous differentiation[2]. However, animal-derived proteins contain undefined biological components varying from lot to lot and can be time consuming to prepare. Also, animal-origin reagents may contain adventitious agents and may not be suitable for applications where use of animal-free reagents is desirable. Recombinant Laminin-521 (rLaminin-521) has been shown to support long-term self-renewal of human pluripotent stem cells in a chemically defined and xeno-free environment[3].

In this study, we demonstrate the undifferentiated expansion of hNSCs on Corning rLaminin-521 (Human). Human NSCs cultured on rLaminin-521 exhibited doubling time and viability comparable to cells cultured on poly-L-ornithine with Laminin (PLO/Laminin), a commonly used surface coating for hNSC culture. Human NSCs expressed undifferentiated NSC markers Nestin and SOX2 over three passages. Post-expansion, cells were successfully differentiated into astrocytes, oligodendrocytes, and neurons. Corning rLaminin-521 (human) offers an animal-free culture substrate for hNSC expansion and differentiation.

Materials/Methods

Coating and Culture

Corning Tissue Culture (TC)-treated 6-well plates (Corning Cat. No. 3516) were coated with either rLaminin-521 or PLO/Laminin prior to initiating cell culture. For rLaminin-521 coating, 1 mL of a 10 µg/mL solution of rLaminin-521 (Corning Cat. No. 354221) in Dulbecco's phosphate buffered saline (DPBS) containing calcium and magnesium (Corning Cat. No. 21-030-CM) was added to each well of the 6-well plates. Plates were stored at 4°C for at least 16 hours and solution was aspirated just prior to use. For PLO/Laminin coating, 2 mL of 20 µg/mL PLO (Sigma Cat. No. P3655) diluted in sterile water (Corning Cat. No. 25-055-CM) was added to each well and incubated at 37°C for 1 hour. After incubation, wells were rinsed with 3 mL sterile water twice before adding 2 mL of 10 µg/mL Laminin (Sigma Cat. No. L2020) diluted in sterile water. Plates were incubated at 37°C for 2 hours, rinsed with 3 mL DPBS without calcium and magnesium (Corning Cat. No. 21-031-CM), and stored at room temperature with 3 mL DPBS without calcium and magnesium at room temperature that was aspirated just prior to use. H9-derived hNSCs (Thermo Fisher Cat. No. N7800-100) were seeded at a density of 4 x 10^4 cells/cm^2 in 2.5 mL per well. Media changes were performed every other day, and cells of StemPro® NSC SFM (Thermo Fisher Cat. No. A1050901) were harvested after 96 hours using TrypLE™ enzyme (Thermo Fisher Cat. No. 12563-029). During harvest, cells were incubated in TrypLE enzyme at room temperature for 30 seconds on the PLO/Laminin surface and for 3 minutes on the rLaminin-521 surface. Harvested cells were diluted in culture medium, centrifuged at 250 x g for 4 minutes, and resuspended in culture medium. Harvested hNSCs were counted on a Vi-CELL® Cell Viability Analyzer (Beckman Coulter) by pooling 2 wells from each plate together to achieve 3 counts per 6-well plate tested. Once counted, the cells were used to re-seed a new 6-well plate of the same surface for continuous passage studies using the seeding and culture protocol described above. The entire 3 passage study was repeated 2 independent times.

Assessment of the Undifferentiated State

Human NSCs were cultured for 3 passages as recommended by the supplier. Expression of undifferentiated cell markers Nestin and SOX2 were assessed at passages 1 and 3 by flow cytometry. Harvested cells were washed with DPBS without calcium and magnesium twice and fixed in 4% paraformaldehyde (PFA) (Boston BioProducts Cat. No. BM-698) for 10 minutes at room temperature. Approximately 1 x 10^6 cells per sample were permeabilized with 0.1% saponin (TCI Cat. No. S0019) in Hank's Balanced Salt Solution (HBSS) (Corning Cat. No. 21-023-CM) containing 0.05% sodium azide (Fisher Cat. No. ZS2271) and centrifuged at 200 x g for 7 minutes. The supernatant was aspirated and replaced with 200 µL of fresh permeabilization buffer per sample. Finally, 10 µL of antibody or isotype control was added to each sample (Table 1). Samples were incubated at room

Table 1. Multipotency Markers

Antibody/Isotype Control	Supplier/Cat. No.
Nestin Fluorescein Mouse IgG1 (1:20)	R&D Systems/IC1259F
Fluorescein Isotype Control Mouse IgG1 (1:20)	R&D Systems/IC002F
SOX2 Allophycocyanin Mouse IgG2A (1:20)	R&D Systems/IC2018A
Allophycocyanin Isotype Control Mouse IgG2A (1:20)	R&D Systems/IC003A

temperature for 30 minutes, washed twice with HBSS, and resuspended in a final volume of 400 µL of HBSS for analysis with a MACSQuant® flow cytometer (Miltenyi Biotec).

Differentiation

After 3 passages, cells were assessed for their ability to differentiate into astrocytes, oligodendrocytes, and neurons. Differentiation was induced following Life Technologies' protocols. For astrocyte differentiation, hNSCs were seeded onto their respective surfaces at a density of 4 x 10^4 cells/cm^2 in 6-well plates in 2.5 mL of growth medium. Two days after cell seeding, the medium was changed to Corning® glutagro™ DMEM (Corning Cat. No. 10-101-CV) supplemented with 1% N2- supplement (Life Technologies Cat. No. 17502-048), and 1% fetal bovine serum (Corning Cat. No. 35-010-CV). The medium was changed again 2 days later, and the cells were stained for marker expression (Table 2) 5 days after seeding. For oligodendrocyte differentiation, hNSCs were seeded under the same conditions at a density of 2.5 x 10^4 cells/cm^2 and differentiation was induced by performing media changes 2 and 4 days after seeding with Neurobasal® medium (Thermo Fisher Cat. No. 21103-049) supplemented with 2% Gibco B-27 supplement (Thermo Fisher Cat. No. 17504-044), 1X GlutaMAX™ (Thermo Fisher Cat. No. 35050-061), and 30 ng/mL of T3 supplement (Sigma Cat. No. T5516). Cells were stained for marker expression (Table 2) 6 days after cell seeding. For neuron differentiation, cells were seeded using the same protocol and seeding density as the oligodendrocytes and differentiation was induced two days after seeding changing the medium to Neurobasal medium with 2% B-27 supplement, and 1X GlutaMAX. Additional media changes were performed 4 and 6 days after seeding with the addition of dcAMP (Sigma Cat. No. D0627) to the differentiation medium at a final concentration of 1 mM. Neurons were stained for marker expression (Table 2) 7 days after seeding. For cellular staining, cells were fixed with 4% PFA in DPBS. After 15 minutes, cells were washed with DPBS containing calcium and magnesium, then permeabilized and blocked with a 0.1% Triton™ X-100 (Integra Cat. No. T756.30.30), 1% BSA (Sigma Cat. No. A9576), 5% FBS solution in HBSS for at least 1 hour at room temperature. After fixation and permeabilization, the buffer was removed and replaced with 1 mL HBSS containing 1% BSA. To each well, 100 µL of conjugated antibody or isotype control was added (Table 2). In addition, 5 µL Hoechst 34580 stain (Molecular Probes Cat. No. H21486) was added to each well to counterstain cell nuclei. Samples were stored at 4°C for 40 minutes, then washed twice with HBSS and visualized using an EVOS® FL Cell Imaging System (Thermo Fisher).

Results and Discussion

In order to assess the ability of Corning rLaminin-521 (Human) surface to support hNSC expansion, hNSCs were cultured on 6-well plates coated with rLaminin-521 and compared to 6-well plates coated with PLO/Laminin. During the study, typical hNSC morphology was observed and similar confluence was achieved on both of the surfaces after 96 hours of culture (Figure 1). Throughout the 3 passage study, hNSCs maintained a consistent doubling time of 41.1 ± 5.5 hours on both surfaces, without significant differences (Figure 2). Human NSCs also maintained high cell viability (>96%) on both surfaces throughout the duration of the study and no significant differences were observed between passages and surfaces (Figure 3).

To confirm the undifferentiated state of hNSCs over 3 passages, Nestin and SOX2 marker expression were quantified via flow cytometry after passages 1 and 3. Representative flow cytometry plots from passage 1 (Figure 4) show high expression levels of Nestin (>99%) and SOX2 (>93%). Average expression levels across

Table 2. Differentiation Markers

Cell Type	Antibody/Isotype Control	Vendor/Cat. No.
Astrocyte	GLAST Phycoerythrin Mouse IgG2a (1:10)	Miltenyi Biotec/130-095-821
	Phycoerythrin Isotype Control Mouse IgG2a (1:10)	Miltenyi Biotec/130-091-835
Oligodendrocyte	A2B5 Phycoerythrin Mouse IgM (1:10)	Miltenyi Biotec/130-093-581
	Phycoerythrin Isotype Control Mouse IgM (1:10)	Miltenyi Biotec/130-093-177
Neuron	BD Pharmingen™ Alexa Fluor® 488 Mouse anti-MAP2B IgG1, K (1:10)	BD Biosciences/560399
	BD Pharmingen Alexa Fluor 488 Mouse IgG1 K Isotype Control K (1:10)	BD Biosciences/557721

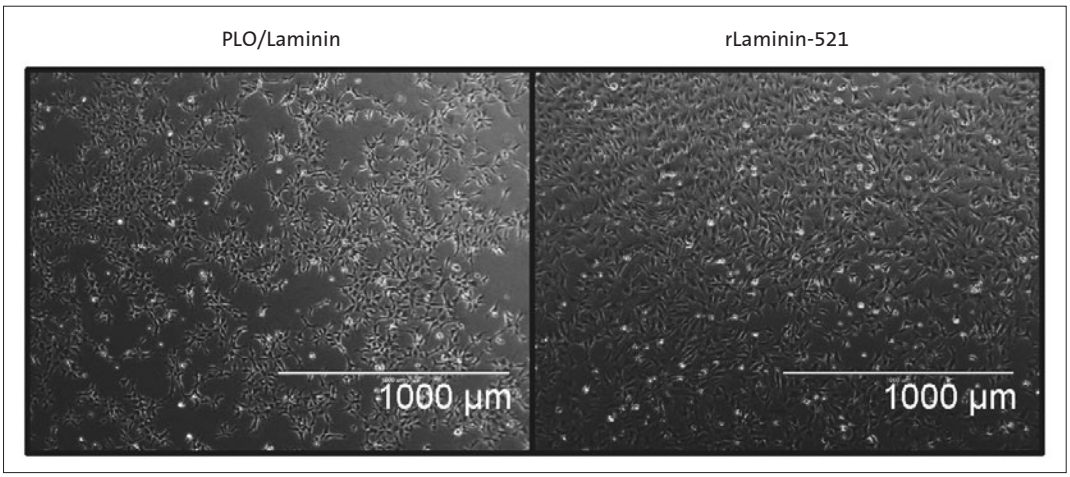

Figure 1. Human NSC morphology and confluence was comparable across rLaminin-521 and PLO/Laminin surfaces. 4X representative images of hNSCs after 72 hours in culture on 6-well plates coated with PLO/Laminin or rLaminin-521.

the three passage study (Figure 5) demonstrate the undifferentiated state was maintained on rLaminin-521 and PLO/Laminin. There were no significant differences in the expression levels of Nestin and SOX2 between the two surfaces.

After hNSCs were cultured on the rLaminin-521 for three passages, their multipotency was assessed by their ability to differentiate into astrocytes, oligodendrocytes, and neurons. Cells from passage 3 were seeded onto their respective surfaces, differentiated, and stained for lineage-specific differentiation markers.

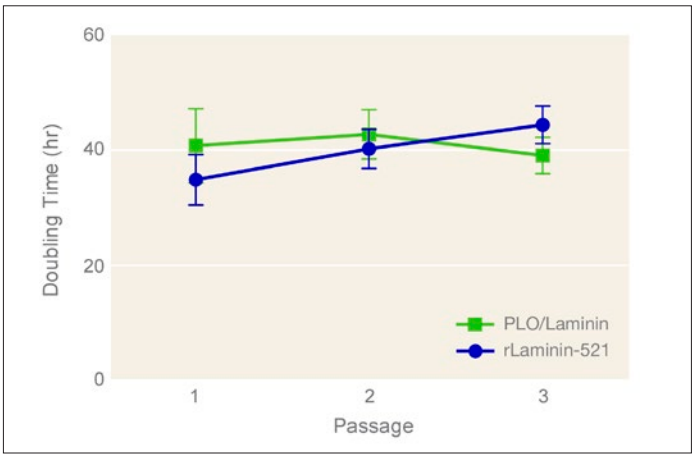

Figure 2. Doubling time of hNSCs was consistent across three passages and comparable between rLaminin-521 and PLO/Laminin coated surfaces. Average hNSC doubling time on each surface was 41.1 ± 5.5 hours across 3 passages. No significant difference was observed in doubling time across the two surfaces tested using a two-tailed unpaired t-test. N = 18 wells for each surface.

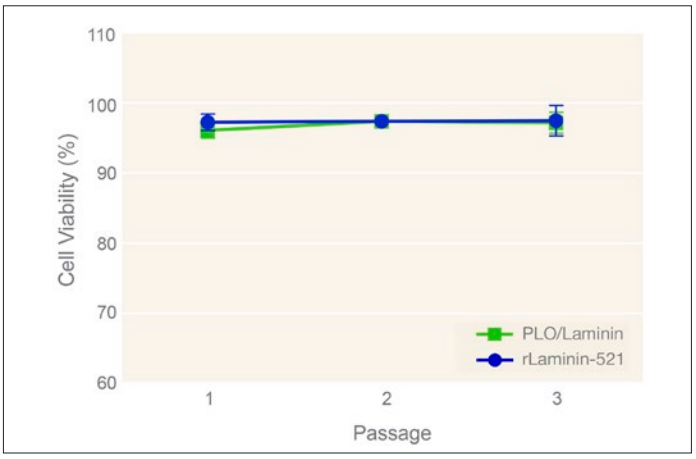

Figure 3. Viability of hNSCs was maintained on rLaminin-521 and PLO/Laminin surfaces. Viability of >96% was maintained on each surface across 3 passages without significant differences using a two-tailed unpaired t-test. N = 18 wells for each surface.

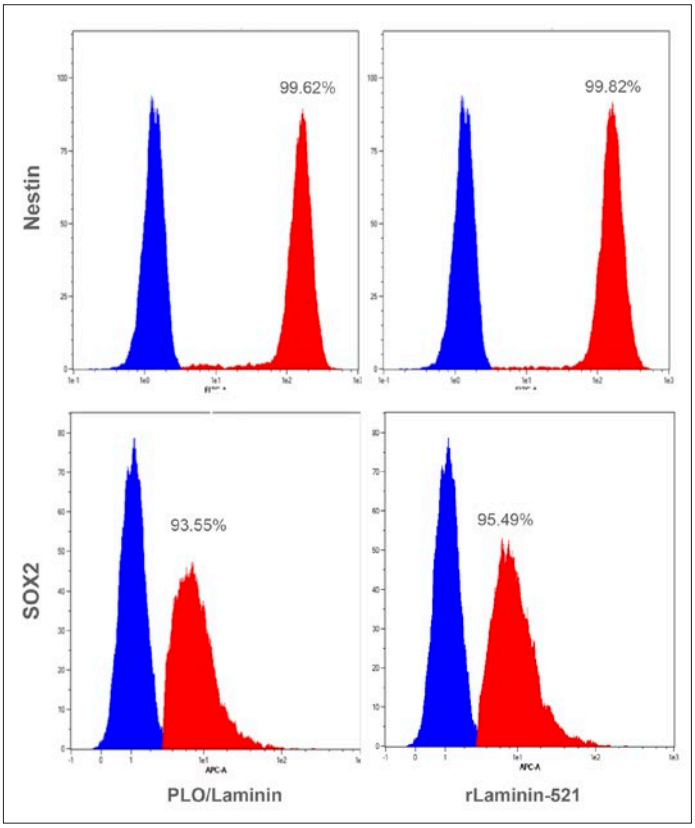

Figure 4. Expression of multipotency markers on rLaminin-521 and PLO/Laminin coated surfaces. Representative histograms of Nestin and SOX2 expression from passage 1 hNSCs cultured on 6-well plates coated with PLO/Laminin or rLaminin-521 as assessed via flow cytometry.

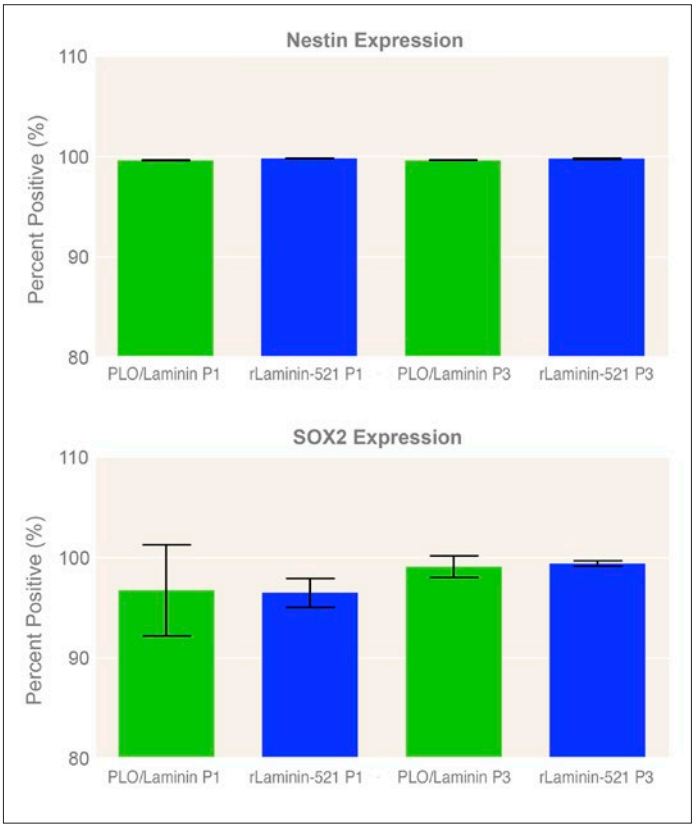

Figure 5. Expression levels of multipotency markers Nestin and SOX2 were maintained on rLaminin-521 and PLO/Laminin surfaces. SOX2 expression was >93.5% on each surface at both passage 1 (P1) and passage 3 (P3) without significant differences in expression level using ANOVA with Bonferroni post-test. Nestin expression was >99.6% on each surface at both P1 and P3 without significant differences in expression level using ANOVA with Bonferroni post-test.

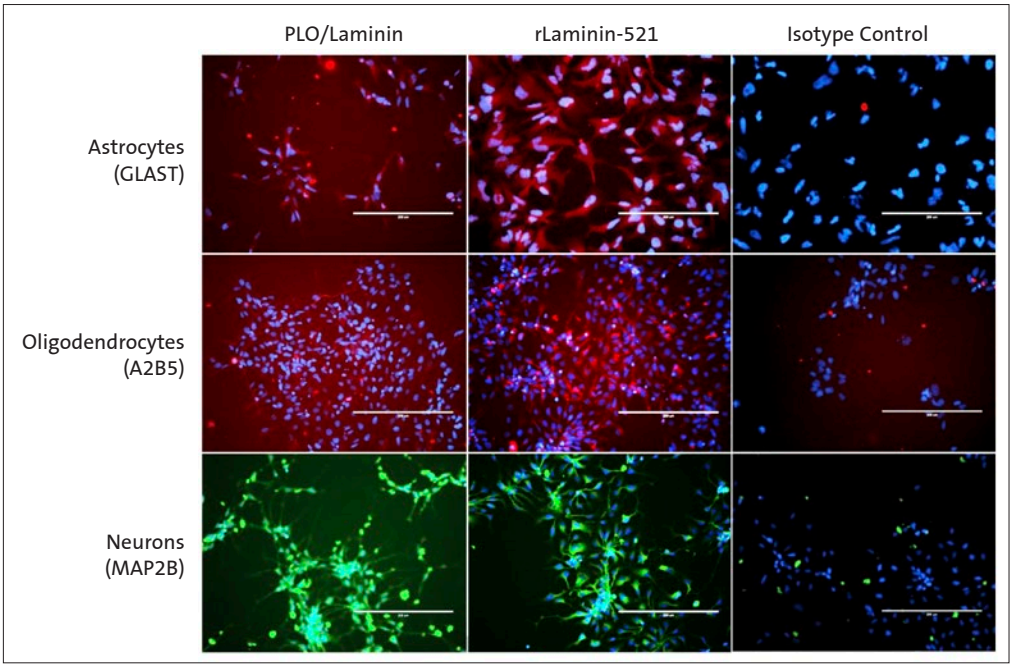

Figure 6. Human NSCs differentiated into astrocytes, oligodendrocytes, and neurons on rLaminin-521 and PLO/Laminin surfaces. Representative 20X images of astrocytes (GLAST), oligodendrocytes (A2B5), and neurons (MAP2B) with Hoechst 34580 nuclei counterstain, as well as the appropriate isotype controls. These cells were obtained by differentiating hNSCs after three passages on PLO/Laminin and rLaminin-521 surfaces.

Figure 6 shows representative immunocytochemistry images of the differentiated cells on rLaminin-521 and PLO/Laminin coated surfaces for the different lineages, supporting the ability of hNSCs cultured for three passages on rLaminin-521 to differentiate into the three lineages examined.

Conclusions

- Corning® recombinant Laminin-521 (Human) is an animal-free recombinant protein that supports the culture, expansion, and differentiation of human neural stem cells in a serum-free medium.
- Over the course of three passages, hNSCs cultured on rLaminin-521 maintained high viability and a consistent doubling time that was comparable with the PLO/Laminin surface.
- The rLaminin-521 surface maintained undifferentiated phenotype of hNSCs and the cells expressed neural stem cell-specific markers Nestin and SOX2.
- Human NSCs cultured on the rLaminin-521 retained their multipotency and were able to differentiate into astrocytes, oligodendrocytes, and neurons.

References

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3. Wang Y, et al. Ann Biomed. 42(7):1357-1372 (2013).

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